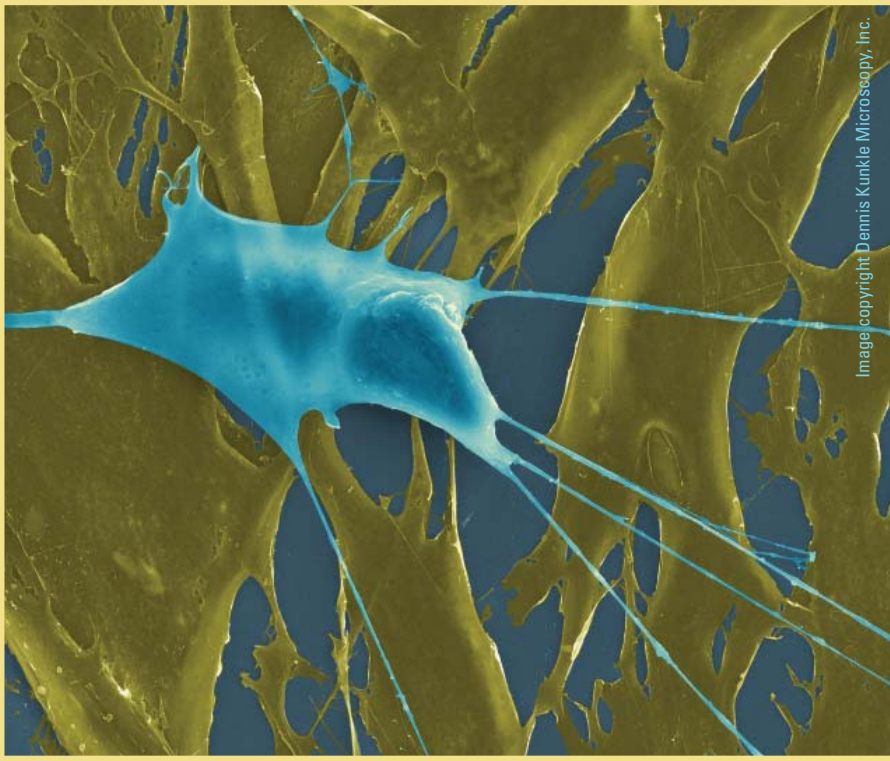
National Cancer Institute

ORGAN SYSTEMS BRANCH

INVESTIGATORS' WORKSHOP

15th SPORE



JULY 7–10, 2007, BALTIMORE MARRIOTT WATERFRONT

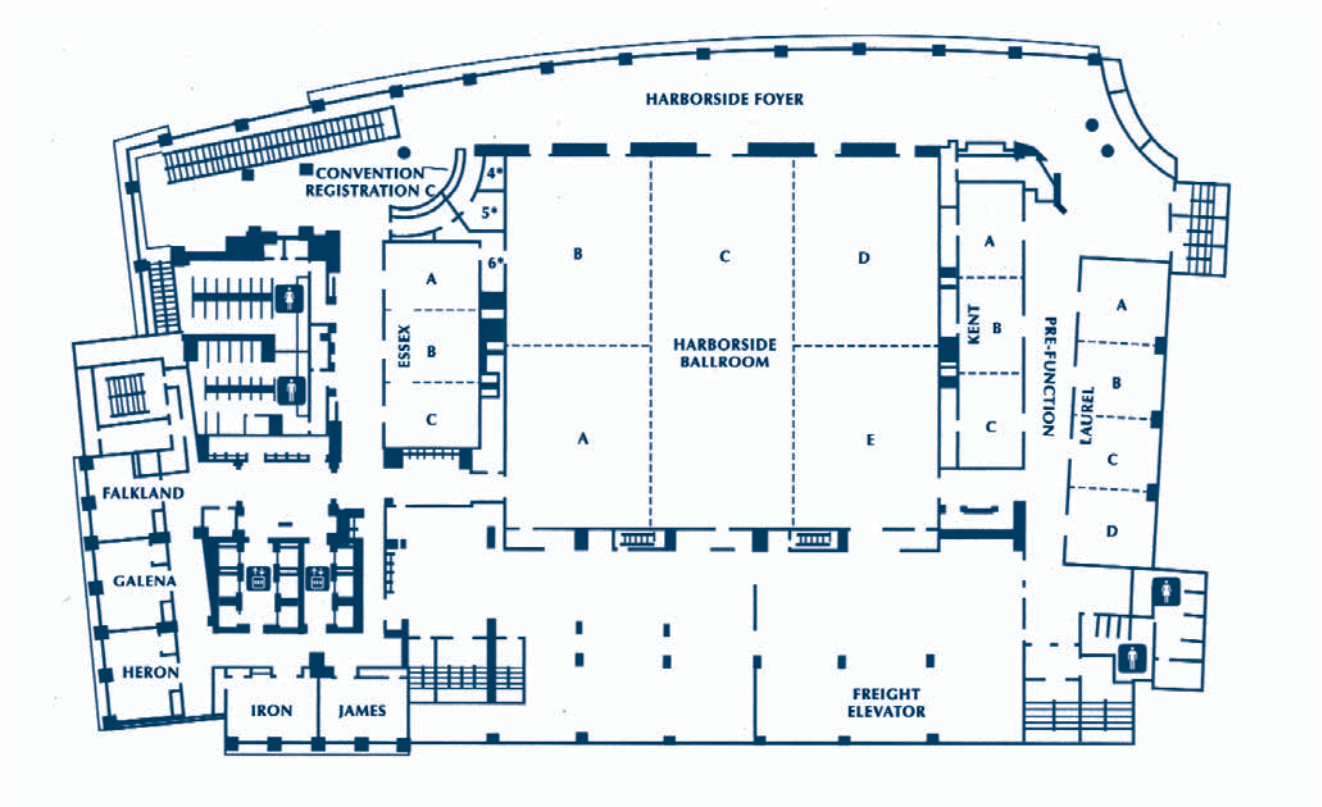
ABSTRACT BOOK

Translational cancer research...
innovation through teamwork

U.S. DEPARTMENT
OF HEALTH AND
HUMAN SERVICES

National Institutes
of Health

Harborside Ballroom



Poster Schedule

Harborside Ballroom

Sunday, July 8	Poster Set-up	11:30 a.m. - 2:00 p.m.
Monday, July 9	Poster Viewing	8:00 a.m. - 12 noon
		2:00 p.m. - 6:00 p.m.
	Poster Session and Meet the Author Buffet Dinner *(prepaid fee event)*	5:30 p.m. - 8:30 p.m.
Tuesday, July 10	Poster Viewing	8:00 a.m. - 6:00 p.m.
	Poster Take-down	8:30 p.m.

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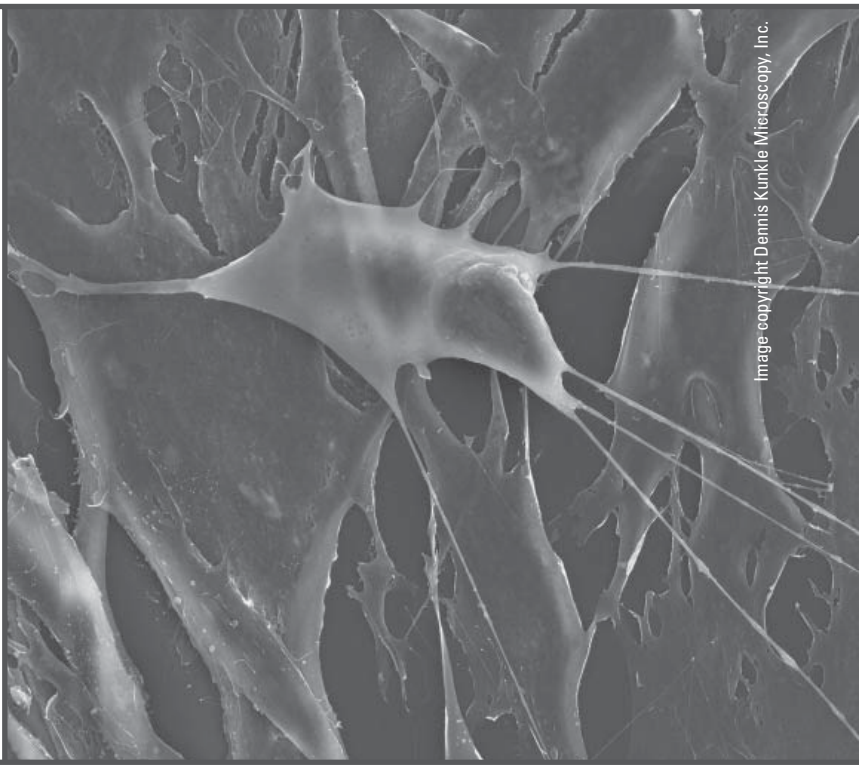

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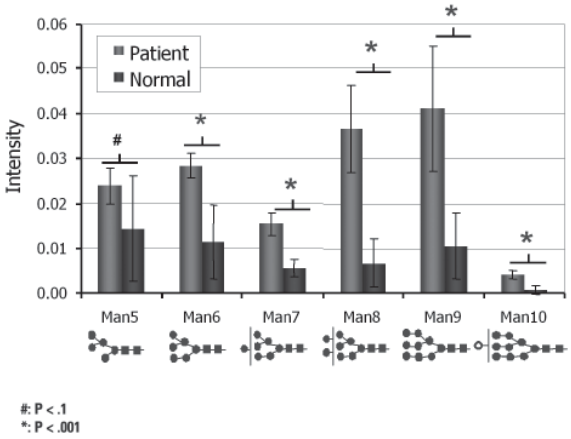
001 Serum Glycan Analysis in Metastatic Breast Cancer

Helen Chew[1], Suzanne Miyamoto[1], Hyunjoo An[2], David Rocke[3], Carlito Lebrilla[2]

[1]Division of Hematology/Oncology, [2]Department of Chemistry, [3]Department of Public Health Sciences, University of California, Davis, Davis, California

Background: There is a need for a reliable breast cancer biomarker that can predict a patient's response to therapy. Serum glycans, or oligosaccharides, are of particular interest as over half of all proteins are glycosylated and alterations in glycosylation influence growth, adhesion, metastasis, and immune surveillance of tumor, among other important functions. Serum glycans can be analyzed by high-resolution mass spectrometry.



Average of signal intensity of quantified mannose-type N-linked glycans from breast cancer patients and control group.

Methods: Sera from patients with known metastatic breast cancer and healthy controls without medical problems were prospectively analyzed by mass spectrometry. Patients older than 18 who were not pregnant or breastfeeding, and without other active cancers, were eligible. Sera were de-identified and analyzed by matrix-assisted laser desorption/ionization (MALDI) and Fourier transform ion-cyclotron resonance mass spectrometry (FT ICR MS). Human serum was treated with PNGase F to release N-linked oligosaccharides (NLO). Released glycans were purified and fractionated using solid-phase extraction and solvents of varying polarity to partition them into highly anionic and neutral components. The fractions were analyzed directly by MALDI-FT ICR MS. The exact compositions with regard to hexoses, N-acetylhexosamines, sialic acids, and fucoses were determined from the accurate masses. For mass quantification, maltohexose was added to SPE fraction prior to MS analysis and all observed peaks were normalized using the intensity of maltohexose.

Results: Sera from six patients with metastatic breast cancer and five controls were evaluated. The NLO were significantly increased in human sera with breast cancer compared to controls as shown in the figure. NLOs were further grouped on the basis of glycan types: high mannose, complex, and hybrid. High-mannose-type oligosaccharides are increased in breast cancer and may potentially be a useful biomarker.

Conclusion: Analysis of serum gylcans by mass spectrometry represents a new paradigm of cancer biomarker studies, focusing on posttranslational modifications of proteins, rather than protein expression. Prospective collection is ongoing and further refinement of this technology may be clinically useful in monitoring response to therapy in metastatic breast cancer.

Keywords: glycans; metastasis

002 Five-Year Stability Study of Free and Total Prostate-Specific Antigen Concentrations in Serum Specimens Collected and Stored at -70 ºC or Less

Dominick A. Scaramuzzino[1], Kelly Schulte[2], Bridgette N. Mack[1], Thomas F. Soriano[1], Herbert A. Fritsche[3]

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The stability of total (t) and free (f) prostate-specific antigen (PSA) in male serum specimens stored at -70 °C or lower temperature for 4.7 to 4.9 years was studied. Until now, the stability of these analytes in serum has not been evaluated systematically beyond 2 years of storage at -70 °C. Aliquots of frozen serum were thawed in 2001 and 2006 and assayed for tPSA and fPSA using a Dade Behring Dimension® RxL analyzer and reagents. TPSA values ranged from 0.07 to 69.94 and 0.00 to 69.83 ng/mL in 2001 and 2006, respectively, whereas fPSA values for the tested specimens ranged from 0.02 to 5.72 and 0.00 to 5.92, respectively. Deming regression analyses showed agreement in assay values over time as tPSA values yielded a slope of 1.0112 and a y-intercept of 0.0195; fPSA values produced a slope 1.0538 and a y-intercept of -0.0442; f/tPSA values yielded a slope of 0.9631

and a y-intercept of 0.1195. A Bland-Altman analysis of the data demonstrated analyte and ratio stability over this time period. We conclude that serum, when collected properly and stored at -70 °C or lower temperature, may be used for tPSA and fPSA clinical studies for at least 5 years after collection.

Keywords: PSA; serum; stability

003 Further Analysis of Serum-Based EPCA-2 as a Specific Prostate Cancer-Associated Biomarker

Eddy S. Leman[1], Grant W. Cannon[1], Bruce J. Trock[1], Lori J. Sokoll[1,2], Daniel W. Chan[1,2], Leslie Mangold[1], Alan W. Partin[1,2], Jordan Dimitrakov[3], Robert H. Getzenberg[1]

[1]Brady Urological Institute, [2]Department of Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland; [3]Urological Diseases Research Center, Children's Hospital and Harvard Medical School, Boston, Massachusetts

Introduction and Objective: Since its introduction in the 1980s, prostate-specific antigen (PSA) has been used for detection of prostate cancer. There is little doubt that PSA has changed the issue of prostate cancer. Today, few men have metastatic disease at time of presentation, largely because of the widespread acceptance and use of PSA as an early diagnostic tool. Despite this apparent success, there is still a need for novel markers for early detection of men with prostate cancer. Utilizing a proteomic approach that focuses only on the structural elements of the nucleus, EPCA-2 was identified as being expressed specifically in prostate cancer. The main objective of this study was to further delineate the ability of an assay to detect EPCA-2 in the serum, which can be utilized as a tool to specifically detect prostate cancer in patients with normal PSA levels, in addition to the more typical patients with elevated PSA levels.

Methods: Using an indirect ELISA, 189 additional serum samples were screened for EPCA-2 using assays constructed to detect an epitope of the protein: EPCA-2.22. These samples consisted of healthy men with PSA <2.5 ng/mL (n = 33), healthy men with PSA ≥2.5 ng/mL (n = 30), men with benign prostatic hyperplasia (BPH) (n = 35), men with chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) (n = 33), as well as men with prostate cancer whose PSAs are less than 2.5 ng/mL (n = 18) and greater than 2.5 ng/mL (n = 40).

Results: Using our previously established cutoff at 30 ng/mL for the immunoassay, we determined the sensitivity and specificity for EPCA-2. At this cutoff, EPCA-2 has a specificity of 94% and sensitivity of 91% in separating the healthy men, men with BPH and CP/CPPS, from those with prostate cancer, regardless of their PSA levels.

Conclusions: The serum ELISA assays produced for the EPCA-2.22 epitope have high specificity in separating normal men and those with BPH and CP/CPPS from two populations of men with prostate cancer (PSA < and ≥ 2.5 ng/mL). Adaptation of these assays to the clinical environment will require further validation and optimization.

Keywords: nuclear structure; prostate cancer; serum marker

004 Multimodality Molecular Imaging of Prostate Cancer

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Departments of [1]Radiology, [2]Urology, [3]Radio-oncology, and [4]Pathology, University of Michigan, Ann Arbor, Michigan

Since there is considerable uncertainty as to the "best" course of prostate cancer management, we currently explore several noninvasive functional imaging techniques regarding their ability to detect cancerous foci and to identify tumor characteristics that may predict subsequent biological behavior. Specifically, we assess whether imaging characteristics correlate with tumor aggressiveness or therapeutic response potential.

This reverse-translational, Phase I imaging trial includes pelvic 3T MRI, endorectal anatomic 1.5T MRI, contrast-enhanced (diffusion-weighted) functional MRI, and C11-choline and F18-FAZA PET/CT imaging. After in vivo imaging, patients undergo radical prostatectomy and ex vivo 3T MRI imaging of the prostatectomy specimen. Imaging results are verified by whole mount histopathology (including local Gleason score) as well as tissue microarray (TMA) analyses for carboanhydrase 9 and MIB-1 (assessing tumor hypoxia and proliferation). Image co-registration is performed using mutual information system (MIAMI fuse) software, which integrates all imaging modalities as well as whole-mount pathology into a three-dimensional voxel space for further analysis.

The table displays preliminary results from 14 tumor foci in 7 patients. The tumor volume, but not the local Gleason score, seems to be the most important parameter for tumor detection. The smallest tumor focus, which was identified by pelvic 3T MRI, had a volume of 0.7 mL. None of the three MRI modalities displayed a clear advantage in identifying tumor foci, indicating that a combination of MRI methodologies might be needed for optimal tumor visualization. Two tumor foci (ranging between 1.4 and 6.8 mL in volume) as well as a pelvic lymph node metastasis were positive on C11-choline, while none of the tumor foci showed increased F18-FAZA uptake. Subsequent TMA analyses will determine whether these molecular PET imaging markers correlate with histochemical markers of proliferation and hypoxia. To date, the lack of enhanced F18-FAZA uptake suggests that tumor hypoxia in primary prostate cancer may not be as prevalent as suggested by previous animal studies.

Pat. No.	Lesion No.	Volume (mL)	Gleason	endo MRI	pelvic MRI	fMRI	Choline	FAZA
1	1	2.85	3+4	j	n	n	n	n
1	2	4.97	3+3	j	n	n	n	n
2	1	8.78	3+4	n	n	j	n	n
2	2	4.48	3+3	n	n	j	n	n
2	3	0.03	3+3	n	n	n	n	n
3	1	0.45	4+3	n	n	n	n	n
3	2	0.01	3+3	n	n	n	n	n
4	1	7.78	4+4	n	j	j	n	n
5	1	6.80	4+4	j	j	j	j	n
6	1	1.36	3+4	n	j	n	j	n
6	2	0.02	3+3	n	n	n	n	n
6	3	0.20	3+3	n	n	n	n	n
7	1	0.72	4+3	n	j	n	n	n

Keywords: molecular imaging; MRI; PET/CT

005 Past, Present, and Future of the Data Acquisition and Management of the M.D. Anderson Head and Neck Tissue Bank

J. Jack Lee[1], Waree Rinsurongkawong[1], Michelle D. William[2], Cynthia Anderson[2], Joie C. Haviland[2], Scott M. Lippman[3], Gary L Clayman[4], Adel K El-Naggar[2]

Departments of [1]Biostatistics, [2]Pathology, [3]Thoracic/Head and Neck Medical Oncology, and [4]Head and Neck Surgery, University of Texas M.D. Anderson Cancer Center, Houston, Texas

Since 1990, a systematic effort has been devoted to acquiring and storing the head and neck tissue specimens at M.D. Anderson Cancer Center for research. Excel spreadsheets and an Access database were created originally for data acquisition. Since being awarded the Head and Neck SPORE in 2002, we have mounted a significant effort in revamping the database infrastructure of the Head and Neck (HN) Tissue Bank. The HN Tissue Bank serves as the central repository for all head/neck cancer-related tissues and fluid samples collected from the Pathology Core of the M.D. Anderson HN SPORE. A comprehensive Web-based database management system was built to facilitate the data entry, tracking, distribution, query, and reporting of all HN tissues. As of May 2007, the database stores over 330,000 tissue samples from more than 28,000 patients, including the following: fresh, 19,951 grids; paraffin, 238,961 blocks; slides, 64,044 slides; and fluid samples, 7,324 grids of NPBL, saliva, serum, sputum, buccal brushing and smear, bronchial washing, and pleural fluid.

From the technical aspect, we have created and posted the first common data element (CDE) for the head and neck site in http://cdebrowser.nci.nih.gov/CDEBrowser. The CDE was developed by adopting standards that are derived from other standards (HL7, ISO, USPS, UPU, W3C, etc.).

The technical specifications of the HN Tissue Bank include an encrypted SSL (secure sockets layer), enabled ASP.Net Web application using VB.Net with a SQL Server database backend. The reports are created with SQL Server 2000 Reporting Services and Crystal Reports. The major components of the database are (1) CDEs, (2) data dictionary, (3) reports, (4) patient visits module, (5) histopathology information module, (6) query tool, (7) help tool, (8) sample tracking and distribution, and (9) Excel file import and export.

Efforts are also ongoing in comparing and integrating the M.D. Anderson HN Tissue Bank with the cancer Biomedical Informatics Grid (caBIG) Tissue Banks and Pathology Tools to facilitate information sharing between the SPORE researchers and the cancer research community.

Keywords: CDE; database; Web-based

006 Urine Cytology for Prostate Cancer Detection: Immunocytostains of Urine Sediment in the Era of New Prostate-Specific Biomarkers

Please see Poster Discussion Session New Technologies and Methodologies I.

007 Biomarker Analysis of a Prospective Multicenter Ovarian Cancer Early Detection Study in Women at Increased Risk

S.J. Skates[1], C.W. Drescher[2], C. Isaacs[3], J.M. Schildkraut[4], D.K. Armstrong[5], S.S. Buys[6], W.R. Brewster[7], M.B. Daly[8], D.M. Finkelstein[9], K.H. Lu[10], ROCA Screening Study Group

[1]Dana-Farber/Harvard Cancer Center, Boston, Massachusetts; [2]Fred Hutchinson Cancer Research Center, Seattle, Washington; [3]Lombardi Cancer Center, Georgetown University Hospital, Washington, D.C.; [4]Duke Comprehensive Cancer Center, Durham, North Carolina; [5]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland; [6]Huntsman Cancer Institute, Salt Lake City, Utah; [7]Chao Family Comprehensive Cancer Center, University of California, Irvine Medical Center, Irvine, California; [8]Fox Chase Cancer Center, Philadelphia, Pennsylvania; [9]Biostatistics Center, Massachusetts General Hospital, Boston, Massachusetts; [10]University of Texas M.D. Anderson Cancer Center, Houston, Texas

Background: No proven ovarian cancer (OC) screening strategy exists for women who are at increased risk for the disease. A risk of ovarian cancer algorithm (ROCA) using serial CA125 values has previously shown greater positive predictive value (PPV) and sensitivity than a single CA125 in screening women at general population risk. We hypothesized that using ROCA would yield a reasonable PPV for ovarian cancer screening in a cohort at increased risk. In addition, the study would provide a biorepository on which to evaluate additional biomarkers.

Methods: Between 7/2001 and 9/2006, 25 sites (14 Cancer Genetics Network, 3 ovarian SPOREs, 1 EDRN, 7 others) prospectively enrolled patients. Inclusion criteria included the following: among self, 1° or 2° relatives in same lineage either (i) BRCA1/2 mutation, (ii) two of OC or early onset (age ≤50) breast cancer (BC), or (iii) Ashkenazi ethnicity and 1 of OC or BC. A previous diagnosis of OC excluded subjects. Subjects underwent CA125 every 3 months and the risk of having ovarian cancer based on the CA125 profile was recalculated after each test. ROCA referred subjects with risk >1% to ultrasound (US), and risk >10% additionally to a gynecologic oncologist. Objectives included PPV for study-indicated surgery, sensitivity, and compliance. Sample size was chosen to observe eight OC endpoints with a power of 80% to rule out PPV ≤10% if the true PPV = 20%. Two additional biomarkers, HE4 and OV110, were evaluated on cases and matched controls.

Results: A total of 2,343 high-risk women were enrolled, with 6,284 woman-years of screening and 19,549 CA125s obtained. There were 628 (10%/year) referrals to US with 414 US performed. 9 OCs were identified during screening, 3 were prevalent (1 early, 2 late stage), and 6 were incident (5/6 = 83% early, 1 late). HE4 and OV110 were evaluated in 6 cases and 6 controls. All controls showed a flat profile for HE4 and OV110, as with CA125, while 4/6 cases showed rising or high levels of HE4 and/or OV110.

Conclusions: Frequent CA125 testing using ROCA results in an acceptable approach to screening women at increased risk for OC. HE4 and OV110 show promise as additional biomarkers for OC and warrant further investigation. A definitive screening study (≥30 incident cases) using ROCA with serial CA125 and additional biomarkers such as HE4 and OV110 is required to define sensitivity for early-stage OC.

Keywords: high risk; longitudinal assessment; screening

008 Genomewide Expression Analysis of Freshly Prepared Formalin-Fixed Paraffin-Embedded Human Prostate Tissues

Thomas Dunn, Helen Fedor, William Isaacs, Angelo DeMarzo, Jun Luo

Departments of Urology and Pathology, Johns Hopkins University, Baltimore, Maryland

Formalin fixation and paraffin embedding (FFPE) is a standard practice for processing surgical specimens in preparation for pathological evaluation. The FFPE procedure introduces chemical modification and cross-linking of biomolecules in the cells, compromising the yield and quality of RNA extracted from such tissues and the ensuing expression microarray analysis. Although multiple studies have demonstrated the feasibility of obtaining reliable expression data from FFPE specimens, it has been a consistent conclusion that global gene expression data derived from FFPE specimens suffered from poor quality and low detection sensitivity, particularly in aged FFPE specimens. In addition, there was no reported study designed to address parallel comparison of FFPE versus frozen specimens by incorporating multiple intermediate quality control measures and array data endpoints. In this study, we designed a series of microarray experiments to determine whether the FFPE procedure compromises genomewide expression analysis, by comparison of freshly prepared FFPE and frozen specimens processed in parallel from the same original specimen. Starting from thin sections of the respective specimens representing prostate cancer and benign prostate tissues, we compared differences in total RNA yield, RNA quality, amplified RNA (aRNA) yield, aRNA quality, labeling efficiency, and the quality and reliability of the whole genome array data, between the two tissue-processing methods (i.e., FFPE vs. frozen). We conclude that the FFPE procedure will not lead to adverse effects on genomewide expression analysis, provided that the specimens are freshly prepared. Therefore, pathological lesions that depend on the FFPE procedure for identification, such as the putative prostate cancer precursor lesions, can be effectively analyzed at the whole genome level, and prospective molecular archiving is possible without compromising standard surgical pathology routines.

Keywords: FFPE; gene expression; prostate

009 Noninvasive Imaging Metrics To Profile Treatment Response in Models of Colorectal Cancer

Please see Poster Discussion Session New Technologies and Methodologies II.

010 Quantitative Analysis of the SPORE Melanoma Progression Array With Ki-67 and HMB-45

David L. Rimm[1], Christopher B. Moeder[1], Bonnie E. Gould Rothberg[2], Harriet Kluger[3], Ruth Halaban[4], David E. Elder[5], George F. Murphy[6], Alexander Lazar[7], Victor Prieto[7], Lyn McDivitt Duncan[8]

Departments of [1]Pathology, [2]Epidemiology and Public Health, [3]Oncology, and [4]Dermatology, Yale University School of Medicine, New Haven, Connecticut; [5]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; [6]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; [7]Departments of Pathology and Dermatology, University of Texas M.D. Anderson Cancer Center, Houston, Texas; [8]Dermatopathology Unit, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts

Background: The progression from a proliferative melanocytic lesion to metastatic melanoma is a complex process that is not completely understood at either the morphologic or molecular level. Toward a goal of understanding the roles of specific proteins in the progression process, a joint group SPORE in Skin Cancer investigators created a multi-institutional tissue microarray with multiple examples of lesions from each category from each institution. Here we report the first systematic quantitative assessment of this array with Ki-67 and HMB-45.

Methods: The SPORE progression array was constructed by the Harvard TMA facility with melanocytic lesions from Harvard, University of Pennsylvania, and M.D. Anderson Cancer Center. It contains 480 0.6 mm cores including 35 nevi represented in 130 cores (superficial, mid, and deep areas of each when available), 60 primary melanomas represented in 200 cores (radial and vertical growth phases of each, when available), and 75 metastases represented in 150 cores. The cases were stained with Ki-67 (BD Pharmingen, mouse monoclonal antibody 1:500) and HMB-45 (BioGenex, a mouse monoclonal antibody to pmel17/gp100, prediluted neat) and quantified using the AQUA technology for quantitative assessment of immunofluorescence. Data were analyzed using Statview, JMP5, and SAS software.

Results: The mean expression levels for Ki-67 showed progression that directly correlated with the morphologic selection, where the lowest mean AQUA scores were seen in thin nevi, followed by thick nevi, thin primaries, thick primaries, lymph node, and visceral metastases. The progression was significant for trend ($p<.0001$) and the malignant cases (excluding thin primaries)

were significantly higher than the nevi (p<.05 adjusted for multiple comparison). Overall HMB-45 antibody staining for pmel17/gp100 showed no significant differences between benign and malignant, nor was any significant difference seen between superficial, intermediate, and deep levels of thick lesions. However, an interesting subcellular distribution was observed. Nearly all nevi had low-level (but significantly above baseline) nuclear staining, while most malignant cases showed exclusively cytoplasmic staining. A ratio was constructed of nuclear over non-nuclear HMB-45, which showed a highly significant difference between nevi (thin and thick) compared to all malignant specimens (ANOVA p<.0001). A ROC curve to assess the value of this finding in distinction of benign from malignant showed an area under the curve of 0.82. A small Yale-based progression array with 20 cases each of nevi, primary melanomas, and metastases confirms this finding.

Conclusions: AQUA-based analysis of the SPORE progression array showed progressive increases in Ki-67 expression corresponding to morphologic stage. HMB-45 analysis revealed low-level nuclear expression in nevi that may have been previously missed on chromogenic stains due to hematoxylin counterstaining. It is also possible that we are detecting a protein other than pmel17/gp100 due to uncharacterized cross-reactivity of the HMB-45 antibody. However, the ratio of nuclear over non-nuclear expression may be valuable in distinguishing benign nevi from malignant melanoma.

Keywords: immunofluorescence; immunohistochemistry; tissue microarray

011 MicroRNAs as Biomarkers in Human Solid Tissues, Blood, and Other Biofluids

Jon Kemppainen[1], Jeffrey Shelton[1], Kevin Kelnar[1], Stephanie Volz[1], Heidi Peltier[1], Anna Szafranska[1], Dmitriy Ovcharenko[1], Bernard Andruss[1], Thomas Illmer[2], Manu Labourier[1], Gary Latham[1], David Brown[1]

[1]Asuragen, Inc., Austin, Texas; [2]Medical Clinic and Policlinic I, University Hospital of the Technical University, Dresden, Germany

The expression of specific microRNAs (miRNAs) can be used to classify cancer patients on the basis of prognosis, cancer type, and genetic abnormality. The diagnostic potential of miRNAs will ultimately depend on their characteristics in readily available biofluids such as serum and plasma and the analytical sensitivity, specificity, and accuracy of detection methods that can be used in diagnostic laboratories. We and others have demonstrated that miRNA expression profiles correlate with disease state and prognosis. We have developed a highly sensitive, quantitative method for miRNA expression analysis and have used it to evaluate miRNAs in a variety of biofluids, as well as frozen and formaldehyde-fixed tissues. Interestingly, miRNAs are abundant in serum, plasma, saliva, and urine, and exhibit surprising stability and donor-to-donor consistency. The ability to detect miRNAs in samples suitable for diagnostic testing supports their potential importance as diagnostic analytes for human cancer and other diseases.

Keywords: gene expression; microRNA; plasma

012 MicroRNA Expression Analysis in Formalin-Fixed Paraffin-Embedded Tissues

Anna Szafranska[1], Martina Doleshal[1], Bhavna Choudhury[1], Timothy Davison[1], Bernard Andruss[1], C. Morisson[2], Scott Jewell[2], Emmanuel Labourier[1]

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For decades, routine clinical practice has resulted in preservation of resected biopsy and autopsy specimens, creating valuable archived tissue collections associated with patient outcome data that include disease progression, therapeutic response, toxicity, and other important information. These collections thus offer enormous potential for discovery and analysis of nucleic acid biomarkers with potential diagnostic and therapeutic value. However, widely used preservation methods, such as formalin-fixed paraffin-embedded (FFPE) preservation, are very harsh on the RNA in a sample. The recovery of quality mRNA from FFPE tissues is challenging because of the cross-linking between nucleic acids and proteins and covalent modifications of RNA that occur during the fixation process. Over the past few years, the involvement of microRNAs (miRNAs) in human diseases, including several cancer types, has become increasingly clear. Expression of specific miRNAs is differentially altered across tumor types, which suggests a direct involvement of miRNAs in oncogenesis. In this study, we established an appropriate procedure for isolation of RNA, including small RNA fraction, from FFPE tissues and evaluated the suitability of miRNAs for molecular characterization of archived human specimens. To unlock the potential of FFPE tissue archives for miRNA analysis, we have evaluated various FFPE RNA isolation products to determine the most appropriate method for recovery of small RNA and mRNA species. We incorporated an internal process control (Armored RNA Quant), which enables monitoring the success of isolation.

This custom procedure allows extraction of high yields of RNA from various archived fixed tissues and offers recovery of miRNA and mRNA species most similar to frozen tissues. Using single-color array analysis of miRNA expression, we compared the miRNA expression profiles of fixed and frozen specimens from human myometrium, B cell lymphoma, and colon tissues. The same miRNAs were found differentially expressed between two tissue types whether using FFPE or frozen samples. Furthermore, we demonstrate the potential for analysis from archives that have been in storage up to 11 years. MicroRNA expression profiling is suitable for retrospective molecular analysis and characterization of archived clinical samples provided that appropriate RNA isolation, quality controls, and microarray methodologies are used.

Keywords: expression profiles; formaldehyde-fixed tissue; microRNA

013 High-Throughput DNA Methylation Profiling Using Promoter Microarrays

Noriyuki Omura, Seung-Mo Hong, Chung-Pin Li, Kimberly Walter, Ang Li, Michael Goggins

Johns Hopkins University, Baltimore, Maryland

Background: Pancreatic cancer is a genetic and epigenetic disease characterized by widespread and profound alterations in DNA methylation. Effective high-throughput genomewide methods for identifying DNA methylation patterns are needed to accelerate the discovery of abnormal DNA methylation patterns. We have developed a novel microarray-based method for detecting differential methylation patterns based on a methylated CpG island amplification (MCA) strategy using SmaI methylation-sensitive restriction enzyme. Sequentially MCA fractions are interrogated on high-resolution promoter microarrays with competitive hybridization.

Methods: Replicate DNA was extracted from the pancreatic cancer cell line Panc-1 and from HPDE, a normal pancreatic epithelial line. Amplicons from Panc-1 and HPDE were hybridized to Agilent human 44K promoter array to profile differential methylation. Candidate methylated genes identified by microarray were further evaluated using conventional bisulfite-modified sequencing (BMS).

Results: After normalization and analysis with chip analytics, we identified 589 probes with a significant higher signal in the pancreatic cancer line relative to the normal line (p-value <.001 in Whitehead Error Model v1.0). A further 493 probes were hypomethylated in Panc-1 relative to HPDE. Replicate analysis of array data showed high correlation value (R^2 = 0.92-0.93). We then performed a similar analysis of four pancreatic cancer cell lines vs. four normal pancreas samples using the Agilent 244K CpG island array. Using a cutoff of >fourfold increased signal, at least one probe methylation was detected in 3,946 genes, and 318 genes were detected as methylated in all four pancreatic cancer cell lines. Of 22 novel genes predicted to be methylated at the CpGs by microarray data, 21 were methylated by BMS.

Conclusion: We describe a DNA restriction method using Agilent CpG island arrays that provides accurate genomewide profiling of DNA methylation in human specimens revealing abundant DNA methylation changes in pancreatic cancer samples.

Keywords: DNA methylation; early detection; pancreatic cancer

014 DNA Hypomethylation in Primary and Metastatic Prostate Cancer

Srinivasan Yegnasubramanian, Michael Haffner, Yonggang Zhang, Bora Guerel, Robert O'Meally, Zhijin Wu, Jeanne Kowalski, Amanda Blackford, G. Steven Bova, William B. Isaacs, Angelo De Marzo, William Nelson

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Hypomethylation of CpG dinucleotides in genomic DNA was one of the first somatic epigenetic alterations discovered in human cancers. It has been postulated that DNA hypomethylation occurs very early in almost all human cancers and that perhaps this epigenetic change facilitates genetic instability and even reactive DNA CpG island hypermethylation changes in cancers. We therefore examined the nature, extent, and timing of DNA hypomethylation changes in human prostate cancer. We hypothesized that DNA hypomethylation can be manifest in at least three ways: (1) reduction in the overall 5-methyl-2′-deoxycytidine (5mC) content in the genome, reflecting reductions of 5mC in sparsely distributed CpG dinucleotides in intragenic and intergenic regions, (2) reduction in the degree of methylation of repetitive elements, such as LINE1 elements, in the genome, and (3) hypomethylation of normally methylated and repressed gene promoter CpG island sequences leading to overexpression of the associated genes. Contrary to the canonical view that DNA hypomethylation changes occur extremely early in all human cancers, we show that reductions in 5mC content in the genome occur very late in prostate cancer progression, appearing at a significant

extent only at the stage of metastatic disease. Furthermore, we found that while some degree of hypomethylation of repetitive elements does occur in primary prostate cancers compared to normal tissues, this hypomethylation of repetitive elements is significantly more pronounced in metastatic prostate cancer. Next, we carried out a tiered, gene-expression microarray-based approach to identify novel genes that are normally repressed by CpG island methylation that become overexpressed in prostate cancer as a result of CpG island hypomethylation. Through this analysis, we show that a class of so-called cancer testis antigen genes undergoes CpG island hypomethylation and overexpression in primary prostate cancers to a small extent, and in metastatic prostate cancers to a significantly larger extent. Finally, we show that unlike CpG island hypermethylation changes in prostate cancers, which appear to be strictly maintained across different metastatic sites within the same patient, DNA hypomethylation patterns are quite heterogeneous across different metastatic sites within the same patients. Taken together, these findings provide preliminary evidence that DNA hypomethylation changes occur late in prostate carcinogenesis, perhaps occurring sporadically and heterogeneously after any major clonal selection steps.

Keywords: DNA methylation; epigenetics; metastasis

015 Use of Vibroacoustography for Imaging Prostate Tissue Structure and Identifying Prostate Lesions

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Vibroacoustography (VA) is an imaging modality that uses the radiation force of ultrasound to vibrate the tissue at a low frequency and records the resulting acoustic field to produce an image related to the stiffness of the tissue. This method provides additional information not available from ultrasound. VA is sensitive to object properties at ultrasound frequencies, as well as audio (low) frequencies. The purpose of this study is to explore the potential of VA in prostate imaging. Tissue stiffness is an important marker of malignancy in prostate and is often used in digital rectal examination to identify prostate cancer. However, deep or small lesions are not accessible to the examining finger. The ultrasound beam of VA can penetrate deep into prostate. In addition, spatial resolution of VA is much better than a palpating finger. In this study, VA imaging and radiography were performed on 12 prostatectomy specimens fixed in formaldehyde. VA images at different depths show normal prostate tissue structure and borders, the urethra, calcification, and lesions. In addition, VA images of the prostates exhibit significant contrast between the central and peripheral zones. The results indicate that, with further development, VA may become a new class of imaging tool for prostate imaging.

This work was supported by National Institutes of Health grant CA91956.

Disclosure: Parts of the techniques presented in the abstract are patented by M. Fatemi and the Mayo Clinic.

Keywords: imaging; ultrasound; vibroacoustography

016 Distinct Classes of Chromosomal Rearrangements Create Oncogenic ETS Gene Fusions in Prostate Cancer

Please see Poster Discussion Session Biomarkers II.

017 Getting Under the Skin of CTCL: Isolation and Study of Malignant T Cells From CTCL Skin Lesions

Rachael Clark, Manoj Muthukuru, Jeffrey Shackelton, Jenny Kim, David Jones, Helen Kuo, Thomas Kupper

In the early stages of cutaneous T cell lymphoma (CTCL), malignant T cells are confined to skin and are difficult to isolate and discriminate from benign infiltrating T cells. We isolated T cells from skin lesions of CTCL patients using short-term explant cultures and found that CTCL skin lesions from all stages of disease contained a unique population of T cells with higher forward and side scatter.

These high-scatter T cells were not seen in either normal skin or skin lesions of psoriasis, contact dermatitis, or atopic dermatitis, suggesting that they do not simply represent antigen-activated T cells. High-scatter T cells were all CD4+ and expressed the skin homing addressins CLA and CCR4 and the activation antigens CD69 and CD25. In later stages of disease, this high-scatter population was clonal. In contrast, the lower scatter population comprised a diverse population of CD8 and CD4 T cells similar to T cells found in normal and inflamed skin. The high-scatter T cell population was also evident in the blood in later stages of

CTCL with known blood involvement, but was absent in earlier stages of disease. In late-stage patients, where malignant T cells could be identified by their particular TCR Vβ expression, the high-scatter T cell population from both skin and blood was clonal and malignant. This ability to discriminate malignant from benign T cells in CTCL makes it now possible to study in detail the malignant T cells in this disorder. Next, we examined the gene expression of T cells isolated from CTCL skin lesions and compared it to T cells isolated from normal human skin. We found that CTCL lesional T cells had upregulation of anti-apoptosis genes (HSPA5, TNFRSF1B, and TGFBI) and downregulation of pro-apoptosis genes (IER-1, DF15, and DCC). Additionally, we found upregulation of LCK and ZAP-70, genes in the TCR signaling pathway, suggesting that proliferation of malignant CTCL T cells may be driven through TCR signaling. To confirm these results, we conducted functional and phenotypic studies of T cells from CTCL skin lesions. T cells from CTCL skin lesions were more resistant to activation-induced cell death in both early- and late-stage disease. T cells from CTCL skin lesions, especially the high-scatter population, expressed higher levels of anti-apoptotic TNFR-II and failed to upregulate the pro-apoptotic TNFR-I after treatment with FAS-ligand and TNF-alpha. Lastly, the high-scatter T cell population expressed lower baseline and induced levels of FAS-ligand. Defective apoptosis has been demonstrated in malignant CTCL T cells isolated from the blood of patients with Sezary syndrome, but this is the first direct demonstration that defective apoptosis is a feature of even early-stage CTCL. In summary, we have directly studied the phenotype and gene expression of T cells isolated from CTCL skin lesions. We have found that CTCL skin lesions contain an identifiable population of malignant T cells that are chronically activated and resistant to apoptosis. Further study of these cells may lead to insights into the pathogenesis of CTCL.

Keywords: CTCL; lymphoma; T cell

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

018 Molecular Etiology of Breast Cancer: Biomarkers of Risk and Prevention

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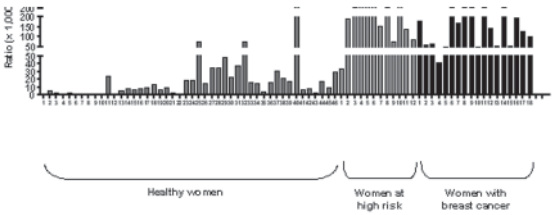
The estrogens estradiol (E_2) and estrone (E_1) can be metabolized to catechol estrogens, which in turn can be oxidized to catechol estrogen quinones, in particular, estrogen-3,4-quinones [$E_1(E_2)$-3,4-Q]. These quinones can react with DNA to form the depurinating 4-OH$E_1(E_2)$-1-N3Ade and 4-OH$E_1(E_2)$-1-N7Gua adducts. The apurinic sites that are generated by their depurination can induce mutations leading to breast cancer initiation. When this occurs in humans, the depurinating N3Ade and N7Gua adducts formed are shed from the tissue of origin and excreted in urine. Thus, purification, identification, and quantification of the estrogen metabolites, estrogen conjugates, and depurinating estrogen-DNA adducts in urine could provide biomarkers of risk for developing breast cancer. Furthermore, the same biomarkers could be used to evaluate the efficacy of possible agents for preventing tumor initiation. To gain evidence for this hypothesis, the estrogen metabolites, estrogen conjugates, and depurinating estrogen-DNA adducts in 2-mL triplicate aliquots of urine from 46 healthy control women, 12 women at high risk for breast cancer (Gail model score > 1.66%), and 18 women with breast cancer were partially purified by solid-phase extraction and analyzed by ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS).

The levels of the ratios of depurinating estrogen-DNA adducts to their respective estrogen metabolites and conjugates were significantly higher in high-risk women ($p<.001$) and women with breast cancer ($p<.001$) than in control subjects. The high-risk and breast cancer groups were not significantly different ($p = 0.62$). After adjusting for subject characteristics, the ratios were still significantly associated with health status. The observation of high ratios of depurinating estrogen-DNA adducts to their corresponding metabolites and conjugates in urine samples from both high-risk women and women with breast cancer supports the hypothesis that formation of estrogen-DNA adducts is the first critical step in the initiation of breast cancer (see reference). Furthermore, these results suggest that this assay may provide a diagnostic tool for early detection of breast cancer risk. In addition, we can hypothesize that the ratio of depurinating estrogen-DNA adducts to their metabolites and conjugates can be used to monitor the efficacy of putative preventing compounds in minimizing formation of catechol estrogen quinones and/or their reaction with DNA, which should reduce the risk of initiating and developing breast cancer. Thus, the depurinating estrogen-DNA adducts are possible biomarkers for early detection of breast cancer risk and response to preventing treatment.



Depurinating estrogen-DNA adducts in the urine of healthy women, high-risk women, and women with breast cancer.

This research was supported by Center of Excellence grant DAMD-17-03-1-0229 and NCI-Avon grant P30-CA 03672722-S2.

Reference

Cavalieri et al. *BBA-Reviews in Cancer* 1766:63, 2006.

Keywords: biomarkers; estrogens; etiology

019 Detection of Bladder Cancer by Proteomics of Urine

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Background: Application of new technologies for detection of cancer could have an important effect on patient survival and overall quality of life. This is particularly urgent in reference to common carcinomas such as those that arise in the bladder and affect a substantial proportion of the population, having a major impact on public health.

Design: We used protein matrix-assisted laser desorption and ionization time of flight (MALDI-TOF) and surface-enhanced laser desorption and ionization time of flight (SELDI-TOF) to study protein profiles in progression of bladder neoplasia from in situ conditions to invasive disease and to detect bladder cancer in urine. We initially analyzed proteomic profiles of bladder cancer development from in situ neoplasia in 18 samples to tumor and adjacent urothelium. The baseline profiles were obtained from urothelial cells prepared from the urethers of nephrectomies. Then we searched for abnormally expressed proteins in voided urine sediments of 94 patients with bladder cancer and validated the findings in a blinded set of 160 voided urine samples from patients with bladder cancer and unaffected controls. Finally we searched for abnormally expressed proteins in voided urine samples of 168 patients with history of bladder cancer and related the findings to pathological, clinical, and followup data. The baseline profiles for urine samples were obtained from 65 age-matched healthy individuals.

Results: Data were preprocessed using Matlab scripts to (a) remove noise, (b) subtract baseline, and (c) identify peak locations. Using the intensities of peaks, we classified urine samples in the training set by defining average "normal" and "tumor" profiles, and computing correlations between samples. Based on their correlation scores, samples were classed as "normal," "tumor," or "ambiguous." Leave-one-out cross-validation was used to define the boundary values for dividing regions in the training set and were subsequently used to classify the samples from the blinded set to assess sensitivity and specificity. Algorithms allowed us to identify cancer and normal samples with specificity 78% and sensitivity 100% with the positive predictive value 81% and negative predictive value 100% for a blinded set.

Conclusions: Protein profiling of voided urine sediments is a promising noninvasive detection tool for bladder cancer.

Keywords: bladder cancer; proteomic profile; statistical analysis

020 Multislice High Spectral and Spatial Resolution Magnetic Resonance Imaging of Entire Human Breast

Please see Poster Discussion Session New Technologies and Methodologies I.

021 Long-Term Effects of Limited p120 Ablation in the Mouse Intestine

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We have established a novel mouse model for determining the long-term effects of p120-ablation in the small intestine and colon. In our first-generation model, p120 ablation was induced by crossing p120 floxed mice to villin-Cre transgenic mice provided by D. Gumucio. A mosaic p120 knockout was observed in approximately half of epithelial cells from the stomach to the anus, with no other tissues affected. The damage, however, was severe, and animals bled to death by 3 weeks of age from widespread epthelial shedding and inflammation. To examine long-term effects of p120 ablation, we have crossed our animals to a new villin-Cre-ER^{T2} transgenic mouse from S. Robine's laboratory, and established tamoxifen dosing conditions to limit p120 loss to less than 10% of epithelial cells in the intestine and colon. Interestingly, morphologic and adhesive defects are similar to those characterized previously but are limited to fields of cells derived from individual p120-null stem cells. E-cadherin is significantly downregulated in p120-null regions, and cell morphology and adhesion are perturbed, but animals do not suffer the early health problems associated with the previous model. By 2 months after tamoxifen injection, however, all animals develop numerous cystic crypts in the small intestine. By 4 months, cystic crypts are visible in the colon as well. This new model will allow long-term analysis of the consequences of p120 loss by itself (e.g., fate of cystic aberrations) and in the presence of additional alterations that might complement p120 ablation.

Keywords: adhesion; colon; p120

022 Overexpression of IGF-II and Activation of Akt in the Endometrium of Neonatal DES-Exposed Rodents

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Neonatal exposure of the rodent to the xenoestrogen diethylstilbestrol (DES) imparts a permanent hormonal imprint on the developing reproductive tract, causing an increase in expression of estrogen-responsive genes and an increased incidence of endometrial hyperplasia (in 58% of rats) and uterine adenocarcinoma. We previously reported that endometrial complex hyperplasia with atypia in postmenopausal women is linked to overexpression of the IGF-I receptor, loss of the negative regulator of the IGF-I pathway (PTEN), and subsequent activation of Akt (see reference). We therefore compared the level of expression of components of the IGF-I signaling pathway (IGF-I, IGF-II, IGFBP3, IGFBP5, IGF-IR, IGF-2R, and IRS-1) as candidate genes involved in endometrial hyperplasia from rodents exposed neonatally to DES compared to vehicle-treated controls in the proestrus/estrus phase. Neonatal DES exposure results in compromised ovarian function; however, developmental exposure to this xenoestrogen resulted in persistent expression of estrogen-responsive genes in adult animals despite their hypoestrogenic milieu. The expression of estrogen-responsive genes IGF-I, and IGFBP3 was consistently high in the endometrium of rats exposed neonatally to DES as compared to proestrus/estrus endometrium of vehicle controls, indicating that these genes have been reprogrammed to be hyper-responsive to estrogen. Additionally, the estrogen-responsive gene IRS-1, a major substrate and mediator of insulin and IGF-I receptor signaling, was overexpressed in the endometrium of rats exposed neonatally to DES as compared to the proestrus/estrus endometrium of vehicle controls. We also evaluated the expression and activation of the downstream signaling component of the IGF-I pathway, Akt. Akt expression and phosphorylation were significantly increased in endometrium of rats exposed neonatally to DES as compared to the endometrium of vehicle controls. Consistent with activation of IGF signaling, there was a significant increase in KI-67 positive cells in endometrium of rats exposed neonatally to DES compared to vehicle-treated controls, indicating that this pathway was promoting cell proliferation. These results suggest that neonatal DES exposure results in developmental reprogramming of components of the IGF signaling pathway in the rodent endometrium. However, activation of this signaling pathway was present in both "normal" endometrium of rats exposed neonatally to DES and the hyperplasias that occurred in these animals, suggesting that additional molecular alterations are required to progress to endometrial hyperplasia in this model system. Our studies indicate that IGF signaling and activation of Akt play a central role in both human endometrial complex hyperplasia with atypia and the rodent model of endometrial hyperplasia induced by neonatal DES exposure.

Reference

McCampbell et al. *Clin Cancer Res*, 2006.

Keywords: Akt; endometrium; IGF-II

023 A Compromised Nuclear Envelope Is Causal for Nuclear Deformation and Aneuploidy in Ovarian Cancer

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Aberrant nuclear shape and polyploidy/aneuploidy are two prominent hallmarks of human cancer, while the enlarged and deformed nuclear shape is used as a diagnostic marker for the malignancy. Polyploidy/aneuploidy is also thought to contribute to carcinogenesis and tumor progression, and recently induced polyploidy has been shown to be causative for tumor development. Often, tetraploidy results from mitotic failure and is thought to precede aneuploidy in the origination of cancer cells. However, the molecular basis for either the deformed nuclear morphology or aneuploidy of cancer cells is not well defined. We found that loss of GATA6 transcription factor led to loss of emerin and nuclear envelope defect in ovarian surface epithelial cells. Suppression of either GATA6 or emerin by siRNA caused nuclear envelope deformation and failure in cytokinesis, which subsequently led to formation of polyploidy and aneuploid cells. We also showed that emerin is missing in ovarian cancer, correlating with nuclear deformation and aneuploidy. Thus, we conclude that diminished expression of GATA6, and subsequently emerin, leads to a compromised nuclear envelope that is causal for nuclear shape deformation and aneuploidy in ovarian tumorigenesis. The current finding that a structural defect in nuclear envelope leads to aberrant nuclear morphology and the formation of tetraploidy in cancer cells suggests a novel mechanism for the development of chromosomal instability in carcinogenesis.

Keywords: chromosome instability; nuclear morphology; premalignant lesion

024 A Profile of the Ovarian Cancer Cell Methylome for Translational Application

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Aberrant promoter hypermethylation is a common mechanism for the inactivation of tumor suppressor genes (TSG) in cancer cells and a promising biomarker for detection of cancer in noninvasive body fluids. In addition, differences in behavior and response to therapy between individual ovarian tumors are likely due to the heterogeneity in the genetic and epigenetic mutations present in particular tumors. To gain insight into the methylome of the ovarian cancer cell, we performed an expression microarray-based analysis of genes reactivated in five ovarian tumor cell lines after treatment with the demethylating drug 5Aza-2 deoxycytidine (5Aza-dC). Between 705 and 3,842 genes were found to have at least twofold upregulation of expression after treatment in each cell line. To identify genes epigenetically silenced by hypermethylation in ovarian cancer cells, a subset of 50 upregulated genes underwent validation. By database analysis, we examined whether each gene was expressed in normal ovarian surface epithelium and harbored a typical CpG island in the promoter. To date, bisulfite sequencing of the promoter region has revealed 13 genes with promoter methylation in tumor cell line DNA but no methylation in normal cell DNA. The majority of the genes have not been previously described in ovarian cancer. Methylation-specific PCR (MSP) of DNA of 35 primary ovarian tumors showed several genes to be frequently methylated in primary ovarian cancer. Informed analysis combined with an evaluation of Gene Ontology and Ingenuity pathway mapping tools as unbiased methods for identifying the molecular pathways and processes supported an important role for the methylated genes in ovarian tumorigenesis. The validation process is tailored to identification of genes that will further our translational aims of testing gene methylation as a target for early detection of ovarian cancer in serum and as a biomarker for prediction of ovarian tumor behavior and response to therapy. The newly identified genes represent some of the most frequently methylated genes yet described in ovarian cancer and are of immediate utility for enhanced coverage and differential diagnosis of ovarian cancer in serum. Biology and correlative studies are used to determine candidate genes where methylation status may aid prognostic and predictive stratification of ovarian cancers. Further study will likely provide insight into the biology of the disease and facilitate more translational studies in ovarian cancer.

Keywords: methylome; serum; tumor suppressor genes

025 Role of Telomere Attrition in Type I Versus Type II Endometrial Carcinogenesis

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Endometrial cancer is not a single disease, but instead is generally recognized to comprise two broad categories of tumors, known as Type I and Type II, with distinct epidemiologic/clinical features and genetic bases. To gain insight into the role of telomere attrition in both subtypes of endometrial cancer, we analyzed telomeres and other markers of telomere status in Type I and Type II tumors. We document significant telomere shortening by PNA-FISH and other methods in both tumor types, but also in the nonmalignant epithelium present in uteri harboring cancers. We also demonstrate that telomere shortening can be associated with significant aneuploidy and, through the use of the marker phospho-H2AX, that this aneuploidy is associated with an increase of double-stranded DNA breaks. To further explore these issues in vivo, we generated and analyzed mice with critically short telomeres, and documented in such mice a distinctive endometrial lesion that closely resembles the accepted in situ precursor of Type II serous carcinomas. Thus this study is the first to demonstrate the feasibility of generating mouse models of Type II endometrial carcinogenesis. Based on these data and previous work, we propose a model in which telomere attrition participates in both the initiation and progression of Type I and Type II endometrial cancers.

Keywords: endometrial (endometrium); mouse model; telomere

026 Improvements in Ultrasound Tomography for Characterization of Breast Masses

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Every 2 minutes a woman in the United States is diagnosed with breast cancer, the leading cancer among women. Despite the great progress that has been made in the detection, diagnosis, and treatment of breast cancer, major limitations in breast imaging remain. Mammography's poor specificity, for example, leads to very high false-positive rates and too many biopsies. Conversely, in women with dense breasts, a high false-negative rate leads to many missed cancers. Furthermore, mammography is not well suited for monitoring response to treatment. Functional imaging techniques that emphasize the injection of contrast agents, such as PET and MRI, can monitor response to treatment but are expensive and time consuming and have limited availability for most women. At the present time, there is no single technique available to provide more specific imaging and inexpensive treatment monitoring.

Our long-term goal is to reduce the false-positive rate associated with diagnostic breast imaging through the development of Computerized Ultrasound Risk Evaluation (CURE), a new technology based on ultrasound tomography. The objective of this project, and a first step in achieving our long-term goal, is to test the concept that ultrasound tomography will allow better differentiation of malignant foci in breast tissue through the use of standard imaging agents. To that end, we present results of a preliminary study that shows a tentative differentiation of cancer based on acoustic properties of tissue. Our results can be summarized as follows:

- Validated ultrasound tomography imaging of tumors: Our preliminary data suggest that a specific combination of acoustic signatures, as measured by CURE, can maximize the differentiation of cancer from benign masses.
- Demonstrated feasibility of functional ultrasound tomography: We present results on the use of FDA-approved contrast agents as imaging agents for ultrasound tomography. Such agents would allow the development of ultrasound tomography in support of breast imaging and the detection of early-stage breast cancer. The initial in vitro data suggest that acoustic properties are enhanced through the use of contrast agents such as Magnevist.

The implications of these results for functional ultrasound tomography and the detection of breast cancer are discussed.

Keywords: breast; detection; imaging

027 Prevention of Mammary Cancers With Newly Synthesized Retinoids and Correlations With Short-Term Biomarkers

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A number of retinoid X receptor (RXR) agonists have proven to be highly effective in preventing methylnitrosourea (MNU)-induced mammary cancers. However, these agonists have side effects, particularly an increase in serum triglyceride levels. A series of ligands for RXR was designed on the basis of computer modeling to the ligand-binding domain (LBD) of the RXR receptors and on structure-activity relationships. The chemopreventive effects of these retinoids were evaluated in the relatively long-term MNU model. As a short-term assay to predict their efficacy, the ability of the retinoids to modulate cell proliferation was also determined in mammary cancers after only 7 days of treatment. The five UAB retinoids evaluated included UAB20, UAB112, UAB30, 4-methyl-UAB30, and the benzosuberone-analog of UAB30. The previously evaluated RXR agonist targretin and the pan-agonist 9-*cis*-retinoic acid (9-*cis*-RA), which interacts with both RAR and RXR receptors, were included as positive agonists known to prevent cancer in the MNU model. In the prevention studies, in which the agents were administered beginning 5 days after MNU until the end of the study, targretin (150 mg/kg diet) and 4-methyl-UAB30 (200 mg/kg diet) were highly effective in decreasing cancer numbers by 75%-85%. UAB30 (200 mg/kg diet) and 9-*cis*-RA (60 mg/kg diet) gave intermediate inhibitions of 60% and 45%, respectively. Targretin (15 mg/kg diet), UAB20 (200 mg/kg diet), and the benzosuberone analog of UAB30 (200 mg/kg diet) showed limited activity by decreasing cancer multiplicity 25%-30%, while UAB112 had no effect on mammary cancer multiplicity.

A direct correlation was observed between the long-term chemopreventive efficacy of these agents and their ability to decrease cell proliferation in mammary cancers after short-term treatment. Furthermore, the highly effective agents (4-methyl-UAB30 and targretin at 150 mg/kg diet) increased apoptosis 3-5 times, while agents with moderate or limited preventive efficacy failed to significantly increase apoptosis. Although the more effective retinoid treatments increased serum triglycerides 2.5-fold to 4.0-fold, one effective agent (UAB30) had no significant effect on lipid levels.

Keywords: breast; proliferation; retinoids

028 KLF4 Blocks Maturation of Human MCF10A Breast Epithelial Cells and Promotes Breast Tumor Formation in MMTV-ErbB2 Transgenic Mice

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KLF4 is a zinc finger transcription factor that was recently implicated in the embryonic stem cell phenotype. KLF4 mRNA and protein expression are increased very early during progression of most human breast cancers. Although KLF4 can function as an oncogene in epithelial models such as mouse skin, its function as an oncogene in breast epithelial cells is less clear. MCF10A cells transduced with KLF4 by retroviral transduction and then mass selected grew at the same rate in two-dimensional culture and showed the same morphology as the vector control. MCF10A cells can be induced to recapitulate the polarized, acinar structures of breast epithelium by culture on a layer of basement membrane components (Matrigel). In three-dimensional culture, KLF4 induced a block to maturation. Instead of the well-organized spheres and smooth, regular basal cell layer characteristic of untransformed cells, KLF4 induced grape-like clusters of cells with irregular basal layers. Clusters retained their roughened, dysplastic morphology throughout the period of observation of 19 days, suggesting that the maturation block is permanent.

To test the activity of KLF4 in vivo, we used the latent tumor model, MMTV-ErbB2. Bitransgenic *MMTV-KLF4*$^{+/-}$;*MMTV-ErbB2*$^{+/-}$ virgin females (n = 12) were compared to mice transgenic for either transgene (n = 9). KLF4 did not appreciably alter the latency of ErbB2-induced breast cancers (range, 23-32 weeks) but bitransgenic animals showed more tumors per animal compared with *MMTV-ErbB2* alone (2.50 vs. 1.44; p = .0119, Mann-Whitney test, two-tailed). No tumors were observed in WT or *MMTV-KLF4*$^{+/-}$ mice. These data suggest that KLF4 acts as an oncogene in breast epithelium.

Keywords: breast; ErbB2; KLF4

029 Circulating Blood Levels of Carbonic Anhydrase 9 in Patients With Renal Cell Carcinoma: Correlations With Disease Activity

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Renal cell carcinoma (RCC) is expected to affect 56,000 individuals in 2007 and its incidence has been steadily increasing over the last 20 years. Biomarkers for RCC may help in diagnosis of minimal residual disease and early relapse in patients at risk for RCC recurrence, could guide antiangiogenic therapy, may help identify patients for adjuvant therapy, and could be used as surrogate markers of disease activity in Phases I/II trials.

Carbonic anhydrase 9 (CA) is an HIF target that could be spliced into a transmembrane and a secreted isoform. It has previously been reported to be upregulated in clear cell RCC. Substantial increases in CA9 may predict response to IL-2 treatment and it has been proposed as a target for RCC imaging.

To evaluate CA9 as a blood circulating biomarker that may correlate with RCC activity, we used an anti-CA9 antibody (m75) based ELISA to measure plasma levels of CA9 in blood obtained prior to and 6-8 weeks after nephrectomy for localized disease in (1) patients with clear cell RCC, (2) patients with papillary and chromophobe RCC or oncocytoma, (3) patients with benign kidney lesions, and (4) normal control individuals.

We observed a significant decrease in the blood levels of CA9, after nephrectomy for localized disease, in the majority of patients with clear cell RCC (73%, or 11/15). In contrast, no patients with RCC of non-clear cell histology or benign disease had a decrease

in CA9 blood levels 6-8 weeks after nephrectomy. Tumor volume correlated with pre-operative levels of CA9 (correlation coefficient r = 0.74). Plasma levels of CA9 in normal controls do overlap with pre-operative levels of CA9 in RCC patients prior to nephrectomy, indicating that it is unlikely that CA9 may be used as a single test for diagnosis of RCC in the general population. Nevertheless our data indicate that it may be a useful biomarker in monitoring disease activity in patients with RCC post-nephrectomy.

Preliminary results indicate that patients with recurrent RCC disease have levels of CA9 significantly higher than the post-nephrectomy values. Currently we are expanding our cohort of patients with localized and recurrent disease to further investigate CA9 as a biomarker for RCC.

Keywords: disease activity; minimal disease; recurrence

030 BioMark™ System: Integrated Fluidic Circuits Enable the Use of Real-Time qPCR for Cancer Gene Expression Panels, Drug Resistance Monitoring, and Circulating Tumor Cell Detection

Please see Poster Discussion Session New Technologies and Methodologies II.

031 Epigenetic Progression of Human Lung Cancer Involves Hallmark Genes and Inhibitors of Wnt Signaling

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While genetic changes are considered central for the genesis of human lung cancer, only a few mutational events occur with great frequency in lung cancer. For example, p53, kras, and EGFR mutations play critical roles in cancer, but are not found in most tumors. In contrast, an increasing number of genes are found to be affected by hypermethylation of the promoter region, leading to gene silencing in lung cancer. The appearance of promoter region methylation for some genes has been examined in early, pre-invasive lung cancer, and the early appearance of these changes has supported the consideration of the use of hypermethylation in early detection strategies for lung cancer. Atypical adenomatous hyperplasias (AAH) are now recognized as a precursor lesion from which lung adenocarcinomas arise and thus represent an ideal target for studying the early genetic and epigenetic alterations associated with lung tumorigenesis. We first assessed the frequency of promoter hypermethylation of genes affecting cell cycle control (p16), evasion of programmed cell death (DAPK), DNA repair (MGMT), retinoic acid signaling (RARβ), Ras signaling (RASSF1A), limitless replicative potential (hTERT), and tissue invasion (TIMP3) in the normal-adjacent, LG-AAH, HG-AAH, and adenocarcinoma samples. A significant increase in the frequency of methylation for all the genes as well as in the number of genes methylated was according to increasing atypia (p-test for trend $p<.01$). We next asked whether epigenetic silencing of inhibitors of Wnt signaling might also be involved in early and preinvasive lung cancer by first assessing the level of Wnt signaling activity in non-small cell lung cancer (NSCLC) cell lines using the active β-catenin antibody. We found that, indeed, many cell lines had evidence of active Wnt signaling in NSCLC cells. We then assessed the level of expression of APC, DKK1, DKK3, LKB1, SFRP 1, 2, 4, 5, WIF-1, and RUNX3 and found that the silencing of these Wnt antagonists correlated with promoter hypermethylation. Using a multiplex nested-methylation specific PCR-based approach, we finally analyzed the methylation status of these genes in lung resections showing the full histopathologic sequence of glandular neoplasia ranging from normal lung parenchyma to low-grade AAH to high-grade AAH to adenocarcinoma. We observed a significant increase in the frequency of methylation for each gene as well as the number of genes methylated with each stage of tumorigenesis (p-test for trend $p<.01$), as had been observed for the "hallmark" genes. These results show that gene silencing of Wnt antagonists by promoter hypermethylation occurs during the earliest stages of glandular neoplasia of the lung and accumulates with progression toward malignancy. Together, our findings demonstrate that epigenetic progression underlies histologic progression and may provide insight into early detection markers using promoter region hypermethylation.

Keywords: DNA methylation; tumor suppressor genes; Wnt signaling

032 Evaluation of the Novel Serum Markers B7-H4, Spondin 2, and DcR3 for Diagnosis and Early Detection of Ovarian Cancer

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Objective: Early detection through regular screening could significantly reduce mortality from ovarian cancer. Advances in biomarkers and imaging continue to improve the sensitivity and specificity of cancer detection, but further improvements are still needed. In this study, we identified and evaluated three new serum biomarkers that may be used to improve detection of ovarian cancer.

Methods: Through genomic analysis, we identified B7-H4, Spondin 2, and DcR3 as overexpressed genes in ovarian cancer tissues. Sensitive sandwich ELISAs were developed to analyze the level of these novel markers in 68 serum samples from patients with ovarian cancer (16 early stage, 52 late stage) and 108 control samples, and 20 healthy women from whom two serum samples were collected 1 year apart. CA125 levels were measured in all samples.

Results: Markers were evaluated for their ability to identify clinical disease. The three novel markers and CA125 were elevated in serum of ovarian cancer patients as compared to normal controls. B7-H4 showed the highest specificity, with the lowest frequency of elevation in all control groups. When all cases were compared against all controls, CA125, Spondin 2, B7-H4, and DcR3 showed areas under the ROC curve of 0.87, 0.78, 0.74, and 0.71, respectively. CA125 and B7-H4 showed the best diagnostic performance for early stage, with AUCs of 0.90 and 0.80, respectively.

Conclusion: This study demonstrates that B7-H4, Spondin 2, and DcR3 are promising new ovarian cancer markers that may improve early detection of cancer when used in combination with traditional diagnostic tests.

Conflict of Interest Statement: I. Simon, K. Krall, R. Wolfert, and N. Kim are employees or a consultant of diaDexus, Inc., which has commercial interests in the development of biomarkers described in this manuscript.

Keywords: diagnosis; PEB; prognosis

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

033 Validation of a Biomarker Panel for Early Detection of Breast Cancer in High-Risk Women

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The power of a panel of markers in predicting the presence of cancer has been demonstrated in many cancers, including ovarian and prostate cancer. Mammography has been shown to decrease breast cancer mortality, but its sensitivity is relatively poor in young women with dense breasts who are typical of the high-risk population. Moreover, mammography can result in a large number of false positives, which can lead to unnecessary surgery and worry. A robust biomarker panel for breast cancer that complements mammography in high-risk women is needed. This study is an interdisciplinary, inter-SPORE collaboration conducted jointly by investigators at the M.D. Anderson Cancer Center, Fox Chase Cancer Center, University of Alabama, and Fred Hutchinson Cancer Research Center to evaluate and validate promising biomarkers for early detection of breast cancer. Our work builds on an ongoing study of women at high risk for ovarian cancer and on substantial preliminary work in breast cancer biomarkers as well as innovative statistical methods for combining complementary markers to maximize their performance. Our goal is to improve outcomes for women at risk for breast cancer, specifically focusing on women with dense breasts or equivocal findings on mammography who may benefit from additional followup using magnetic resonance imaging (MRI) or PET. Marker development, evaluation, and validation will be conducted jointly by the four SPORE sites on a standardized set of specimens selected by the acting coordinating center at Fred Hutchinson. Innovative statistical approaches will be used to evaluate the performance of combined markers and individual markers used in a longitudinal algorithm. Serum specimens will be collected from healthy women undergoing annual mammography and women who have breast biopsies in outpatient clinics. A subset of these specimens was selected for inclusion in the Avon Validation Set. Once validated, our biomarker panel will be used in future work to identify women for whom further imaging studies are indicated and to reduce the rates of missed cancers and unnecessary biopsies in women undergoing mammography.

Keywords: breast cancer; early detection; serum markers

034 Angiogenesis in Bronchial Dysplasia and Angiogenic Squamous Dysplasia Is Associated With the Development of Immature Vasculature

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Background: Angiogenic squamous dysplasia (ASD) is a dysplastic lesion of the bronchial airways that is distinguished from other dysplasias by virtue of the presence of characteristic vascular morphology with projection of microvessels into the overlying dysplastic epithelium. To date we have identified ASD in 167 of 721 patients who have undergone fluorescence microscopy at the University of Colorado Health Sciences Center. ASD has been found in nonmalignant airways of 38 patients with invasive carcinoma, including 28 patients with prevalent carcinoma, 9 patients with incident carcinoma, and 130 patients without carcinoma. These lesions are associated with increased VEGF expression and high microvessel densities (MVD) in comparison to normal bronchial epithelium. In addition, we have recently demonstrated these lesions to be associated with longer periods of persistence in comparison to non-ASD bronchial dysplasia.

Methods: Immunohistochemical stains for CD31 and actin were performed on consecutive sections of bronchoscopically obtained biopsy material. CD31 and actin vessel densities for identical areas within each biopsy were produced by image analysis with collection of area measurements and vessel tags. A microvessel maturation index (MMI) was calculated via division of actin MVDs by CD31 MVDs for each biopsy. In addition, single vessel analyses were performed for microvessel papillary structures in ASD lesions.

Results: The mean MMI for 12 dysplastic lesions was significantly less than that measured for 4 normal bronchial biopsies (MMI 0.62 vs. 1.02, respectively; $p = .04$). When analyzed independently, the vessels that project into the intraepithelial papillae of ASDs showed the lowest MMI of 0.56. This was not statistically different from the MMI for the dysplasia group as a whole but was significantly decreased in comparison to normal MMI ($p = .71$ and .04, respectively). Incomplete vascular maturation appears to be associated with dysplastic change in the bronchial airways and is most striking in the vascular structures of ASDs.

Conclusions: Angiogenesis in bronchial dysplasia is associated with reduced vascular maturation. Immature vasculature may have an impact on the growth and progression of dysplastic lesions. Increased delivery of protumorigenic factors, such as growth-promoting factors and mutagenic substances, may be facilitated by immature vascular networks. Further analysis of ASD-associated vasculature may allow for the identification of angiogenic mediators that control the maturation of microvessels in preneoplastic airway disease.

Keywords: angiogenesis; bronchus; dysplasia

035 Amplification and Overrepresentation of TP73L (p63), EGFR, MYC, and 5p15 Sequences in Premalignant Bronchial Biopsies From Smokers at High Risk for Lung Cancer

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Background: Lung carcinoma development is accompanied by cytogenetic alterations of the airway epithelium that may have diagnostic importance. Our study aimed at identifying changes observed in preinvasive lesions at specific genomic locations known to be aberrant in non-small cell lung cancer. We investigated genomic gains in four specific loci, *TP73L (P63), EGFR, MYC,* and 5p15, as predictors of prevalent or incident lung cancers.

Materials: Bronchial biopsy specimens were obtained by LIFE bronchoscopy from 134 subjects, including 104 males and 30 females, with a mean age of 63.7 years. The population included patients with invasive carcinoma (41 prevalent and 3 incident cases) and 90 subjects who remained free of invasive tumor for 5 years (controls). The histological grade in the premalignant lesions was carcinoma in situ (CIS) in 32, severe dysplasia (SD) in 48, and moderate dysplasia (MD) in 54 subjects.

Methods: 5-micron paraffin sections were submitted to a 4-target FISH assay (LAVysion, Abbott Molecular) including centromere 6, 5p15.2, 7p12 (*EGFR*), and 8q24 (*MYC*) sequences. For 65 subjects, specimens were reprobed with a 2-target FISH assay, including p63 (*TP73L* at 3q28; BAC clones RP11-53D15 and RP11-373I6, digoxigenin-labeled, detected by FITC) and Spectrum Orange-CEP 3 sequences (Abbott Molecular). Spot counts were obtained for 30-50 nuclei per specimen for each probe, over areas of interest marked by the pathologist on adjacent H&E stained sections.

Results and Conclusions: Results for the LAVysion probe set were available for all cases, and the reprobing with the 2-target set was successful in 51 specimens. Genomic alterations at these regions decreased in frequency from high- to low-grade lesions. Overrepresentation, extra copies, of these specific regions were found in >50% of CIS specimens, 30% of SD specimens, and <25% of MD specimens. Clustered *EGFR* amplification was found in 3 CIS and 1 SD specimen; *MYC* amplification was seen in one CIS (clustered) and one SD (diffuse); 5p15 sequences were diffusely amplified in two SD; p63 copy number gain was observed in 16/26 CIS, 1/15 SD, and 1/15 MD. Clustered amplification of p63 was found in 13 of the 26 CIS. Future analysis will attempt to determine the added value of these four FISH probes to identify these genomic alterations in preinvasive lesions and ultimately to determine whether those changes may have a role in predicting the progression of these lesions to an invasive phenotype.

Keywords: FISH; genomics; premalignancy

036 Second Primary Head and Neck Tumor Risk in Cervical Cancer Patients: SEER Data Analysis

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Almost all cases of invasive cancers of the cervix and most other anogenital tract cancers are associated with high-risk HPV types (predominantly, types 16, 18, 31, and 45). Although tobacco and alcohol use are the primary risk factors associated with head and neck squamous cell carcinoma (HNSCC), HPV is also associated with ~25% of these tumors. The prevalence of HPV-positive HNSCC is highest in the oropharynx, especially the tonsils. The source of HPV infection to the oral mucosa is still unclear and the role of HPV infection in the development of HNSCC after a first diagnosis of cervical cancer is unknown. Using SEER data, we compared the risk of second primary cancer (SPC) HNSCC in cervical cancer patients to the general population and to females with other primary cancers. The lifetime risk of developing SPC in the pharynx of cervical cancer patients was significantly higher than in the general population (SIR: oropharynx = 2.7; tonsil only = 3.1, hypopharynx = 2.8). When compared with females with other cancers, the risk of SPC in the pharynx, vagina, vulva, and anus/anal canal was in excess in cervical cancer patients, while the risks of SPC in the other head and neck subsites (oral cavity, nasopharynx, and salivary glands) were not in excess. The relative risk of SPC among females with cervical cancer was compared to that of age-matched female breast and urinary bladder cancer patients, to indirectly assess whether the risk of second primary tumors in head and neck subsites among cervical cancer patients was to be mostly attributed to persistence of smoking. The risk of second primary lung cancer was significantly higher in women who had cervical or bladder cancer, whereas it was not increased in women with breast cancer. As expected, cervical cancer patients were at risk of developing HPV-related second primary tumors arising in the vagina (SIR = 23.7), vulva (SIR = 4.0), and anus/anal canal (SIR = 3.4). Breast and bladder cancer patients were not at risk for these second primary tumors. For the development of SPC in the oropharynx, female breast and bladder cancer patients were not at increased risk (SIR: oropharynx = 1.0 and 1.1, respectively). However, for cervical cancer patients, the risk of SPC in the oropharynx was elevated threefold (SIR = 2.8 for oropharynx, 3.5 for tonsils only). Our findings show that cervical cancer patients develop an excess HNSCC SPC in comparison to females with other cancers. A possible role of HPV is suggested.

Keywords: epidemiology; etiology; viral infection

037 HRQOL and Satisfaction With Cancer Treatment Outcome From the Perspective of Early-Stage Prostate Cancer Patients and their Spouse/Partners: Results From a Prospective, Multicenter Study

Please see Poster Discussion Session Therapy II.

038 A Survey of Patient Advocates Within the National Cancer Institute's Prostate Cancer SPORE Program: Who Are They? What Motivates Them? What Might They Tell Us?

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Patient advocates are involved in medical care, research, national policy, and legislation. They appear often to be cancer survivors and "bring a wide variety of experiences and cancer patient perspectives...." Some government health agencies have created advisory boards that solicit patient advocates, and advocates often go to Washington and to their State legislatures to press their viewpoint. Yet, few studies have focused on the advocates themselves. Who are they? What motivates them? What might they tell us?

In an effort to answer these questions, a four-page survey was developed and sent to advocates within the National Cancer Institute's Specialized Programs of Research Excellence (SPOREs) in Prostate Cancer.

Of 19 advocated, 10 responded to this mailed survey. The median age of respondents was 72 years (range, 63-79). Most were retired (80%) with previous/current professions including attorney, engineer, professor, school district superintendent, airline pilot, military officer, government executive, physician, and manager. All had faced a diagnosis of prostate cancer. The advocates expressed a variety of views on subjects ranging from education to research funding to their own personal medical experiences. They participate in several activities, often involving education, such as conducting patient support groups. Some educational efforts are directed toward health care providers and researchers, advising them that their ultimate goals should be patient

oriented. Advocates appear highly engaged in the research agenda and try to influence it by direct contact with SPORE researchers and lobbying efforts in Congress.

Although this survey had a low response rate, findings suggest that advocates are involved in important ways in support of prostate cancer patients and prostate cancer research. Other researchers may want to attempt this kind of study to involve patient advocates involved in other cancer types and other advocacy organizations.

Keywords: advocate; prostate; survey

039 Projecting Individualized Probabilities of Developing Bladder Cancer in Caucasians: An Integrative Epidemiology Approach

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Purpose: Cancer risk prediction models have mostly relied on traditional epidemiologic risk factors. We developed the first such model for bladder cancer; more significantly, we incorporated mutagen sensitivity—a constitutive genetic instability marker—to build an enhanced epidemiologic-genetic risk prediction model for bladder cancer.

Methods: We used extensive epidemiologic and genetic data from a large case-control study of bladder cancer. We applied a series of sophisticated statistical tools to build multivariate risk models and evaluate high-order interaction among these risk factors. We calculated the specificity and sensitivity of the model by constructing receiver operating characteristic (ROC) curves and calculating the area under the curve (AUC). We also estimated the absolute risk of developing bladder cancer of representative individuals at 5, 10, and 15 years.

Results: The significant risk factors included in the basic epidemiologic model included pack-years smoked and past exposures to diesel, aromatic amine, dry cleaning fluids, radioactive materials, and arsenic. This model yielded a good discriminatory ability with an AUC of 0.70 (95% CI, 0.67-0.73). When mutagen sensitivity data were incorporated into the model, the AUC increased to 0.80 (95% CI, 0.72-0.82), a substantial improvement in prediction accuracy. These two models both showed excellent concordance in our internal validation. Classification and regression tree (CART) revealed complex interplay between risk factors, which categorized individuals into subgroups with varying degrees of risk for bladder cancer. The subgroup with the highest risk exhibited a 67-fold increased risk over the reference subgroup. We also computed an ordinal risk score based on regression coefficients from the multivariate risk models. As the score increased, the percentage of controls in each category decreased while the percentage of cases increased; for example, the percentage of cases in the lowest risk group (scores ≤2) derived from the enhanced model was 9.88%, whereas the percentage of cases in the highest risk group (scores >25) was 84.42%, indicating that our model had a high discriminatory ability.

Conclusions: We developed the first risk prediction model for bladder cancer. The enhanced epidemiologic-genetic model with the addition of a single genetic component exhibits excellent discriminatory ability, demonstrating the promising future of cancer risk prediction by incorporating multiple genetic factors.

Keywords: genetic marker; integrative epidemiology; risk assessment model

040 Metastatic Outcomes and MC1R Genotype Status in a Cohort of Melanoma Patients

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Background: Polymorphic variation in the melanocortin-1 receptor (*MC1R*) gene and particularly the R-variants (D84E, R151C, R160W, and D294H) previously have been shown to increase risk of melanoma. While one report noted an association between *MC1R* variants and tumor thickness, little is known about the contribution of *MC1R* to tumor progression and, importantly, whether inherited variation at this locus impacts upon metastatic potential.

Objective: We sought to preliminarily investigate the impact of *MC1R* variants on risk of metastasis in a cohort of melanoma patients previously enrolled in an ongoing case-control study of low-penetrance genetic susceptibility of melanoma.

Methods: Patients with newly diagnosed single invasive primary melanoma seen at the University of Pennsylvania's Pigmented Lesion Clinic since 1997 and who enrolled in the parent case-control study constitute the melanoma cohort. All study participants gave a buccal swab, answered a brief questionnaire, and had a skin examination. The complete coding region for *MC1R* was genotyped by direct sequencing using genomic DNA collected from buccal swabs. Among cohort members, time-to-event is defined as time between date of first melanoma diagnosis and date of melanoma recurrence or date of last followup for those without metastases. Logistic regression models were built to determine the association between *MC1R* R-variants and progression to metastasis (yes/no). Time-to-event was modeled using Cox proportional hazards models. Age at first primary melanoma diagnosis, sex, and thickness of the primary melanoma were adjusted for in the model.

Results: Among the 955 patients in the melanoma cohort, 49 (5.1%) had a metastatic event. Average time-to-event was 3.9 (±2.4) years. *MC1R* genotyping was completed on a total of 706 members of cohort, 39 (5.5%) of whom had a metastatic event. There were no statistically significant differences ($p>.05$) between those with and without *MC1R* genotype comparing metastatic events, sex, age at first melanoma diagnosis, and tumor thickness; however, those with a completed genotype had longer average time-to-event compared to those without (4.1 vs. 3.6 years; $p = .003$). Carriage of at least one R-variant was inversely associated with having a metastatic event, although this association was not statistically significant in the crude or adjusted analyses (aOR = 0.76, 95% CI, 0.38-1.5). Results from time-to-event analyses also failed to show a statistically significant association with risk of metastasis (aRR = 0.85, 95% CI, 0.44-1.7). When persons with less than 6 months of time-to-event (n = 7 with and n = 50 without metastatic events, respectively) were excluded from the analyses, associations between R-variants and having a metastatic event and time-to-event became stronger, but still did not reach statistical significance (aOR = 0.63, 95% CI, 0.29-1.4; aRR = 0.73, 95% CI, 0.35-1.5).

Conclusion: Our data suggest that inherited *MC1R* variants may be a genetic marker for modest protection against melanoma progression, although presently there are too few metastatic events in our cohort to draw a more definitive conclusion. We will continue to follow cohort members for future outcomes.

Keywords: inherited variation; *MC1R*; metastasis

041 Bladder Cancer Patient Support Team: M.D. Anderson Cancer Center SPORE in Genitourinary Cancer

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Bladder cancer is the fifth most common cancer diagnosed in the United States. The American Cancer Society estimates 60,240 new cases of bladder cancer will be diagnosed in 2007, and 12,710 patients will die of their disease. Bladder cancer occurs about 3 times more often in men than in women and is twice as common among whites than among African Americans and Hispanics. Cigarette smokers are 2 to 3 times more likely than nonsmokers to develop bladder cancer. When bladder cancer is diagnosed and treated in a localized stage, the 5-year survival rate approaches 95%. Therefore, education and awareness are critical to early diagnosis and treatment.

The Bladder Cancer Support Team (BCST) was developed through a supplement to the M.D. Anderson SPORE in Genitourinary Cancer and has effectively leveraged its support and available resources at M.D. Anderson to promote its mission. The mission of the BCST is to (1) improve public awareness of bladder cancer and its causes, (2) enhance access to SPORE-initiated bladder cancer research, (3) ensure minority representation in SPORE-initiated research, (4) inform patients of clinical trials, and (5) contribute to the establishment of a national advocacy group. In the 5 years since the program's inception, we have developed the administrative structure including appointment of a new SPORE Patient Advocate; developed a HIPAA-compliant bladder cancer patient database and advocacy group; collaborated with institutional components to establish Bladder Cancer Awareness Week; aligned bladder cancer programs with smoking cessation, wellness, and patient network programs; developed a panel of SPORE investigators and experts to speak at patient conferences and educational seminars including the annual Anderson Network-sponsored "Living Fully with Cancer and Beyond" Conference; organized and participated in local and national events; and established an educational Web site for our patients. We will continue to expand the educational and awareness component of the program, including access to information about emerging clinical trials in bladder cancer.

Keywords: advocacy; education; support

042 Clinical Classification of BRCA1 and BRCA2 Variants of Uncertain Significance

Please see Poster Discussion Session Biomarkers I.

043 Potential Gene-Environment Interactions in Ovarian Cancer Risk: Talc and NAT2

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Background: Weak or inconsistent associations observed between modifiable risk factors for ovarian cancer such as talc may be explained, in part, by interactions with genetic variants. Thus, environmental factors may modify ovarian cancer risk in some women but not others based on their genotype. Genital exposure to talc may result in chronic inflammation and free radical damage that could be modified by genes involved in detoxification or oxidation pathways. The NAT2 gene produces the enzyme N-acetyltransferase 2, which is involved in metabolism of carcinogens and other chemicals and can act either to activate or deactivate specific substrates. Several common polymorphisms in the NAT2 gene result in reduced enzyme activity. We evaluated functional polymorphisms in the NAT2 gene, as well as gene-environment interactions involving NAT2, in relation to ovarian cancer risk in a population-based case-control study (NECC) and a prospective nested case-control study (NHS).

Methods: We genotyped three SNPs that together identify the slow acetylator phenotype in the NAT2 gene (rs1041983, rs1801280, rs1799930) in 1,176 cases and 1,201 controls in NECC and 161 incident cases and 458 controls in the NHS. We calculated odds ratios and 95% confidence intervals using conditional and unconditional logistic regression.

Results: We observed an inverse association between the NAT2 slow acetylator phenotype and ovarian cancer risk in the NHS (covariate-adjusted OR = 0.53; 95% CI, 0.34-0.84) but not the NECC (covariate-adjusted OR = 0.90; 95% CI, 0.75-1.08). However, in the NECC the association between talc and ovarian cancer appeared to differ by acetylator phenotype. Rapid acetylators with a history of regular talc use had an increased risk of ovarian cancer compared to rapid acetylators who did not use talc (covariate-adjusted OR = 1.48; 95% CI, 1.16-1.90), while slow acetylators had no difference in ovarian cancer risk by talc use (p-value for interaction = .12). In the NECC, there were also significant positive trends with increasing frequency (p-trend = .001) and duration (p-trend = .002) of talc use among the rapid acetylators, but not the slow acetylators (p = .36 and .86, for frequency and duration, respectively).

Conclusion: The NAT2 slow acetylator phenotype may decrease ovarian cancer risk and negate the risk associated with talc while genital exposure to talc may increase the risk of ovarian cancer among individuals with the rapid acetylator phenotype.

Keywords: NAT 2; single nucleotide polymorphism; talc

044 Selective Gene Upregulation by Talc-Exposed Vaginal Epithelium May Link Talc in Genital Hygiene With Risk of Ovarian Cancer

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Cumulative epidemiologic evidence links regular use of talc in genital hygiene with an increased risk for ovarian cancer; however, underlying biologic mechanisms remain unclear to date. Recently we proposed that many risk factors for ovarian cancer might operate by raising or lowering immunity to human mucin 1 (MUC1), a gene in the same family as CA125 that is normally expressed in genital tract epithelium and overexpressed in ovarian cancer. We found that regular use of talc in genital hygiene was associated with a lower level of antibodies and greater risk for ovarian cancer, leading us to hypothesize that talc might affect risk for ovarian cancer by altering immune events initiated in the lower genital tract mucosa. We began in vitro and in vivo studies to test this hypothesis. First we surveyed women presenting for routine annual Pap testing about talc use in the genital area and received their permission to study residual Pap test material. Immunocytochemical staining of ThinPrep Pap slides demonstrated that in the talc users, MUC1 was co-expressed with heat shock proteins known to modulate MHC Class I antigen presentation, suggesting the possibility of talc-related altered immune response. Applying a novel nuclease protection chemiluminescent assay for mRNA, we found significantly higher mRNA expression of a number of stress genes involved in

mucosal immune regulation including Ets2, FOS, Cox-2, MAPK-1, and GSCF in cytologic Pap test material from talc users as compared to nonusers. The same genes were induced by talc in a dose-dependent manner in a physiological in vitro model of the vaginal epithelium. A preliminary analysis of endometrial and cervical tissue obtained from an ovarian cancer case of a talc user showed similar selective gene upregulation as compared to a matched nonuser cancer case. Moreover, proteomic analysis by Meso Scale Discovery electrochemiluminescent assays revealed that talc induced in the vaginal culture model a dose-dependent phosphorylation of stress proteins, e.g., heat shock protein hsp27 and mitogen-activated protein kinases, known to affect RNA stability of cytokines and Cox-2. Finally, scanning electron microscopy and x-ray spectroscopy identified talc in pelvic lymph nodes of a woman with ovarian cancer who was a chronic talc user, suggesting another pathway for immune dysregulation by talc use. We believe that, collectively, our experimental results provide new insights into the mechanisms by which use of talc in the genital area may increase risk of ovarian cancer. An estimated 10% of the ovarian cancer cases in this country might be prevented by avoidance of talc in genital hygiene, and our studies may provide a basis for stronger warnings or regulatory action on the use of cosmetic talc powders.

Keywords: genital epithelium; lymph nodes; talc

045 Leukemia Patient Advocate Group: Continued Development of the Leukemia SPORE Support Group at M.D. Anderson Cancer Center

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Leukemia is a heterogeneous group of hematologic disorders. An estimated 59,240 new cases will be diagnosed in 2007. The continued development of a patient advocate support group has enhanced the research effort of the SPORE and has provided benefits to leukemia patients.

Through the M.D. Anderson Leukemia SPORE, we have developed the Leukemia Patient Advocate Group (LPAG). The members include two patients with different types of leukemia, the administrator of the SPORE Grant, and one investigator. The mission of the LPAG is to continue obtaining the support needed to continue this program as a component of the ongoing SPORE. Our goals are (1) to expand the number of members to include caregivers as well as additional patients, (2) to facilitate access to leukemia SPORE-initiated research throughout the country, (3) to educate community and health professionals on patient care and treatment options, and (4) to enhance patient services.

In summary, with the support of the SPORE and our institutional commitment, we hope to enhance access to leukemia SPORE-initiated research throughout the country and to educate and inform patients and caregivers alike on new treatment advances in leukemia.

Keywords: access; education; support

046 8q24 Is a Major Prostate Cancer Risk Locus

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Linkage analysis of hereditary prostate cancer families has resulted in many candidate loci for prostate cancer risk; however, few have been validated and replicated. Over the past year, a chromosomal region on 8q24 has emerged as playing a major role in prostate cancer etiology. Two groups (including ours), using independent methods and populations, have implicated this locus.

Initially, we performed an admixture genomewide scan in a population of African American men largely derived from the Multiethnic Cohort. Admixture scanning is a particularly efficient way of performing a whole genome scan because the process of admixture (e.g., European and African ancestral admixture leading to an African American population) creates long, contiguous, alternating stretches of the genome that can be identified as African or European in origin. The goal of admixture mapping is to identify regions of the genome that are significantly enriched in one ancestry. Since prostate cancer incidence is much higher in men of African ancestry than in men of European ancestry, we looked for genomic regions that are elevated in African ancestry in African American men with prostate cancer.

A 3.8 megabase region on chromosome 8q24 (125.68-129.48 Mb in build 35 of the reference sequence) was significantly associated with prostate cancer. We genotyped close to 1,300 ancestry informative markers across the genome in 1,597 cases of prostate cancer. The region on 8q24 was significantly enriched in African ancestry and achieved genomewide significance. This area contains nine annotated genes, including the MYC oncogene.

Followup fine mapping in this region identified seven alleles that independently contribute to prostate cancer risk across multiple ethnic populations. A total of 2,973 SNPs were genotyped in up to 7,518 prostate cancer cases and controls. Seven risk variants (of which five were previously undiscovered) were needed to fully describe the genetic risk of developing prostate cancer. These seven variants span about 450 kilobases on chromosome 8q24. Surprisingly, these polymorphisms account for a substantial proportion of population attributable risk in each of the five populations tested to date (32%-68%). Interestingly, the variants do not align with any known genes or micro-RNAs.

The 8q24 findings indicate one of the first validated genetic risk factors for prostate cancer. Many questions, however, still exist and much important work remains to be done. Active investigations into uncovering the actual causal alleles as well as understanding how these alleles are exerting their effect on risk are ongoing. How or if this information will be clinically useful also remains an open question and active area of investigation.

Keywords: genomics; risk; whole genome scan

047 Genetic Variation in UGT2B Gene Cluster and Breast Cancer Risk

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Breast cancer is a complex disease caused by the progressive accumulation of multiple gene mutations combined with epigenetic dysregulation of critical genes and protein pathways. Since the steroid hormones play a critical role in the regulation of breast proliferation and apoptosis [1], it is reasonable to hypothesize that variation in the levels and activity of enzymes that can metabolize steroid hormones may contribute to inter-individual variation in breast cancer risk. Glucuronidation is a major pathway of steroid elimination in the liver and steroid target tissues and is catalyzed by the UDP-glucuronosyltransferases (UGTs: [2]). One of the two major subfamilies of UGTs in humans, UGT2B, includes seven active genes located on chromosome 4q13 [3]. Except for two members, 2B10 and 2B11, whose substrate is still unknown, the other five genes, 2B4, 2B7, 2B15, 2B17, and 2B28, play an important role in steroid hormone metabolism [2].

To uncover the effect of UGT2B variation in breast cancer risk, we have started a full resequencing survey in this gene cluster in ethnically diverse samples. So far, we have resequenced the promoter and coding regions of major UGT2B members. In addition, we have used comparative genomics and computational approaches to identify conserved clusters of predicted binding sites for transcription factors enriched in the liver as well as nuclear hormone receptors, such as glucocorticoid receptor and estrogen receptor. These sequence elements together with the coding and upstream regions have been sequenced in 44 HapMap individuals (22 CEU, 12 YRI, and 10 Asian). So far, we have identified 119 SNPs, of which 70 were not previously described (not in dbSNP); 33 of the 70 newly discovered SNPs have minor allele frequency greater than 5% in our global sample. These findings underscore the importance of performing SNP discovery in clusters of duplicated genes, such as UGT2B, which may have low SNP coverage in public databases. Bioinformatics approaches will be used to predict the function of all variants. Population genetics analysis will be performed to explore the role of natural selection in shaping the observed patterns of variation. The tag SNPs will also be selected on the basis of the resequencing result.

In the future, we will measure the expression of UGT2B genes in normal liver and breast tissues by real-time PCR and genotype the tag SNPs in the same individuals. The association between expression and genetic variation will be investigated to identify regulatory variants. These candidate risk variations will be tested in breast cancer cases and controls.

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Keywords: genetic susceptibility; SNP discovery; steroid metabolism

048 Evaluation of the Risk of Incident Pancreatic Cancers in a High-Risk Family Registry

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Pancreatic Cancer is the fourth leading cause of cancer death in the United States, with a 5-year survival rate of <5%, the poorest of any major tumor type. Cigarette exposure and having a family history of pancreatic cancer are the only well-established risk factors for this disease. In 1994, the National Familial Pancreatic Tumor Registry (NFPTR) was established to better understand the inherited basis of pancreatic cancer. A total of 2,308 families have enrolled as of May 1, 2007. Of these, 844 are "Familial Pancreatic Cancer Kindreds," defined as families in which a pair of first-degree relatives (parent-offspring, sibling pairs) has developed adenocarcinoma of the pancreas. All families are prospectively followed. The aim of this study was to quantify an individual's risk of developing pancreatic cancer according to the family history of the disease using prospectively collected incidence data from our Registry.

To date, 55 family members who were free of pancreatic cancer at enrollment in to the NFPTR have developed adenocarcinoma of the pancreas. Of these 55 incident cancers, 51 developed in individuals genetically related to the index cases. One of the incident patients was detected by screening as part of our ongoing NCI-sponsored, Cancer of the Pancreas Screening Trial (CAPS). Time-to event analysis using Cox proportional hazards modeling was performed to examine the risk of pancreatic cancer according to family history (e.g., member of familial vs. sporadic pancreatic cancer kindred, or unrelated individual) and cigarette exposure. Approximately 32,000 person-years of followup were available, with a mean length of followup of 3.4 years (range, 0-12.5). Cigarette smoking did not appear to be a major risk factor for pancreatic cancer in these data (relative hazard [RH] = 1.05; 95% CI, 0.57-1.96). While members of sporadic pancreatic cancer kindreds did not have a significantly increased relative hazard (RH = 1.14; 95% CI, 0.32-4.04), members of familial pancreatic cancer kindreds were at a significantly increased risk of developing pancreatic cancer (RH = 3.34; 95% CI, 1.17-9.8) compared to unrelated individuals. This is comparable to the ~3.7-fold difference between members of familial pancreatic cancer kindred and unrelated individuals we previously reported in a smaller series. We have previously demonstrated that unrelated individuals in this cohort are at higher risk of pancreatic cancer than the general population, most likely due to household clustering of environmental risk factors. Hence, these risk estimates underestimate risk of pancreatic cancer in this cohort compared to the general population. We will also present the incidence of pancreatic cancer in this cohort relative to the incidence of pancreatic cancer in the general U.S. population. The use of unrelated household members as comparison group does control for unmeasured confounders such as registry referral patterns etc.

In summary, individuals with a family history of pancreatic cancer are at higher risk of developing pancreatic cancer themselves. Individualized risk assessment using risk prediction tools such as PancPRO and early detection screening represent important steps in the management of individuals with a family history of familial pancreatic cancer.

Keywords: epidemiology; family history; pancreatic cancer

049 Comparing the Reliability of Responses to Telephone-Administered vs. Self-Administered Web-Based Surveys in a Case-Control Study of Adult Malignant Brain Cancer

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To determine whether a self-administered Web-based survey was an acceptable method of data collection for a case-control study of adult brain cancer, we compared the reliability of paired responses to a main survey and a short resurvey for participants completing both surveys either by telephone (n = 79) or self-administered on the Web (n = 469). The resurvey contained a subset of 25 variables from the main survey including smoking and oral contraceptive histories, residence histories, water sources, meat preparation, fruit and vegetable consumption, and pesticide use. Weighted kappas were estimated for categorical variables (including recoded continuous variables) and simple kappas were estimated for binary variables. Concordant paired responses were summed to create a normally distributed variable for linear regression. Respondents were 95% white, with 85% having post-secondary education. Kappas for individual questions ranged from 0.30 (duration of residence in a single-family house) to 0.96 (ever-smoker vs. never-smoker); across variables, the median kappa was 0.57 (95% CI, 0.38-0.91). Of 25 paired responses, median number of concordant responses was 18 (95% CI, 14-22). Reliability was greater for controls than for cases, and for Web-based vs. telephone-administered surveys. More online sessions to complete the survey, female gender, and higher income were associated with greater reliability. Frequency of e-mail and Internet use was not associated with reliability. By allowing the respondent to determine the timing and pace of the survey, Web-based surveys could be a reliable method for data collection in samples with higher socioeconomic status.

Keywords: brain tumors; epidemiology; validation

050 The Cancer Genetics Network: A Resource for Research

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In 1998 the National Cancer Institute (NCI) established the Cancer Genetics Network (CGN) to support collaborative investigations into the genetic basis of cancer susceptibility, explore mechanisms to integrate this new knowledge into medical practice, and identify ways of addressing the associated psychosocial, ethical, legal, and public health issues. Eight recruitment centers enrolled individuals with cancer and/or a family history of cancer and collected data on family cancer history, health history, and environmental and behavioral risk factors that are stored in a Core Registry. These Core data can be queried to identify eligible individuals for proposed studies. Investigators within and outside the CGN are encouraged to utilize this infrastructure for research. A Data Coordinating Center is available to assist in designing, conducting, and analyzing studies and provides a Web-based data entry system (Trial/DB).

The CGN has conducted a variety of pilot studies: Ovarian Cancer Screening (using longitudinal biomarker levels), Inherited Colon Cancer Among Sibling Pairs, Families with Prostate Cancer, Genetic and Environmental Modifiers of Cancer Risk Among Women With BRCA1/2 Mutations, Validation of Models Estimating Risk of Being a BRCA1/2 Carrier, and Breast Screening Study. As of May 2007, the CGN Core Registry contained data on 26,746 probands (participants) and 500,068 family members. Among the probands are 21,056 cancer diagnoses: 3,676 prostate, 7,915 breast, 1,956 colorectal, 368 lung, and 736 ovarian, as well as many other less prevalent cancers. In addition, many of the families in the CGN have familial or hereditary cancers. Most notably, there are 9,127 families with familial breast cancer and 843 families with familial ovarian cancer. There were also 3,707 instances of familial prostate cancers, and 2,706 familial colorectal cancers.

Future plans include collection of biospecimens from participants and family members with multiple and/or early-onset cancer diagnoses to facilitate genetic linkage and association studies. The CGN is a rich resource for collaborative projects in cancer genetic susceptibility, translational research, screening and prevention studies, and behavioral research. For further information see http://hedwig.mgh.harvard.edu/cgnpub/ or contact dfinkelstein@partners.org.

Keywords: genetic susceptibility; registry; statistical coordinating center

051 A Collaborative Binational Study of Arsenic Exposure: The BAsES Study in Arizona and Sonora, Mexico

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Arsenic is recognized as a human skin, lung, and bladder carcinogen and has been associated with other solid tumors following chronic ingestion or inhalation. Only limited attention has been paid to the potential for an effect on gastrointestinal cancers. Elevated levels of As are found in groundwater reserves of Arizona and northern Mexico. This project is seeking to evaluate associations between elevated arsenic exposure and selected genetic polymorphisms that are considered either to be part of carcinogenesis pathways or to potentially modulate arsenic metabolism. There are two distinct study approaches.

Project 1 focuses on determining effect(s) of environmentally relevant doses of As and As metabolites on cell proliferation and cancer-related signaling pathways in normal and colon cancer cell lines. Using stably transfected colon cancer and colon cell lines where APC expression is under experimental control, cell variants were exposed to arsenic (as sodium arsenite) at 50 and 100 ppb for up to 7 days. Data suggest functional status of APC affects the interaction of colon cancer cells with arsenic, and ingestion of arsenic may further exacerbate the effects of mutated APC in the development of colon cancer.

Project 2 is the Binational Arsenic Exposure Survey (BAsES). This population study seeks to evaluate the association between current As exposures and metabolite profiles at a variety of groundwater As concentrations and to determine whether these associations are modulated by genetic polymorphisms in critical pathways identified in Project 1. Households were recruited (152 in Arizona, 225 in Sonora) and adults older than age 18 were asked to provide urine, blood, buccal cell, and toenail samples and complete questionnaires regarding historical exposures and diet. Water samples were collected from all water sources in each household, including tap water (filtered and unfiltered) and bottled water. Preliminary household results in Arizona show total As concentration in drinking and unfiltered water ranged from nondetectable to 1,004 parts per billion (ppb), with 28% of households exceeding US-EPA standard of 10 ppb. Total As concentration from 116 wells in Sonora ranged from 0.80 ppb to 45.2 ppb and over 57% of households exceeded current US-EPA standards. In Arizona, weighted total As exposure from drinking water was 13.47 ppb/person/day (range: nondetectable-576.94 ppb). Mean inorganic urinary As concentration per person was 38.51 ppb (range: 1.64 ppb-756.08 ppb). The Spearman correlation between weighted total As consumption and urinary inorganic As was 0.30 ($p<.001$). Further analyses will contrast Mexico and Arizona and include potential arsenic metabolism and carcinogenesis genes.

Keywords: arsenic; gastrointestinal cancer; genetic risk

052 Genetic Biomarkers of Prostate Cancer Risk in African Americans

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Although the origin of the disparity is unknown, the incidence of prostate cancer in African Americans is 1.6 times that in Caucasians. Data from animal studies and from examination of prostatectomy specimens suggest that oxidative stress is an early contributor to prostate cancer risk. African Americans commonly inherit genotypes associated with increased oxidative stress, including G6PD deficiency, alpha thalassemia, and sickle trait. In addition, African mitochondrial haplotypes exhibit tightly coupled oxidative phosphorylation, which increases energy production and production of superoxide radicals. Preliminary data suggest that, in African Americans, inheritance of G6PD A- (which is specific for Africans and results in enzyme deficiency) or the mitochondrial 10398A allele (which alters the structure of Complex 1 of the electron transport chain and is associated with increased free-radical generation) may be linked to prostate cancer. We hypothesize that in African American men, inherited mutations that increase generation of reactive oxygen species or decrease cellular capacity to manage oxidative stress may contribute to development of prostate cancer. We are extending our preliminary studies to evaluate germline single-nucleotide polymorphisms in a series of nuclear and mitochondrial genes associated with generation of or protection from reactive oxygen species in a much larger group of African American prostate cancer patients and healthy, age-matched African American controls. We will present early results from the case-control study.

Keywords: African American; biomarkers; SNP

053 New-Onset Diabetes Precedes Onset of Symptoms and Diagnosis of Pancreatic Cancer: Implications for Early Diagnosis of Pancreatic Cancer

Please see Poster Discussion Session Biomarkers II.

054 Patient Choice in Clinical Trials: Does it Exist?

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For many patients with difficult to treat or rare cancers, treatment choices can be quite limited no matter where they look. For example, their choices of FDA-approved treatments in a nonclinical trial setting might be slim to none; their standard of care treatment (if it exists) might not be curative; and therefore their remaining treatment options might be regimens not approved, not proven effective, or approved and marginally effective for their condition based on controlled clinical studies. This is a very difficult set of circumstances for patients to choose from, even in the best of scenarios.

Making a choice generally involves a process of evaluating or reviewing options and then making a selection. A 2001 Harris Survey of cancer patients reported 85% of patients surveyed were not aware that a clinical trial was a treatment option. This begs the question—did these patients really have a choice or not (see reference)? Patients become informed about clinical trials from myriad sources—nurses, other health care professionals, the Internet, and, most commonly, their doctor. In the Harris study, two-thirds of trial participants said that a physician strongly influenced their decision to participate in the clinical trial.

Patient accrual rates on cancer clinical trials for adults hover at a lowly 4%-6%. This low rate of accrual seemingly tells us that many patients are choosing not to participate in clinical trials; however, we do not have a clear understanding of what choices they are making and why. There is also a significant need and a lack of data on the health professional side of the coin about the practices, data collection methods, and timing of the recruitment and accrual process.

Much emphasis and attention are placed on accrual results; for example, total number of patients accrued in a given timeframe is a routinely reported metric. Much less attention is given to the process and methodology involved with the recruitment process itself and given the low rates of accrual, further examination of the details and intricacies of these processes is warranted.

Accrual is simply the result of a recruitment process aimed at accrual; it is not a process aimed at providing patients with all their choices. Improve the process and the choices and improved accrual will follow.

Reference

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Keywords: choice; clinical trials; research

055 Patient Advocacy in Kidney and Adrenal Cancer

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From the perspective of the laboratory, the mechanisms of kidney cancer have much in common. Research focuses on the pathways to kidney cancer initiation, development, and proliferation, and ways to constrain the proliferation, shrink tumors, and control the disease. Biomarkers are sought to help identify people who might be carrying an asymptomatic tumor, or to measure total tumor burden, providing one measure of the success of treatment or the danger of relapse.

From the perspective of the patient, there are slightly different but complementary goals. We want earlier diagnosis of kidney cancer. For people with sporadic tumors, a biomarker test would be ideal. For families at risk for kidney cancer, ways of identifying family members at risk, education of family members to help monitor for signals of trouble, and strategies for surveillance and treatment are extremely important.

Most kidney tumors today are diagnosed at relatively advanced stages, often after metastasis has occurred. Most current drug development is appropriately focused at this stage. However, with better diagnostic tools and better family risk assessment and surveillance, tumors could be found at increasingly earlier stages. Drugs and strategies for dealing with smaller tumors will be different from those for advanced tumors. In addition to measuring "cancer-free survival" there should be measures for quality of life.

As one example, 15 years ago the "standard of care" for kidney cancer was that if you saw one tumor, you removed the entire kidney. While that was a good way to ward off recurrence, it was also a quick way to wind up on dialysis. We were focused on cancer-free survival and not on quality of life. We have already come a long way. But it is time to move yet another step forward in our thinking—from chemotherapy to targeted therapy and progressively greater quality of life.

Even worse diagnostic challenges face people with pheochromocytoma. The symptoms are unclear, and are all too often attributed to teenage or female hormones, stress, attention deficit (sometimes labeled ADHD or dyslexia), or psychological issues. We need better tools to assist doctors in looking beyond the surface to deduce any underlying medical problems.

As patient advocates, we keenly appreciate our partnership with physicians and researchers working to improve diagnosis, treatment, and quality of life.

Keywords: kidney cancer; pheochromocytoma; renal cell carcinoma

056 Recent Advances in Risk Prediction for BRCA1 and BRCA2 Carriers

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BRCAPRO is a widely used statistical tool in counseling individuals at risk of the hereditary breast and ovarian cancer syndrome (HBOC). It uses family history and clinical information to predict the probability that an individual carries a BRCA mutation. BRCAPRO is regularly updated to include the latest findings on markers, penetrance, and prevalence. Here we describe three different projects that led to significant modifications of the algorithm.

We incorporated a molecular profile consisting of ER status, HER2 status, PR status, and CK5/6 and CK14 status into the BRCAPRO model to enhance its ability to distinguish BRCA1 mutation carriers. We show what impact these markers have on the BRCAPRO probability prediction.

Because different penetrance estimates lead to different risk assessment, which is currently problematic in counseling, we performed a comprehensive meta-analysis to draw on all eligible penetrance studies to date to produce one set of penetrance. This set of penetrance, once incorporated into BRCAPRO, can be used by counselors and clinicians who are interested in advising patients on the basis of a comprehensive set of studies.

Male breast carcinomas are important features of HBOC families. We estimate the risk of developing breast cancer for male mutation carriers from a large U.S. cohort. These risk estimates help establish the association of male breast cancer with both BRCA1 and BRCA2 mutations. Adopted by BRCAPRO, they will assist in counseling and managing male mutation carriers and their relatives.

Keywords: biostatistics; prediction; risk

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

057 Breast MR Cancer Volume Measurements for Prediction of Outcome in Patients on Neoadjuvant Chemotherapy

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Purpose: To determine whether automated measurements of breast MRI tumor volumes using in-house software and commercially available software are predictive of length of recurrence-free survival (LRFS).

Methods and Materials: A total of 69 patients (mean age, 48 years) receiving neoadjuvant chemotherapy for locally advanced breast cancer underwent dynamic breast MRI at 1.5T before and after treatment. Tumor volumes were automatically determined using in-house software and using a commercial package (CADSTREAM™) based on summing voxels with an enhancement threshold of 70% over pre-contrast signal intensity. Volume response was calculated as percentage change in tumor volume pre- and post-treatment. LRFS was calculated as the time between surgery and occurrence of local or distant metastasis or death. Agreement between the two methods was assessed with Bland-Altman regression analysis. Cox regression model was used to assess relative risk (RR) of baseline and changes in tumor volume to predict disease-free survival for each of the two volumes. Bootstrap method was used to compare differences in derived RR by the two methods.

Results: Volumes measured by the two methods were highly correlated. Correlation coefficients between visits ranged from 0.83 to 0.94 ($p<.0001$ for all). Change in volume from baseline to last visit was also highly correlated ($r = 0.89$; $p<.0001$). Bland-Altman regression analysis suggested the two methods were exchangeable ($p>.99$ for all visits as well as percentage changes). Both baseline volumes and percentage changes measured by both methods were significantly predictive of LRFS. RR per cm^3 increase in tumor volume was 1.023 and 1.019 for baseline volume ($p<.001$) of CADSTREAM™ and our software, respectively. Similarly, the RRs for 1% change in volumes were 1.0178 and 1.0194 respectively for the two modalities.

Conclusions: Automated volume measurements using a commercial package were predictive of length of time to recurrence-free survival and produced results similar to those reported previously using in-house software. Our results support the robustness of volumetric measurements of tumor response as a surrogate endpoint for survival outcomes.

Clinical Significance: Automated serial breast MR tumor volume measurements are reproducible and may prove to be a valuable surrogate endpoint for survival outcomes.

Keywords: breast; recurrence; volumetric

058 Genomewide Approach to Identifying Genetic Variants That Contribute to Chemotherapy-Induced Cytotoxicity

Please see Poster Discussion Session New Technologies and Methodologies I.

059 New Model for Studying Invasion and Angiogenesis in Glioblastoma Multiforme

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Mitochondria play an important role in cellular energy metabolism, free radical generation, and apoptosis. Defects in mitochondrial function have long been suspected to contribute to the development and progression of all types of cancer. In particular, cancer cells are highly glycolytic. Previously, we described and characterized two different bioenergetic phenotypes in human and mouse glioma cells (see reference). In particular, one cell line, U-251MG, was found to be heterogeneous, with 20% of the cell population having a functional oxidative phosphorylation pathway (OXPHOS), indicating that these cells can switch from glycolysis to OXPHOS to generate their main source of energy. In the present study, we investigated the role of mitochondria in tumor growth, invasion, and angiogenesis in vitro and in vivo. For this, we generated a mitochondria-depleted glioma cell line, U-251MG rho, from isogenic counterparts with mitochondria. U-251MG rho cells exhibit a higher rate of glucose consumption and a lower rate of oxygen consumption compared to their parental counterparts. Moreover, they display a higher number of

forming colonies, indicating significant increase of anchorage-independent growth. The in vitro invasion assay indicated that U-251MG rho invaded significantly more than their parental cell line. Finally, U-251MG rho cells implanted into the brains of nude mice significantly increased in the angiogenic profile as determined by an increase in blood vessels, particularly at the edges of the tumor, with a resulting increase in tumor size. These data (1) indicated that depleting mitochondria in glycolytic glioma cells revealed a major role in increasing tumor invasion and angiogenesis and (2) constitute a new tumor model to study invasion and angiogenesis in glioma.

Reference

Griguer et al., 2005.

Keywords: angiogenesis; gliomal; invasion

060 Induction of Osteogenesis in Osseous and Non-Osseous Sites by an Androgen Receptor-Negative Human Prostate Cancer Xenograft

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In prostate cancer, androgen-blockade strategies are commonly used to treat osteoblastic bone metastases, but responses to these therapies are of short duration and eventually tumor progression occurs. The mechanism for androgen-independent progression in bone is not clear. We established two prostate cancer xenografts (MDA PCa 118a and MDA PCa 118b) from an osteoblastic bone metastasis in a man with castration-resistant prostate cancer. These xenografts are androgen receptor negative and grow more in castrated than in intact male mice. Also, these cells induce a robust osteoblastic reaction in bone and in the subcutis of immunodeficient mice. We found that these cells display a pattern of gene expression similar to that of the osteolytic prostate cancer cell line PC3, in that both MDA PCa 118 and PC3 express bone morphogenetic proteins, Wnt, and endothelin-1 as well as the Wnt inhibitor Dickkopf-1 and the osteoclast-activating factor parathyroid hormone-related peptide, suggesting that other factors are involved in MDA PCa 118-induced osteogenesis. In a gene array analysis, we identified FGF9 as being overexpressed in the xenografts relative to other bone-derived prostate cancer cells of mildly blastic (MDA PCa 2b) or lytic (PC3) phenotypes and discovered that FGF9 induced osteoblast proliferation and new bone formation in a bone organ assay. Collectively, these results suggest that FGF9 contributes to MDA PCa 118-induced osteogenesis and may participate in the osteoblastic progression of prostate cancer in bone. MDA PCa 118 is the first human prostate cancer xenograft that does not express androgen receptor, yet it induces strong osteogenic response in both bone and the subcutis of immunodeficient mice. These observations suggest that androgen receptor-null cells contribute to the castration-resistant osteoblastic progression of prostate cancer and imply that targeting these cells will be critical in the treatment of prostate cancer bone metastases. In addition, this new osteogenic xenograft will be an important preclinical model for therapy testing.

Keywords: androgen receptor; bone metastases; prostate cancer

061 Validation of Chromogenic In Situ Hybridization for Detection of EGFR Copy Number Amplification in Non-Small Cell Lung Carcinoma

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Epidermal growth factor receptor (EGFR) gene copy number has been found to correlate with response to tyrosine kinase inhibitors (TKIs) in patients with non-small cell lung carcinoma (NSCLC). Fluorescence in situ hybridization (FISH), a standard methodology to detect EGFR copy number abnormalities in NSCLC, is limited by instrumentation and cost. Chromogenic in situ hybridization (CISH) is an emerging alternative hybridization detection technique using light microscopy, but its utility in assessing EGFR copy

number in lung cancer is not established. To address the utility of CISH, we studied paraffin-embedded NSCLC specimens from 77 Taiwanese nonsmoking women treated by surgery alone. We recorded the number of signals per tumor cell nucleus for each case, correlated EGFR copy number by CISH with FISH results, and used receiver operating characteristics to identify cutoff points for the CISH results. Tumors were classified according to WHO criteria as adenocarcinoma (n = 28), mixed adenocarcinoma with bronchioloalveolar features (n = 25), bronchioloalveolar carcinoma (BAC) (n = 2), squamous cell carcinoma (n = 15), and adenosquamous carcinoma (n = 7). By FISH, 28.5% of cases had no amplification, 18% had low polysomy, 35% had high polysomy, and 12% had gene amplification. EGFR copy number detected by CISH highly correlated with FISH (Spearman r = 0.81; p<.0001). We determined the optimal EGFR CISH cutoff points able to discriminate between no amplification and low polysomy (3.2 signals; p = .08); no amplification plus low polysomy and high polysomy plus gene amplification (4.5 signals; p<.0001); and high polysomy and gene amplification (7.9 signals; p = .0003). CISH is an alternative assay to FISH in determining EGFR copy number status that may contribute to stratification of patients with NSCLC for clinical trials and identify a subset of patients that should be treated with TKIs.

Keywords: adenocarcinoma; chromogenic in situ hybridization; EGFR

062 KRAS and BRAF Mutational Activation Does Not Correlate With ERK Mitogen-Activated Protein Kinase Activation or to Sensitivity to MEK Inhibitor Treatment in Colorectal Cancers

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A critical role for mutational activation of Ras in colorectal cancer (CRC) progression and maintenance is well established. Therefore, much emphasis has been placed on the development of anti-Ras therapies, with current efforts mainly focused on the development of inhibitors of the Raf-MEK-ERK mitogen-activated protein kinase cascade, a key downstream effector of Ras-mediated oncogenesis. However, the therapeutic value of inhibitors of the ERK pathway is complicated by the fact that Ras also utilizes Raf-independent effectors to promote oncogenesis. Therefore, it is unclear whether inhibitors of the Raf-MEK-ERK pathway will be effective for CRC treatment. To assess this issue, we first evaluated a panel of 10 CRC cell lines and unexpectedly found no correlation between *KRAS* or *BRAF* mutation status and ERK activation. Second, elevated ERK activation did not correlate with sensitivity to U0126 and CI-1040 MEK inhibitor-mediated growth inhibition. Third, MEK inhibitor reduction of ERK activity did not correlate with inhibition of growth. Finally, we validated our cell line observations and found that ERK activation did not correlate with *KRAS* or *BRAF* mutation status in patient CRC tissue. Surprisingly, we also found that ERK activation was elevated in normal colon tissue. Our results suggest that *KRAS* or *BRAF* mutation status or ERK activation will not be useful biomarkers for MEK inhibitor sensitivity, that ERK activation will not provide an accurate biomarker for patient selection for MEK inhibitor treatment, and that normal colonic tissue toxicity may compromise the therapeutic efficacy of these inhibitors.

Keywords: colorectal cancer; ERK; MEK inhibitors

063 Similar Regions of Allelic Losses in Low-Grade Serous Ovarian Tumors With and Without an LMP Background

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Objectives: A two-tier system for classifying invasive serous ovarian tumors into low-grade and high-grade groups is gaining more acceptance. It has been hypothesized that low-grade and high-grade serous ovarian tumors develop from two different pathways. We hypothesized that low-grade serous ovarian tumors exist along a continuum with low malignant potential (LMP) tumors of the ovary. To test this hypothesis, we investigated the allelic imbalances in low-grade serous ovarian tumors with and without an LMP background. We also included a few high-grade tumors.

Methods: To maximize detection of allelic imbalance, we used laser capture microdissection to obtain pure tumor cells from frozen sections. Fifteen low-grade serous ovarian tumor samples (2 stage 1, 1 stage 2, and 21 stage 3C) were analyzed using single

nucleotide polymorphism (SNP) arrays. Eight of the stage 3C low-grade tumors have an LMP background. In addition, 13 stage 3C high-grade tumors were also included in this study. SNP genotyping data were processed using dChip software to detect allelic losses. Selected SNP loci with allelic losses or loss of heterozygosity were further validated by sequencing of the polymerase chain reaction products amplified from the corresponding SNP loci in DNA extracted from microdissected stromal and tumor cells.

Results: There was an increase in allelic losses from early-stage to late-stage tumors. Allelic losses were detected in chromosomes 1, 4, 5, 6, 8, 9, 10, 13, 14, 15, 16, 17, 18, 19, and 22. In contrast to high-grade serous ovarian tumors analyzed in this study, no allelic losses were detected in chromosomes 12 and 21. The most common regions with LOH in low-grade ovarian serous carcinomas are located at 1p (15 of 24), 6q (3 of 24), 9p (7 of 24), 18q (5 of 24), and 22q (8 of 24). Allelic losses at 1p, 6q, and 22q were detected in tumors both with and without an LMP background. A homozygous deletion at 9p21 was also detected in one of the tumors.

Conclusions: Low-grade serous ovarian tumors, with and without an LMP background, have similar regions of allelic losses, supporting the hypothesis that low-grade serous ovarian tumors exist along a continuum with LMP tumors of the ovary. The novel regions shown to have allelic losses may harbor tumor suppressor genes.

Keywords: allelic loss; low malignant potential; ovarian cancer

064 Podoplanin Expression Is a Novel Marker of Oral Cancer Risk in Patients With Oral Premalignancy

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Background: Oral leukoplakia (OL) is a heterogeneous oral lesion that confers an increased oral cancer risk. However, clinical assessment is not sufficient to distinguish premalignant and benign conditions in OL lesions. We recently reported that podoplanin, a lymphatic endothelial factor, is highly expressed in oral cancer and some oral premalignancies. The purpose of this study is to evaluate podoplanin as a biomarker in assessing oral cancer risk in patients with OL.

Methods: Podoplanin expression was determined in 150 OL patients with long-term followup using immunohistochemistry. Associations between the protein expression patterns and clinicopathologic parameters including cancer development during followup were analyzed.

Results: Of the 150 OL specimens, 108 (72%) expressed podoplanin including 56 (37%) with expression that extended above the basal layer (podoplanin-positive cases). Podoplanin positivity was more frequent in older patients ($p = .016$), females ($p = .020$), and dysplastic histology ($p = .040$). Patients whose OL showed podoplanin positivity developed significantly more invasive oral cancers than those whose OL were podoplanin-negative ($p = .0002$). At 5 years, 92% (95% CI, 0.86-0.98) of the podoplanin-negative patients were oral cancer-free, compared to only 63% (95% CI, 0.51-0.77) of the podoplanin-positive patients. In the multivariate analysis, podoplanin positivity and histology were the only independent factors for oral cancer development (hazard ratios: 2.81, CI, 1.4-5.68, $p = .004$; 2.01, CI, 1.02-3.96, $p = .04$, respectively). Oral cancer risk can be better predicted by considering the two factors together.

Conclusions: Podoplanin is frequently expressed in OL. Together with histology, podoplanin may serve as a powerful biomarker to predict the risk for oral cancer development in patients with OL.

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Keywords: leukoplakia; podoplanin; premalignancy

065 A Tissue Biomarker Panel That Predicts Which Men With a Rising Prostate-Specific Antigen Postdefinitive Prostate Cancer Therapy Will Have Systemic Progression

Please see Poster Discussion Session Biomarkers I.

066 Blast Transformation in Chronic Myeloid Leukemia: Genomic Mapping and Mutation Tracking Supports the Presence of Multiple, Distinct Clones at Time of Therapy Resistance

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Background: Leukemia transformation in chronic myeloid leukemia (CML) provides a model for understanding both resistance to tyrosine kinase inhibitors (TKIs) and the genetic changes associated with acute leukemia. CML usually proceeds through accelerated phase (AP) to blast crisis (BC), which is frequently associated with major genetic changes (e.g., trisomy 8 and loss of chromosome 17q). Similarly, TKI resistance and blast transformation are correlated with the development of bcr-abl kinase domain mutations (KDM) in approximately half of cases. However, in 20%-40% of AP/BC-CML, the factor(s) contributing to transformation are not apparent.

Methods: We studied three categories of *imatinib-resistant* AP-BC CML: Type A included cases with major chromosomal losses/gains apparent by routine G-banded karyotype; type B included cases with a dominant KDM documented by direct DNA sequencing of bcr-abl transcripts; type C had neither karyotypic abnormalities nor KDM detected. Array comparative genomic hybridization (aCGH) was done using a 44,000-feature 60-mer oligonucleotide whole genome array (Agilent Technologies), with total genomic tumor DNA probed against normal reference DNA. Data were analyzed using CGH Analytics software. Comparison was made with G-banded karyotype on parallel samples, with confirmation of some aCGH copy number alterations using quantitative genomic PCR (TaqMan method, Applied Biosystems). In most cases, we also profiled a second resistant AP/BC tumor that emerged after switch to a second therapy, which was usually another TKI with or without cytotoxic chemotherapy. To track the fate of KDM, we used quantitative pyrosequencing and mutation-specific quantitative PCR.

Results: 58 AP/BC samples from 29 patients with resistance type a (n= 10), b (n = 10), and c (n = 9) were studied by aCGH. The median number of different, unequivocal genetic alterations detected by aCGH were 9, 4, and 7 in types A, B, and C, respectively. Aside from trisomy 19 and iso17q/del17q, chromosomal gains at chromosome 1p36 were the most frequent alteration in AP/BC-CML seen in 37% of all samples analyzed; gains of material at chromosome 12q12 were seen in six patients (21%). Initial and secondary resistant samples were clonally related in 62% of cases; however, rapid shifts over 3-6 months to unrelated clones with loss or gain KDM were seen in 38% and occurred in all resistance types.

Conclusions: Array CGH identifies commonly altered genetic loci in transformed CML that were not seen by G-banded karyotype and could serve as markers of progression. Blast transformation in CML appears clonally complex with rapid shifts to distinct subclones apparent by simultaneously tracking fate of KDM and genetic changes by aCGH. These findings suggest that therapeutic strategies that reduce clonal complexity and cross-resistance may help slow the rapid outgrowth of multiple, distinct resistant clones.

Keywords: genomics; kinase inhibitors; therapy resistance

067 Elevated Expression of Activation-Induced Cytidine Deaminase in Peripheral Blood Mononuclear Cells Precedes AIDS-NHL Diagnosis

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Background: AIDS-associated non-Hodgkin's B cell lymphoma (AIDS-NHL) is a common cancer in HIV infection. Many NHLs are thought to result from errors in class switch recombination (CSR) and/or somatic hypermutation (SHM), processes that occur in germinal center (GC) B cells and require the activity of activation-induced cytidine deaminase (AID). Since NHL is a common cancer in HIV infection, and expression of AID can contribute to the molecular lesions that result in the development of NHL, we quantified AID expression in peripheral blood mononuclear cells (PBMCs) collected prior to the diagnosis of AIDS-NHL.

Methods: AID mRNA levels were measured by TaqMan RT-PCR in viably frozen PBMC, obtained prior to AIDS-NHL diagnosis, from 16 HIV-infected subjects who developed AIDS-NHL, and from control subjects (AIDS but no NHL, and HIV-negative subjects), from the Multicenter AIDS Cohort Study (MACS).

Results: PBMC AID expression was seen to be markedly elevated in those who developed AIDS-NHL, when compared to AIDS and HIV-negative controls. In some cases, elevated AID expression was seen over a period of several years pre-NHL diagnosis. Additionally, AID expression was seen to differ depending on NHL subtype, with the highest levels of expression seen in those who developed Burkitt's lymphoma, and little AID expression seen in those who developed primary CNS NHL. B cells were isolated from PBMCs of one subject who went on to develop Burkitt's lymphoma, by positive selection using CD19 beads. AID expression was seen to be 25-fold higher in these enriched B cells than in the PBMCs from which they were purified.

Conclusions: The AID-expressing B cells seen in circulation prior to NHL diagnosis may be undergoing IgH CSR and SHM, events that normally occur in GC B cells, and not in circulating B cells. Errors in these DNA modifying events are known to be able to lead to the development of NHL. It is interesting to note that elevated AID expression was generally not seen in those who developed CNS lymphomas, tumors that are virtually all EBV+ and are associated with the loss of EBV-specific cellular immunity, rather than with chronic B cell hyperactivation. It is not known whether the cells that express AID pre-NHL are premalignant B cells or simply circulating activated or GC-like B cells. Studies to better characterize these AID-expressing B cells are planned. Overall, these results indicate that elevated levels of AID expression in circulating B cells precede lymphoma in HIV-infected persons and support the hypothesis that aberrant AID expression may play a significant role in the development of non-CNS AIDS-associated NHL.

Keywords: etiology; HIV/AIDS; lymphoma

068 Comparison of Sample Preparation and Solubilization Techniques for Shotgun Proteomic Analysis of Laser Capture Microdissected Cells From Human Breast Tumors

Please see Poster Discussion Session New Technologies and Methodologies I.

069 Phenotypic Differences Between NRAS-Mutated, BRAF-Mutated, and Wild-Type Human Melanomas

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Introduction: Mutations of *NRAS* and *BRAF*, components of the mitogen-activated protein kinase pathway, have been well described in human melanoma. *NRAS* mutations are found in 15%-20% of tumors, the majority occurring in codon 61 (nucleotides 181, 182, and 183). *BRAF* mutations, identified in approximately 60% of melanomas, are found almost exclusively in exon 15 as a T1799A mutation. Despite the frequency of these mutations, a distinct clinical phenotype has not been described that distinguishes the behavior of *NRAS* or *BRAF* mutation-bearing tumors from those carrying wild-type (WT) genes. The relatively small number of samples in most studies, and DNA purification techniques that often rely upon macrodissection, may have limited previous attempts to accurately identify associated phenotypes. Accordingly, we have initiated a study, the primary objective of which is to utilize microdissection of human primary melanomas to obtain pure tumor DNA from a large sample cohort, in order to define biologic and clinical phenotypes associated with these various genotypes.

Methods: Cells from the invasive components of primary melanomas were harvested by laser capture microdissection. After lysis, DNA was purified, amplified with primers specific for *NRAS* exon 2 and *BRAF* exon 15, and sequenced in both directions. Sequences were interpreted by two observers (JAE and VRG). Mutations were identified on both forward and reverse sequences in all cases. Clinical information obtained for each case included Breslow thickness, thickness ≤1 mm, ulceration status, and the presence or absence of nodal involvement (usually identified in sentinel nodes).

Comparison of Mutated and WT Melanomas

	NRAS mutant	*BRAF* mutant	WT	*p* value
Number	13	68	38	
Breslow mm (median)	1.70	1.40	0.98	0.020, *NRAS* vs. WT
Thickness ≤1mm (%)	15.4	37.3	55.3	0.013, *NRAS* vs. WT
Ulceration (%)	15.4	16.2	10.5	not significant
Nodal involvement (%)	46.2	33.8	18.4	0.048, *NRAS* vs. WT

Results: To date, the *NRAS* and *BRAF* status has been determined for 119 patients. Thirteen cases (11%) carried *NRAS* mutations, 68 (57%) had *BRAF* mutations, and 38 (32%) were WT. The pathologic and clinical features for each group and the results of statistical analysis are described in the table.

NRAS mutants displayed the most aggressive phenotype and were significantly different from WT tumors in terms of Breslow thickness, the percentage of tumors ≤1 mm thick, and the rate of progression to regional lymph nodes. Values for *BRAF* mutants generally fell between those for *NRAS* mutants and WT tumors. Ulceration rates did not differ among the three groups.

Conclusion: Melanomas harboring mutations in *NRAS* codon 61 display an aggressive phenotype, with a greater capacity for dermal invasion and regional metastasis. WT tumors appear to be more indolent. Melanomas with *BRAF* mutations demonstrate a phenotype intermediate to *NRAS* mutants and WT tumors, a finding that argues against the general assumption that *NRAS* and *BRAF* mutations are biologically equivalent.

Keywords: microdissection; mutations; phenotype

070 Molecular Analysis of Metaplastic Breast Cancer: Increased EGFR Gene Copy Number via Aneusomy

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Purpose: Metaplastic breast carcinoma, a rare tumor composed of adenocarcinomatous and nonglandular growth patterns, is characterized by a propensity for distant metastases and resistance to standard anticancer therapies. We sought confirmation that this tumor is a basal-like breast cancer, expressing epidermal growth factor receptor (EGFR) and stem cell factor receptor (KIT). Furthermore, we investigated *EGFR* activating mutations and high copy number (associated with response to tyrosine kinase inhibitor gefitinib) and *KIT* activating mutations (associated with response to imatinib).

Methods: A total of 77 metaplastic cases were identified (1976-2004); 38 with tumor blocks available underwent pathologic confirmation prior to EGFR and KIT immunohistochemical analyses. A tissue microarray of malignant glandular and metaplastic elements was constructed and analyzed immunohistochemically for cytokeratin 5/6, estrogen receptor (ER), progesterone receptor (PR), and p63, and by fluorescence in situ hybridization for *EGFR* and *HER2/neu*. DNA isolated from individual elements was assessed for *EGFR* and *KIT* activating mutations.

Results: All 38 cases were negative for ER, PR, and (except one) HER2. The majority were positive for cytokeratin 5/6 (58%), p63 (59%), and EGFR overexpression (66%); 24% were KIT positive. No *EGFR* or *KIT* activating mutations were present; 26% of the primary metaplastic breast carcinomas displayed high *EGFR* copy number, secondary to aneusomy (22%) and amplification (4%).

Conclusion: Metaplastic breast carcinomas belong to the basal subtype of breast cancer. While absent *EGFR* and *KIT* activating mutations, the presence of high *EGFR* copy number suggests that EGFR tyrosine kinase inhibitors should be evaluated as a primary therapy in this disease.

Keywords: basal; EGFR; metaplastic

071 Chromosomewide Methylation Analysis in Normal Melanocytes and Melanoma Cells Gives New Insights Into the Effectiveness of DNA Promoter Methylation in Transcriptional Repression

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Aberrant changes in gene activity due to chromatin remodeling are frequent in cancer cells. One such process involves methylation/demethylation of cytosine at cytosine-guanine (CpG) pair-rich islands in promoter regions and first exons of genes. Aberrant gain or loss of DNA methylation is associated with altered expression of genes involved in tumorigenesis and maintenance of the malignant phenotype.

To evaluate the predictive and therapeutic utilities of targeting epigenetic changes in chromatin in melanoma, in particular methylation of CpG-rich islands, we employed genomewide DNA promoter methylation analysis coupled with gene expression analysis in response to 5-Aza-2′-deoxycytidine.

A preliminary analysis of the entire X-chromosome allowed us to evaluate the complexity of the DNA methylation patterns across the different coding and noncoding genome regions in melanoma cells compared to normal melanocytes, and highlighted several interesting regions of differential methylation. In general, melanoma cells seem to be hypermethylated in the upstream, 5'UTR, and coding exon regions compared to normal melanocytes. Moreover, hypermethylation of upstream and 5'UTR in high CpG content regions seems to be highly indicative of transcriptional repression. Following these indications, we further analyzed the DNA methylation level of several melanoma cell strains from early passage cultures and normal melanocytes by means of genomewide promoter tiling arrays.

Our analyses showed that estimates of DNA methylation level at specific genes can be combined with sample type, CpG content, and genome annotation to evaluate the ability of DNA methylation to predict gene transcription. This statistical framework can, for example, be applied to the identification of genes that have been deregulated after change of DNA methylation level in a specific region (e.g., promoter, 5'UTR) and whose expression has been restored after treatment with a DNA demethylation agent like 5-Aza-2'-deoxycytidine. These may pinpoint potential tumor suppression genes.

Keywords: chromatin modification; DNA methylation; genomewide methodology

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

072 Multiplexed In Situ Analysis of Protein Expression (AQUA™) To Predict Breast Cancer Prognosis and Response to Trastuzumab

Please see Poster Discussion Session Biomarkers I.

073 Identification of microRNAs That Modulate Sensitivity to Paclitaxel of Non-Small Cell Lung Cancer

Please see Poster Discussion Session Biomarkers I.

074 Validation and Streamlining of Ovarian Cancer Prognostic Profiles With a Customized Genechip

Please see Poster Discussion Session Biomarkers I.

075 Methylation of CpG89 Within the MGMT Promoter is a Superior Predictor of Temozolomide Sensitivity in the Mayo Glioblastoma Multiforme Xenograft Panel

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CpG methylation within the O6-methylguanine methyltransferase (MGMT) promoter is associated with enhanced survival of glioblastoma multiforme (GBM) patients treated with temozolomide (TMZ). MGMT methylation is routinely assessed by methylation-specific PCR (MS-PCR). Although this assay provides important prognostic information, the accuracy of MS-PCR as a predictive assay is limited. With the aim of identifying a more robust predictive assay, TMZ responsiveness of 13 GBM xenograft lines was correlated with MGMT protein expression, MGMT MS-PCR, and MGMT promoter methylation-specific sequencing. For each xenograft line, mice with established intracranial xenografts were treated with vehicle control or TMZ (66 mg/kg × 5 days), and TMZ response was defined as relative prolongation in median survival for TMZ-treated vs. control-treated mice. The relative survival benefit with TMZ was inversely related to MGMT protein expression ($r = -0.75$; $p = .003$), and survival was marginally higher in MGMT hypermethylated tumors (median survival benefit, 253%; range, 82%-579%) relative to nonmethylated tumors (median survival benefit, 65%; range, 21%-558%; $p = .13$). However, similar to clinical observations, neither assay was sufficient to accurately discriminate between sensitive and resistant xenograft lines. In contrast, methylation sequencing of the nine CpG sites queried by MS-PCR demonstrated that methylation at CpG89 was highly associated with increased TMZ sensitivity (median survival benefit, 277%; range, 82%-579%) as compared to tumors lacking CpG89 methylation (median survival benefit, 60%; range, 21%-110%; $p = .004$). These data support the idea that MGMT promoter methylation is an important determinant of TMZ sensitivity and suggest that more precise delineation of MGMT promoter methylation may provide greater predictive accuracy.

Keywords: glioblastoma; MGMT methylation; temozolomide

076 Gene Expression Signatures of Drug Sensitivity in Lung Cancer

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Lung cancer patients have different responses to chemotherapy because of patient inter-individual variation (pharmacogenomics) and also tumor autonomous differences. We are identifying tumor mRNA (microarrays), protein (reverse phase protein arrays, RPPA, ~100 proteins), and secreted cytokine angiogenic factors (CAFs, ~25) expression signatures that predict for drug sensitivity and resistance prior to treatment using an integrated approach of lung cancer cell lines, xenografts, and patient tumor and blood specimens. To start, ~50 lung cancer cell lines (DNA fingerprinted for provenance) are tested in vitro with a 96-well plate MTS colorimetric assay to determine the growth inhibitory concentration (IC_{50}) of multiple chemotherapeutic drugs (n~20) (drug response phenotype). Large datasets are generated, consisting of a collection of 8 × 12 matrices from each 96-well plate assay (more than three replicates per tumor line and drug). We designed a high-throughput database software called DIVISA (Database of In Vitro Sensitivity Assays) for the purpose of storing and handling these data. The database has a server-client structure and includes various functionalities such as import of new assays, dose-response graphs, calculation of IC_{50}s, and data summaries. User-selected subsets of assay data can be exported to a text file and analyzed with other applications. One such analysis involves a novel clustering method of selecting classes of sensitive and resistant cell lines. For each assay, dose response data are averaged into a single vector, of length equal to the number of doses. For each drug, we can thus perform hierarchical clustering on these assays and different clusters correspond to different sensitivity classes to the drug. Further refinement, using principal component analysis, allows the selection of two or three classes: sensitive, resistant, and intermediate. These classes can then be used to derive mRNA, protein, or CAF signatures of sensitivity to the various drugs. Expression arrays using Affymetrix HG-U133 and Illumina WG6-v2 (all probe sets verified by BLAST) were performed on the cell lines before drug treatment. After background correction, normalization, and transformation, the expression data are analyzed by different algorithms including t-test, correlation, and meta-gene approach to select the signature genes that correlate with the drug sensitivity. We also use a variety of classification methods such as support vector machines, prediction analysis of microarrays (PAM), and decision tree-based methods. Cross-validation is used to evaluate the predicted accuracy rate. Our main conclusions are as follows: (1) Drug responses among the cell lines are tremendously different; for example, IC_{50} of paclitaxel ranges from 1 to 900. (2) Gene expression data can be effectively used to predict the drug sensitivity; still using the paclitaxel example, the cross-validation accuracy rate is 80%. The predicted accuracy rates vary among different drugs, indicating the correlation between drug sensitivity and the gene expression profiles varies. (3) There is no universal best statistical algorithm for feature selection and prediction; further methodology development and validation are needed in this area. (4) Hierarchical clustering of Pearson correlations between IC_{50}s and gene expression can classify the drugs by their mechanisms of action. These signatures are being used to predict the response to therapy of untested lung cancer lines, lung cancer xenografts, and patient tumor and blood samples.

Keywords: drug response phenotype; genomics; prediction signatures

077 Adenovirus-Mediated Gene Expression Imaging To Directly Detect Sentinel Lymph Node Metastasis of Prostate Cancer

Please see Poster Discussion Session New Technologies and Methodologies II.

078 Treatment With Combination Celecoxib and Erlotinib Therapy in Non-Small Cell Lung Cancer Is Associated With a Decline in Serum-Soluble E-Cadherin and Low Baseline Matrix Metalloproteinase-9

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Background: Cyclooxygenase-2 (COX-2) overexpression may mediate resistance to epidermal growth factor receptor (EGFR) tyrosine kinase (TK) inhibition through prostaglandin E2 (PGE2)-dependent promotion of epithelial to mesenchymal transition (EMT) in non-small cell lung cancer (NSCLC). Suppression of epithelial markers such as E-cadherin can lead to resistance to erlotinib, an EGFR TK inhibitor. PGE2 downregulates E-cadherin expression by upregulating transcriptional repressors including

ZEB1 and Snail. Furthermore, loss of E-cadherin can be modulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which stimulate tumor invasion. Markers of EMT and tumor invasion were evaluated from patient serum from a Phase I clinical trial investigating the combination of celecoxib and erlotinib in patients with advanced NSCLC.

Methods: Samples from 22 of 22 subjects were evaluated. Soluble E-cadherin (sEC) was evaluated by ELISA in pt serum at baseline and weeks 4 and 8 of treatment. Other markers of COX-2 gene expression were evaluated by ELISA, including matrix (MMP)-9, TIMP-1, and CCL15.

Results: Serum sEC, MMP-9, TIMP-1, and CCL15 levels were determined at baseline and after 8 weeks of treatment. Levels were analyzed according to best response, partial response (PR), stable disease (SD), or progressive disease (PD) by RECIST guidelines. Patients with PR had a significant decrease in sEC, TIMP-1, and CCL15 between baseline and week 8 when compared to those with SD or PD (p = .021, .047, and .016, respectively). In patients who responded to the combination therapy, baseline MMP-9 was significantly lower compared to that for nonresponders (p = .006).

Conclusions: SEC, MMP-9, TIMP-1, and CCL15 may be useful for assessing response to combination celecoxib and erlotinib in patients with advanced NSCLC. A randomized Phase II trial is planned comparing erlotinib and celecoxib with erlotinib plus placebo in advanced NSCLC. This study will evaluate the efficacy of the combination therapy and assess these and other biomarkers in both serum and tumor tissue.

This research was supported by University of California, Los Angeles Lung Cancer SPORE NCI P50 CA90388, ASCO Young Investigator Award, Nickoll Fund, and GLAVAHS Career Development Award.

Keywords: cyclooxygenase-2; EGFR; epithelial to mesenchymal transition (EMT)

079 HLA Class II Alleles Predict Recurrence and Pattern of Failure in 1,241 Early-Stage Melanoma Patients

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Background. Prior reports have suggested a link between specific HLA Class II alleles, including *HLA-DRB1*1101*, and melanoma recurrence. We therefore investigated the relationship between HLA Class II alleles and clinical outcome in a large population of patients who presented with localized melanoma.

Methods: HLA Class II alleles were determined by typing of genomic DNA from patients who presented with localized melanoma (AJCC stage 1A-2C). Patients found to have occult regional disease by sentinel node biopsy were excluded. Log-rank and Cox analysis of outcome versus standard prognostic factors and common HLA Class II alleles (≥5% of the population) was performed. Because of the large and mature nature of the dataset, a two-step analysis was performed in which primary statistical associations were identified by analysis of a random subset of one-half the patient population (exploratory dataset) and then confirmed by analysis of the remaining half of the population (confirmatory dataset).

Results: A total of 1,241 patients underwent HLA typing. The median primary tumor thickness was 1.1 mm, 16% were ulcerated, and the median followup was 56 months. Results of analysis of the exploratory dataset were in agreement with those of the confirmatory dataset. Tumor thickness, ulceration, and Clark level were independent predictors of recurrence. The HLA Class II allele *HLA-DRB1*1101* (11% of the population) independently predicted a higher risk of recurrence (hazard ratio [HR], 2.04; p = .004) while *HLA-DRB1*0401* (20% of the population) predicted a lower risk of recurrence (HR, 0.42; p = .004). *HLA-DRB1*1101* was a particularly strong predictor of recurrence in stage 1 patients (HR, 2.73; p<.0001). Among patients who recurred, *HLA-DRB1*1101* was an independent predictor of local-regional vs. distant recurrence (HR, 2.7; p = .007), and was more common in those whose first recurrence was isolated to the regional nodal basin, compared to those whose first recurrence was elsewhere or in multiple locations (24% *HLA-DRB1*1101*-positive vs. 14%, p<.009).

Conclusions: Results in this large, early-stage melanoma patient population confirm that *HLA-DRB1*1101* is a genetic marker of increased risk of melanoma recurrence, while *HLA-DRB1*0401* is a marker of decreased risk of recurrence. *HLA-DRB1*1101* is a particularly strong marker among the group of patients predicted clinically to be at the lowest overall risk for recurrence, and independently predicts pattern of recurrence (regional nodal failure). This information is of value in the development of surveillance strategies for melanoma patients, as well as the selection, stratification, and randomization of early-stage melanoma patients for clinical trials.

Keywords: alleles; HLA; recurrence

080 Genomewide Expression Profiles Associated With 5-Aza-2′-Deoxycytidine-Induced Apoptosis in Melanoma Cells

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Aberrant changes in gene activity due to chromatin remodeling are frequent in cancer cells. One such process involves methylation/demethylation of cytosine at cytosine-guanine (CpG) pair-rich islands in promoter regions. Aberrant gain or loss of DNA methylation causes altered expression of genes involved in tumorigenesis and maintenance of the malignant phenotype. The reversible nature of epigenetic changes in chromatin is the rationale for Phase I and II clinical trials with the DNA methylation agent 5-Aza-2′-deoxycytidine (5-Aza-CdR) in the treatment of cancer patients including those with melanoma. However, patient response is variable and molecular markers that can predict or monitor responses are needed to improve the efficiency of this treatment. Indeed, dose-response analysis of several melanoma cell strains revealed differential sensitivity to 5-Aza-CdR in proliferation and apoptotic assays. To assess the molecular basis for this variability, we performed whole-genome differential gene expression studies in response to 5-Aza-CdR employing several resistant and sensitive melanoma cell strains. The results corroborated previous findings, such as IGFBP3, DKK3, and H19 dependent expression on promoter-methylation. Sequencing of bisulfite-modified DNA of promoter regions confirmed the CpG methylation status of several genes. Western blot analysis validated increased protein expression of apoptotic genes in 5-Aza-CdR-sensitive melanoma cells. Finally, bioinformatic gene-enrichment analysis of the data led to the identification of candidate genes and gene classes that can further elucidate the molecular differences between the responsive and nonresponsive melanoma cell strains.

Keywords: gene expression; genomics; promoter methylation

081 FGF2 and FGFRs Comprise an Auxiliary Autocrine Pathway in Non-Small Cell Lung Cancer Cell Lines

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Background: Despite broad co-expression of EGFR and EGF family ligands in non-small cell lung cancer (NSCLC), only a subset of patients respond to EGFR inhibitors. On the basis of the observed multiple growth loops in both NSCLC and SCLC, we proposed that resistance to EGFR inhibitors derives from co-expression of additional receptors that function as auxiliary autocrine loops. When Affymetrix GeneChip data from a panel of 31 NSCLC and 16 SCLC lines were mined for co-expression of additional growth factors and their cognate receptors, frequent co-expression of the FGF2 and its receptors FGFR1 and/or 2 was observed. We hypothesized that FGF2 and its cognate receptors FGFR1 and 2 provide an additional autocrine/paracrine growth factor pathway in lung cancer that contributes to EGFR-TKI resistance.

Methods: FGF2, FGFR1, and FGFR2 expression was assessed in a panel of 16 SCLC and 17 NSCLC lines by qRTPCR analysis. Secretion of FGF2 and TGFα was assessed by ELISA. FGFR1 and 2 protein expression was confirmed by Western blot. PCR analysis of FGFR1 and 2 splice variants confirmed expression of the FGFR1c and FGFR2c isoforms which bind FGF2. FGF2 receptor-stimulated ERK phosphorylation was analyzed by Western blot. The role of FGF2 and FGFR1 in proliferation/anchorage-independent growth was evaluated through shRNA FGF2 silencing, expression of dominant negative FGFR1, and RO4383596, a pharmacological small-molecule inhibitor of FGFRs, VEGFRs, and PDGFRs. Combination effects of RO4383596 plus gefitinib on proliferation were evaluated by MTS assay, on anchorage-independent growth using soft agar colony formation and on ERK-MAP kinase signaling by Western blot. The correlations between continuous variables such as gender and Affymetrix data were evaluated using the Spearman correlation coefficient. The Wilcoxon rank sum test was used to compare the difference in FGF2, FGFR1, and FGFR2 expression, clinical features, and RO4383596 sensitivity.

Results: Increased FGF2 expression was found in 12 of 31 NSCLC lines by Affymetrix gene expression data, in 5 of 9 lines by ELISA, and 10 of 17 by qRTPCR. Affymetrix data also showed increased FGF2 cognate receptor expression of FGFR1 (11/31) and FGFR2 (10/31). By qRTPCR increased expression of FGFR1 was observed in 7 of 17 and FGFR2 in 13 of 17 lines. Protein expression was confirmed by Western blot in all positive cell lines. There was no significant association between the clinical features of gender, histology, or smoking history and FGF2, FGFR1, or FGFR2 gene expression in the panel of 31 NSCLC lines.

There was a statistically significant correlation between expression predicted by Affymetrix data and mRNA expression by qRTPCR for FGF2 (r = .68; p = .005), FGFR1 (r = .71; p = .003), and FGFR2 (r = .65; p = .009). Regarding ligand expression and cognate receptor expression, there was a significant correlation between FGF2 and FGFR1 expression by both qRTPCR (r = 0.57; p = .017) and Affymetrix data (r = .62; p = .013), suggesting the presence of an FGF2 autocrine pathway in the cell lines that express both ligand and receptor. FGF2 secretion abrogated in one line by shRNA inhibited proliferation and anchorage-independent growth compared to empty vector. Expression of a dominant-negative FGFR1 also inhibited proliferation and anchorage-independent growth. There was no association between FGF2 expression and FGFR2 expression by either method. In FGFR1 and FGFR2, the third Ig-like domain is alternatively spliced to yield FGFRb and FGFRc splice variants. The FGFR1c and 2c isoforms bind FGF2. By PCR, all FGFR1-positive lines expressed the FGFR1c isoform and seven of the FGFR2-expressing lines were positive for the FGFR2c isoforms. Downstream ERK phosphorylation by FGF2 stimulation was limited to lines expressing FGFR1c or FGFR2c.

Growth inhibition by the FGFR inhibitor RO4383596 correlated with FGF2 expression by ELISA (p = .045) and by qRTPCR, although the qRTPCR failed to reach significance in the small sample (p = .063). RO4383596 sensitivity did not correlate with FGFR1 and FGFR2 expression by either Affymetrix data or qRTPCR. FGFR1 mRNA expression was high in 5 of 7 RO4383596 sensitive and 3 of 10 resistant lines, demonstrating that, as with EGFR inhibitors, receptor expression alone does not predict drug sensitivity. RO4383596 inhibited FGF2- but not EGF-stimulated ERK activation and also inhibited proliferation and anchorage-independent growth in FGF2-secreting lines.

Of the 17 NSCLC lines tested for FGFR-TKI growth inhibition, 8 were sensitive to gefitinib and 8 were gefitinib resistant. Interestingly, there was a statistically significant correlation between gefitinib IC_{50} values and FGF2 expression by Affymetrix expression data (r = .52; p = .047) borderline by qRTPCR (r = .49; p = .054) but not by ELISA (r = .48; p = .19). Gefitinib IC_{50} values in NSCLC lines were positively correlated with FGFR1 expression by both QPCR (r = .64; p = .0008) and Affymetrix data (r = .8; p = .0004) and negatively correlated with FGFR2 by both methods (r = -0.54, p = .03; r = -0.53, p = .04). In 16 lines with sensitivity data for both gefitinib and RO4383596, 3 lines were sensitive to both drugs. Resistance to both drugs was observed in four lines. Five lines were sensitive to gefitinib alone and four were sensitive to RO4383596 alone. This suggests that we can identify more sensitive lines to TKIs by examining more then one growth factor pathway and that identification of more then two pathways will be necessary to inhibit the growth of all cell lines. In combination experiments, synergistic growth inhibition was observed in cell lines that secreted both FGF2 and TGFα and were sensitive to both drugs alone. RO4383596 induced little or no growth inhibition in gefitinib-resistant lines that secreted both FGF2 and TGFα. Additive growth inhibition was followed by addition of gefitinib. In TGFα alone, positive NSCLC cell lines, RO4383596 alone, and the combination had no benefit.

In SCLC lines, FGF2 expression was observed in 5 of 16 lines by Affymetrix data and in 8 of 13 lines by qRTPCR and there was a statistically significant correlation between the two methods (r = .88; p = .008). Increased FGFR1 expression was found in 15 of 16 lines by Affymetrix data and qRTPCR confirmed expression in 11 of 13 lines; however, the correlation between predicted gene expression and mRNA was not significant (r = .64; p = .12). FGFR2-increased gene expression by Affymetrix data was found in 6 of 16 lines. qRTPCR confirmed FGFR2 mRNA expression in 8 of 13 SCLC lines. Interestingly, APCR analysis revealed frequent expression of FGF7 and FGF9 in SCLC lines, two FGF family members that are not expressed in NSCLC.

Conclusions: We provide evidence for FGF2 signaling pathways in NSCLC, which may contribute to primary gefitinib resistance. Therapeutic strategies of combining approved EGFR inhibitors with novel FGFR inhibitors may enhance tumor growth inhibition in NSCLC patients.

Keywords: FGF2; FGFR; NSCLC cell lines

082 A Systems Approach to Marker Guided Therapy

Please see Poster Discussion Session Biomarkers II.

083 Targeting Apoptosis To Overcome Cisplatin Resistance: A Translational Study in Head and Neck Cancer

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Cisplatin (CDDP) is the primary agent for organ-sparing treatment of advanced head and neck squamous cell cancers (HNSCC). Cisplatin resistance remains a significant barrier to organ-sparing therapy and to the survival of patients with advanced HNSCC. HNSCC cell lines and cisplatin-resistant progeny were studied to further investigate this phenomenon. The parental lines had mutant p53 and low or moderate levels of Bcl-xL. In contrast, the cisplatin-resistant cells contained wild-type p53 and high expression of Bcl-xL, suggesting that wild-type p53 and Bcl-xL are responsible for the resistant phenotype. We used lentiviral vectors to modify p53 status and Bcl-xL expression in these tumor cell lines. Expression of wild-type p53 in tumor cell lines with low Bcl-xL resulted in enhanced sensitivity to cisplatin-induced apoptosis. Expression of Bcl-xL alone did not significantly increase cisplatin resistance in cells with mutant p53; however, expression of Bcl-xL and wild-type p53 together caused tumor cells to become highly cisplatin resistant, via a mechanism involving cell cycle arrest, DNA repair, and escape from apoptosis. Pretreatment biopsies from clinical trials confirm that tumor p53 and Bcl-xL status predict outcome in patients. Patients whose tumors had wild-type p53 and low Bcl-xL enjoyed the best overall and disease specific-survival, whereas those with wild-type p53 and high Bcl-xL tumors had the worst survival. We conclude that targeting Bcl-xL and wild-type p53 is a rational concept for treating HNSCC to overcome escape from cisplatin-based therapy. (-)-Gossypol, a molecule that binds to the BH3 pocket of Bcl-2 and Bcl-xL and displaces proapoptotic BH3 proteins, induces high levels of apoptosis in cisplatin-resistant cells. (-)-Gossypol also suppresses tumor growth in HNSCC cells with wild-type p53 and Bcl-xL in a xenograft model. Recently, we have begun testing another novel therapeutic strategy to target resistant HNSCC cells: inhibition of the MDM2-p53 interaction. In HNSCC cells with wild-type p53, p53 is effectively inhibited by its endogenous cellular inhibitor, the MDM2 (or HDM2) protein. MDM2 is transcriptionally activated by p53, and in turn inhibits p53 activity through multiple mechanisms mediated by direct binding to p53. MDM2 thus functions as a potent and highly effective endogenous inhibitor of p53. Blocking the interaction between MDM2 and p53 using a potent small-molecule inhibitor can effectively reactivate p53 function in HNSCC cells with wild-type p53, which leads to cell cycle arrest and apoptosis. Design and development of nonpeptide small-molecule inhibitors to target the MDM2-p53 interaction is an exciting and new therapeutic strategy for the treatment of CDDP-resistant HNSCC with wild-type p53.

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Keywords: apoptosis; cisplatin resistance; head and neck cancer

084 Evidence From Transgenic Mouse Models, Preclinical Studies, and In Silico Data Analysis Point to a role for HOXB7 in Tamoxifen Resistance and Poor Disease Outcome

Please see Poster Discussion Session Biomarkers II.

085 MR Parameters as Noninvasive Biomarkers for Predicting Survival in Newly Diagnosed Patients With Glioblastoma Multiforme

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Introduction: The objective of this study was to determine whether MR perfusion, diffusion, and spectroscopy values that are measured either prior to surgery or after surgery and prior to radiation therapy were predictive of outcome for patients with newly diagnosed glioblastoma multiforme (GBM). Having such noninvasive markers would be valuable for determining which patients might benefit from more aggressive therapy and assigning them to specific treatment regimens.

Methods: A total of 100 patients were entered into the study, with 56 examined prior to surgery and 70 examined after surgery but prior to radiation therapy. MR data acquired included anatomic images, diffusion weighted images, dynamic perfusion

weighted images, and three-dimensional H-1 spectroscopic imaging. Enhancing (CEL), non-enhancing (NEL), and necrotic (NEC) regions were delineated and applied to maps of relative cerebral blood volume (rCBV), apparent diffusion coefficient (ADC), and fractional anisotropy (FA). For the spectroscopic data, voxels within the PRESS localized region comprising predominantly white matter were used to normalize the intensities of the metabolites choline (Cho), creatine (Cr), N-acetylasparate (NAA), lactate (Lac), and lipid (Lip). Parameter values were adjusted for age and subjected to proportional hazards analysis to determine which were associated with survival.

Results: For the patients studied pre-surgery, 55 showed contrast enhancement, and 49 patients had macroscopic necrosis visible on the T1-weighted images. Median volumes of the anatomical lesions for these patients were as follows: CEL 15.1 ± 14.1 cc, NEL 43.4 ± 33.5 cc, and NEC 3.46 ± 8.5 cc. Although the absolute volumes of the anatomical lesions did not predict outcome, the lesions with large percentage of the overall T2 hyperintensity being in the CEL (HR = 20.19; p = .005; n = 56, censored = 23) and large percentage of NEC (HR = 45.58; p = .01, n = 56; censored = 23) were correlated with worse survival. Regions with low ADC, increased lipid, and increased lactate were also associated with worse survival. The patients studied post-surgery and prior to radiation had lesions with relatively lower rCBV, Cho, and Cre in the residual CEL, and relatively lower ADC, Cho, and Cre in the residual NEL than prior to surgery. All of the absolute measures of residual tumor burden, including the volumes of the CEL, overall T2 hyperintensity, volume of CNI2 (number of pixels within the T2 lesion having CNI >2), volume of CBV3 (number of pixels within the T2 lesion having CBV >3), and volume of nADC15 (number of pixels within the T2 lesion having nADC <1.5), had a higher risk for poor outcome. High lactate and CBV values in the residual T2 hyperintensity were also associated with poor survival.

Conclusion: We have shown that a number of anatomic, physiologic, and metabolic measures of tumor burden are important for predicting survival in patients with newly diagnosed GBM. The pre-surgery markers associated with more malignant behavior are thought to correspond to high cell density and regions of hypoxia or necrosis. Of particular interest in understanding the effects of different treatments is that the residual (post-surgical) measures of tumor volume are more closely associated with survival than are initial tumor volumes. This information is likely to be important in defining noninvasive biomarkers for stratifying patients to specific treatment protocols and for planning focal therapy.

Keywords: biomarkers; imaging; survival

086 Preliminary Results From I-SPY (CALGB150007/150012/ACRIN6675): Contrast-Enhanced MRI and Correlative Science Studies to Characterize Tumor Response to Neoadjuvant Therapy for Locally Advanced Breast Cancer

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The I-SPY Trial is a multi-institutional correlative science trial of locally advanced breast cancer integrating serial biopsy to characterize biology and MRI to measure response of tumors to neoadjuvant chemotherapy.

Methods: Eligible patients had histologically confirmed new primary breast cancer, with at least 3 cm measurable disease within the breast following diagnostic procedure. Initial treatment was required to consist of an anthracycline (AC)-based neoadjuvant chemotherapy regimen; taxane (T) or additional agents (including trastuzumab) were allowed at the discretion of the treating physician. Surgical treatment was individualized on the basis of surgeon and patient preference. Serial MRI and core biopsies were performed at baseline (T1), after one cycle of AC (T2), intra-regimen (T3), and pre-surgery (T4) to identify markers of tumor response.

Results: The first phase of the study completed accrual in March 2006, with 237 patients enrolled at 10 institutions. A total of 222 patients were available for analysis as of 4/17/07. Enrolling institutions included University of California, San Francisco (30%), University of Alabama at Birmingham (21%), University of Pennsylvania (16%), University of North Carolina (14%), Memorial Sloan Kettering Cancer Center (8%), University of Texas Southwestern Medical Center (5%), University of Washington (3%), Georgetown University (2%), and University of Chicago (1%). The median patient age was 49 years, with a range of 27-69. The distribution by age was 20%, 35%, 36%, and 10% for women ages ≤40, 41-50, 51-60, and 61-70 respectively. Distribution by menopausal status was 48% premenopausal, 35% postmenopausal, and 17% unknown. The majority of patients enrolled in the study were Caucasian (74%), and ≤19% were African American, 4% Asian, and 3% other.

At study entry, the median tumor size was 6 cm (range, 0-25 cm), and 70% had clinically positive nodes; 22% had low, 38% intermediate, and 39% high-grade tumors prechemotherapy; 54% were estrogen receptor-positive, 43% were progesterone receptor-positive, and 28% had HER2/neu overexpression. Preoperative chemotherapy data are available on 207 subjects. The most common chemotherapy regimen given was AC followed by T (91%), although 6% received an AC-based regimen without T and 2% received additional chemotherapy beyond AC +/- T. Response to therapy was measured by clinical response, MR response, and pathological assessment. The distribution by complete response, partial response, and stable or progressive disease was 46%, 39%, and 15% measured by clinical exam, and 7.7%, 41.4%, and 24% by MR LD measurement, while 27% have yet to be assigned. 35% had complete pathological response (no invasive tumor), 10% had ≤1 cm residual, 34% had >1 cm residual on pathological examination, and 20% have yet to be assigned.

Of 222 patients eligible for analysis, MRI scans at the pretreatment timepoint are available for 219. Morphologic analysis by MRI revealed that 16% of patients had a single unicentric mass with well-defined margins, 30% had a multilobulated but well-defined mass, and the remaining 54% of patients had increasingly less organized areas of diffuse enhancement (30% multifocal, 14% diffuse, and 8% septal pattern). A total of 1,040 frozen cores were obtained on 197 patients, and 1,309 paraffin cores were obtained from 222 patients at all four timepoints. Biological assays completed on baseline samples include 120 Agilent expression arrays, 159 cDNA microarrays, 140 BAC arrays, 126 MIP arrays, 195 reverse-phase protein arrays (47 phosphoprotein endpoints), and 195 IHC assays (8 endpoints).

Exploration is ongoing using the caINTEGRATOR platform to enable development as a prototype in the I-SPY TRIAL to conduct cross-platform analysis.

Conclusion: These results demonstrate the feasibility of performing a multicenter clinical/translational trial of neoadjuvant therapy with successful collection of imaging and specimen data for biomarker assessment. Molecular studies, imaging endpoint assessment, and further clinical followup are ongoing and should provide a rich source of biological information about locally advanced breast cancers.

Keywords: response to therapy; tumor biology

087 PSA Dynamics Do Not Improve the Predictive Accuracy of a Single Prostate-Specific Antigen Measurement in Either Long-Term Prediction of Prostate Cancer Diagnosis or Outcome From Radical Prostatectomy

Please see Poster Discussion Session Biomarkers II.

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

088 The *Ella* Binational Breast Cancer Study

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Breast cancer is a poorly understood and understudied disease in Hispanic/Latina women in the United States. In Mexico breast cancer is currently the most commonly diagnosed cancer among women in the more industrialized and the northern regions of the country. We recently initiated a binational study of breast cancer to accomplish two primary specific aims: (1) to compare profiles of tumor markers of prognostic and/or predictive importance (ER, PR, HER2/neu, and Ki-67) and basal/luminal subtype markers between women in Mexico and Mexican American women living in the United States and (2) to assess whether differences in markers are more pronounced in postmenopausal women compared to premenopausal women and whether these are explained by factors associated with acquisition of lifestyles more representative of the United States (low parity, late age at first birth, adult weight gain pattern, etc.). Our goal is to recruit 1,225 women with breast cancer. Challenges identified relate to securing approval from national agencies in Mexico and managing the IRB approval process. Key issues identified to facilitate such binational collaborations include the involvement of bilingual and bicultural investigators and staff in the United States, the integration of clinicians and other officials from the health care setting in Mexico, and frequent communications that include face-to-face meetings. Our collaboration and early successes with database construction, cross-site training, and questionnaire development are a critical first step in describing and potentially capturing emerging changes in breast cancer in women of Mexican descent that may arise with lifestyle changes associated with migration to the United States.

089 Immunostaining With Antibody 8F1 Does Not Measure Cellular ERCC1

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ERCC1 binds to the xeroderma pigmentosum group F (XPF) protein, forming an endonuclease that participates in DNA repair. Recent reports have suggested that in non-small cell cancer, ERCC1 protein levels are a prognosticator of tumor responsiveness to platinum-based chemotherapy and clinical outcome. These conclusions were based on immunostaining of tumors using the monoclonal antibody 8F1. However, the specificity of this antibody in immunostaining was not demonstrated. We compared 8F1 and a second commercially available antibody (FL-297) for specificity in detecting ERCC1 in immunoblots and immunofluorescence using normal human fibroblasts and cells from patients with inherited mutations in ERCC1 (165TOR) or XPF (XP2YO) causing deficiency of the ERCC1-XPF nuclease. Immunoblotting of normal fibroblast lysates with FL-297 detected a single band that co-migrated with recombinant ERCC1. The band was greatly attenuated in ERCC1-deficient XP2YO and 165TOR cells. 8F1 antibody detected ERCC1 and at least one other protein in whole cell extracts. The cross-reacting protein is equally abundant in both normal and ERCC1-deficient cells. XP2YO cells and normal human fibroblasts were labeled with different-sized cytoplasmic beads and co-cultured. FL-297 was able to discriminate between ERCC1-positive and ERCC1-deficient cells by immunofluorescence. In contrast, 8F1 stained the nuclei of both cell types equally. Immunostaining of fibroblasts locally irradiated with ultraviolet light revealed co-localization of signals produced by FL-297 and an antibody that recognizes UV photoproducts in DNA, indicating that FL-297 recognizes a protein that specifically associates with DNA damage. In contrast, the signal produced by 8F1 did not co-localize with sites of UV irradiation or the antigen recognized by FL-297. These experiments show that ERCC1 is not the primary antigen recognized by the 8F1 antibody in human cells and that 8F1 cannot discriminate between ERCC1-positive and ERCC1-deficient cells. Therefore, currently there is no support for the conclusion that ERCC1 protein level is a useful prognosticator in non-small cell lung cancer.

Keywords: antibodies; cisplatin; immunostaining

090 Influence of Race on the Prevalence of PIK3CA, TGFBRII Mutations, and Microsatellite Instability in Sporadic Colorectal Cancer

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Background: Microsatellite instability (MSI) is one form of genomic instability that is common in sporadic colorectal cancers. PIK3CA encodes for PI3-K oncogenic protein and is frequently mutated at hotspots in cancer cell lines and colorectal cancer. PIK3CA somatic mutations and microsatellite instability (MSI) status in colorectal cancer have been widely studied in Asians and Caucasians. However, there are few data on the rate and spectrum of these mutations in colorectal tumors from African Americans. We evaluated somatic mutations in PIK3CA, TGFBRII, and MSI instability in colorectal tumors from subjects (148 Whites and 44 African Americans) enrolled in a population-based study of colorectal cancer (CanCORS) and assessed whether these tumor molecular characteristics could be useful as prognostic and predictive factors of colorectal cancer.

Methods: DNA was extracted from tissue microarrays (TMAs). MSI status was determined using the NCI panel of microsatellite markers (BAT25, BAT26, D5S346, D2S123, and D17S250). PIK3CA exon 9 and 20 mutations and TGFBRII poly A tract deletion were assessed from the same tumor DNA used for MSI analyses.

Results: Overall the frequency of MSI-high (MSI-H) was 20.9% (38/192), PIK3CA mutation was 14.3% (18/126), and the TGFBRII poly A tract deletion was 4%. Thirty-two MSI-H tumors were from Whites and six from African Americans. MSI-H was associated with PIK3CA mutations (41%) compared to MSI-H tumors without mutations (23%). The distribution of MSI-H tumors by stage of disease was 40.9% local, 50% regional, and 9.1% distant. PIK3CA mutated tumors were more frequent in Whites (15.2%) than in African Americans (9.5%). There was an association between PI3-K mutations and local and regional stage but not distant stage. Seven of eight TGFBRII mutations were found in tumors without PI3-K mutations and in one tumor harboring PI3-K mutations.

Conclusions: We observed that MSI and PIK3CA mutations were more frequent in Whites than in African Americans and also both mutations were associated with early stage of disease. These results suggest that PIK3CA and MSI may influence tumorigenesis and progression of colorectal cancer at the early stage of disease. The combination of MSI and PIK3CA mutation status could be useful as prognostic and predictive factors for early-stage colorectal cancer.

Keywords: African Americans; colorectal cancer; microsatellite instability

091 IL-6 Predicts Recurrence and Survival Among Head and Neck Carcinoma Patients

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Purpose: Increased pretreatment serum interleukin (IL)-6 levels among patients with head and neck squamous cell carcinoma (HNSCC) have been shown to be correlated with poor prognosis, but sample sizes have been small and thus unable to control for other prognostic variables.

Methods: This cohort study prospectively determined the correlation between pretreatment serum IL-6 levels and tumor recurrence and all-cause survival in a large population (n = 444) of HNSCC patients. Control variables were age, gender, smoking, cancer site and stage, and comorbidities. Kaplan-Meier plots and univariate and multivariate Cox proportional hazards models were used to study the association between IL-6, control variables, and times to recurrence and survival.

Results: The median IL-6 level was 13 pg/mL. (range, 0 to 453). The 2-year recurrence rate was 35.2% (standard error = 2.67%). The 2-year death rate was 26.5% (standard error = 2.26%). Multivariate analyses showed that IL-6 levels independently predicted recurrence at statistically significant levels ($p<.01$) as did cancer site (oral/sinus) ($p<.05$) and cancer stage (marginally significant $p<.09$). IL-6 level was also a statistically significant independent predictor of survival ($p<.05$), as well as older age ($p<.001$), current and past smoking ($p<.05$), cancer site (oral/sinus) ($p<.05$), cancer stage ($p<.05$), and comorbidities ($p<.05$).

Conclusion: Pretreatment serum IL-6 may be a valuable biomarker for predicting recurrence and survival among HNSCC patients. Using IL-6 as a biomarker for recurrence and survival may allow for earlier identification and treatment of disease relapse.

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Keywords: IL-6; recurrence; survival

092 Automated, Whole-Slide-Based Multiplexed Molecular Marker Assessment in Formalin-Fixed, Paraffin-Embedded Tissues

Please see Poster Discussion Session New Technologies and Methodologies I.

093 Using Quantitative Analysis of Estrogen-Regulated Gene Expression to Subclassify Endometrial Carcinomas

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Objectives: In a prior study, we examined endometrial gene expression in postmenopausal women before and 3 months after treatment with estrogen replacement therapy. We identified a panel of four known genes that were significantly induced by estrogen treatment (IGF-1, RALDH2, sFRP1, sFRP4) and one gene involved in an estrogen-regulated pathway (IGF-1R). Given the

relationship between estrogen exposure and endometrioid-type endometrial cancer, we hypothesized that genes induced by estrogen might be relevant to endometrial carcinogenesis. Furthermore, we hypothesized that analysis of these genes could form the basis of a molecular classification of endometrial carcinoma.

Methods: RNA prepared from 72 frozen endometrial carcinomas (58 endometrioid, 14 nonendometrioid) was analyzed by quantitative RT-PCR for expression of the estrogen-regulated genes. These data were compared to patient demographic and pathologic characteristics. An unsupervised cluster analysis was performed to assess for correlations in gene expression. Fisher's Exact, Mann-Whitney, Jonckheere-Terpstra, and Student's t-tests were utilized for statistical analysis.

Results: The expression of IGF-1R ($p = .001$) was significantly increased in endometrioid versus nonendometrioid tumors. Among endometrioid adenocarcinomas, there was decreasing expression of IGF-1 ($p = .046$), IGF-1R ($p<.001$), RALDH2 ($p = .002$) with increasing grade of tumor. When compared to nonendometrioid carcinomas, grade 1 endometrioid carcinomas had significantly higher expression of all genes except sFRP1. Conversely, grade 3 endometrioid tumors and nonendometrioid tumors had similar profiles of gene expression. In the cluster analysis, there were two distinct groups based on expression of IGF-1, IGF-1R, RALDH2, sFRP1, and sFRP4, with an agglomerative coefficient of 0.79. The first cluster had overall lower expression of the five estrogen-regulated genes. Clinical correlation revealed that patients in this cluster had higher median age ($p = .012$), lower BMI ($p = .044$), and higher grade tumors ($p = .038$), and were more likely to be of grade 3 endometrioid or nonendometrioid histology ($p = .004$). Interestingly, grade 2 endometrioid tumors were divided equally among the first and second cluster, and there were no clinical or pathologic factors associated with their inclusion in either cluster.

Conclusions: Quantitative analysis of genes known to be regulated by estrogen demonstrates clear distinctions in the molecular profiles of the different histologies of endometrial carcinoma. Interestingly, while grade 1 endometrioid tumors demonstrate a profile consistent with the "hyper-estrogenization" theory of endometrial carcinogenesis, the gene expression profile of grade 3 endometrioid tumors is more similar to that for nonendometrioid tumors. Grade 2 endometrioid tumors demonstrate an equivalent number in each molecular profile, suggesting an overlap of the molecular carcinogenic pathways of endometrial carcinoma development.

Keywords: biomarker; endometrium; estrogen

094 Arterial Spin Labeling MR Perfusion Provides Radiologic-Pathologic Correlation for Monitoring Responses to Antiangiogenic Therapy in Renal Cell Cancer Animal Models

Please see Poster Discussion Session New Technologies and Methodologies I.

095 Epithelial-Mesenchymal Transition in Head and Neck Squamous Cell Carcinoma: Association With Src Activation and E-Cadherin Downregulation and Aggressive Tumor Features

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The role of the epithelial-mesenchymal transition (EMT) in the invasion and progression of head and neck squamous cell carcinoma (HNSCC) remains unexplored. In this study, the expression of cell adhesion, motility, and signaling molecules was examined in 11 HNSCC cell lines and 50 primary tumor tissues to determine which are associated with the squamous-mesenchymal modification and aggressive tumor features. Our results showed paradoxical expression of p-Src and E-cadherin in the majority of cell lines and in primary tumor tissues relative to normal squamous mucosa. In most cell lines and tumor tissues, elevation of activated Src was accompanied by downregulation of E-cadherin and the detection of vimentin. In vitro inhibition of Src led to elevation of E-cadherin expression and increased cell adhesion in squamous carcinoma cell lines. Immunophenotypic analysis of the protein products of these genes in primary tumor tissues was conducted and correlated with different clinicopathologic factors. High levels of activated Src correlated significantly with pattern of invasion ($p>.001$), tumor differentiation ($p = .02$), and lymph node metastasis ($p = .05$). E-cadherin downregulation correlated only with lymph node ($p = .05$) metastasis. In this study, the expression of vimentin in tumor epithelial cells was significantly correlated with individual cell and finger-like invasive pattern ($p = .004$) and poor tumor differentiation ($p = .01$). Our study indicates that Src and E-cadherin play a major role in the epithelial-mesenchymal transition, invasion, and progression of HNSCC. These proteins may be targeted for the therapeutic intervention of patients with HNSCC.

Correlation of p-Src, E-Cadherin, and Vimentin and Clinicopathologic Factors in HNSCC									
Feature	p-Src		P-Value	E-Cadherin		P-Value	Vimentin		P-Value
	Low[1]	High[2]		Low	High		-	+	
Invasion									
Finger-like[1]	7	22	>.001	4	13	=.35	10	20	=.004
Broad[2]	17	3		13	20		16	5	
Differentiation									
Well and Moderate[1]	10	7	=.02	25	25	.72	13	4	=.01
Poor and Sarcomatoid[2]	8	25		14	14		13	21	
LN Mets									
+[1]	17	20	=.05	11	11	=.05	10	20	=.08
-[2]	11	3		26	26		8	4	
Stage									
I & II[1]	8	3	=.08	19	19	=.38	9	5	=.09
III & IV[2]	15	24		12	12		9	19	

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Keywords: epithelial-mesenchymal; signaling; squamous

096 Translational Studies of Thromboxane Synthase in Non-Small Cell Lung Cancer

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Recently thromboxane (TX) A_2, the product of TX synthase (TXS), has been shown to be a mediator of cyclooxygenase (COX)-2-dependent angiogenesis. TXS regulates tumor cell motility in prostate cancer cells through a COX-2 pathway. TXS and COX-2 expression is high in non-small cell lung cancer (NSCLC) compared to normal lung tissue. In contrast, pulmonary prostacyclin synthase (PGIS) overexpression reduces the development of lung cancer in transgenic mice models, suggesting that the balance between TXS and PGIS may play a role in lung tumor development and survival.

Thromboembolic disease affects approximately 15% of all cancer patients and is the second leading cause of death for cancer patients. TXA_2 is a strong inducer of platelet aggregation. Activated platelets may contribute to tumor angiogenesis. Serum concentrations of vascular endothelial growth factor (VEGF) correlate with platelet counts in cancer patients and aggregation causes VEGF release from α-granules.

In this project we will evaluate the role TXS may play in NSCLC pathogenesis by correlating (a) TXS expression in retrospective paraffin-embedded and fresh human lung cancers with thromboembolic events, and thrombosis and angiogenesis within the tumor, as well as clinico-pathological variables including histology, grade, stage, nodal status, age, gender, time-to-disease progression, and overall survival, (b) TXB_2 levels in sera from NSCLC patients, both clinico-pathological variables and thromboembolic complications, and (c) through transfection experiments, the effects of overexpression or silencing of TXS in lung cancer cell lines on tumor cell survival, migration, and cell cycle progression under normoxic and hypoxic conditions. The relationship with PGIS expression in tumor samples and cell lines will also be explored.

In summary, this work will determine the pathogenic role of TXS in NSCLC and establish the enzyme as a potential novel target for therapy in this disease.

Keywords: angiogenesis; thrombosis; thromboxane

097 Expression of the Novel Survival Peptide, Humanin Protein, Is Associated With Prostate Cancer Recurrence

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Humanin (HN) is a mitochondrial-encoded 24 amino acid polypeptide originally simultaneously discovered as a neuronal survival factor in Alzheimer's disease models and as an antagonistic binding partner for the pro-apoptotic molecules Bax and IGFBP-3. HN is secreted from cells and also acts by activating cell surface receptors and kinase cascades. We decided to investigate the

potential significance of humanin in human prostate cancer by examining its in situ expression across a wide spectrum of primary tumors by tissue microarray analysis. Western immunoblotting was performed on frozen tissues from 10 cases of morphologically normal human prostate tissue and 10 samples of prostate cancer and revealed a single 3.5 kD band in both normal and cancer prostate samples with a fourfold stronger staining in prostate cancer than in normal prostates (p<.001). Similarly, TRAMP tumors stained 4-5 times stronger than normal 20-week-old mouse prostates. Immunohistochemistry was performed on tissue microarrays constructed from paraffin-embedded primary prostate cancer specimens from 226 hormone naive patients who underwent radical retropubic prostatectomy. In total, 979 tissue microarray spots were informative, including morphologically normal prostate (NL; n = 257), benign prostatic hyperplasia (BPH; n = 107), prostatic intraepithelial neoplasia (PIN; n = 41), and invasive prostate cancer (cancer; n = 574). Both cytoplasmic and nuclear HN expression was scored in a semiquantitative fashion using an integrated intensity measure (0.0-3.0) and positivity (0%-100%), respectively. The protein expression distribution was examined across the spectrum of epithelial tissues and its association with standard clinicopathological covariates and tumor recurrence was examined in 184 outcome and marker-informative patients. HN expression was low overall in prostate tissues; 97% of spots had negative to weak cytoplasmic staining (<1.0), and 92% of spots had infrequent nuclear staining (<25%). Nonetheless, the mean cytoplasmic HN expression was significantly higher in cancer (intensity = 0.11) compared to normal (intensity = 0.045; p = .0028), and the mean nuclear HN expression was also significantly higher in cancer (10.48% positive) compared to normal tissue (2.24% positive; p<.0001). Nuclear HN expression was a significant and negative prognosticator in low-grade (Gleason Score 2-6) prostate cancers when used as either a continuous variable (Cox Proportional Hazards p = .006) or dichotomized variable cut at 15% positive (p = .01). However, HN was not an independent predictor of tumor recurrence in multivariate analysis in this patient substratum, including seminal vesicle and capsular involvement, and preoperative PSA as covariates. We conclude that HN is expressed at higher levels in prostate cancers compared to matched normal tissues. High nuclear HN expression is strongly associated with an increased risk of tumor recurrence. This is the first report examining HN in prostate cancer and our findings suggest that it may be a prognostic marker and therapeutic target and is most informative in patients with low-grade tumors, providing further stratification of this category as a potential guide for clinical followup and clinical trials.

Keywords: humanin; recurrence; tissue array

098 Detection of Promoter Hypermethylation in Salivary Rinses as a Biomarker for Head and Neck Squamous Cell Carcinoma Surveillance

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Purpose: Hypermethylation of tumor suppressor gene promoters has been found in head and neck squamous cell carcinoma (HNSCC) and other solid tumors. We evaluated these alterations in salivary rinse pre- and post-treatment from HNSCC patients in a quantitative fashion using real-time quantitative MSP (Q-MSP).

Experimental Design: Pre- and post-treatment salivary rinse DNA samples from HNSCC patients were evaluated for patterns of hypermethylation using Q-MSP. Evaluated tumor suppressor gene promoter regions were selected on the basis of a previous study regarding building of a screening panel for HNSCC in high-risk population subjects. Q-MSP was normalized using a β-actin control and was considered as a binary variable and analyzed using appropriate statistical analyses (chi-square and Kaplan-Meier). The selected genes were *DAPK, DCC, MINT-31, TIMP-3, p16, MGMT,* and *Cyclin-A1.*

Results: We analyzed the panel in a cohort of 117 HNSCC patients. The majority were male (79.5%), with median age (range, 41-89) of 61 years; 80% reported regular tobacco consumption and 73.2% alcohol consumption. Tumors were sited mainly in the oral cavity and oropharynx, but include larynx/hypopharynx cases as well (19 patients had recurrent tumors). Fifty-three of the analyzed patients (49.5%) presented with methylation of at least one of the selected genes in the salivary rinse DNA pre-treatment and 22 (29.7% of the analyzed patients) presented with methylated post-treatment salivary rinses. Local recurrence-free survival was significantly lower in patients presenting with methylated pre-treatment salivary rinse (p = .019) but not post-treatment salivary rinse (p = .540). Pre-treatment methylated salivary rinse was not significantly associated with tumor site (p = .264) or clinical stage (p = .140).

Conclusion: We were able to confirm an elevated rate of promoter hypermethylation detected in HNSCC patients' salivary rinses using a panel of gene promoters previously described as methylated in HNSCC but not in control subjects. Detection of

hypermethylation in pre-treatment salivary rinse DNA seems to be predictive of local recurrence. This finding has the potential to influence treatment and surveillance of HNSCC patients.

Keywords: detection; promoter hypermethylation; salivary rinse

099 Bone Microenvironment and Androgen Status Modulate Cellular Localization of ErbB3 in Prostate Cancer Cells

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Background and Objectives: Prostate cancer (PCa) becomes incurable once it metastasizes to bone. Delineating the mechanisms involved in promoting metastatic prostate tumor progression in the bone will lead to better treatment strategies for bone metastasis. ErbB3, an ErbB receptor tyrosine kinase, has been implicated in the pathogenesis of several malignancies, including PCa. In this study, we investigated the role of ErbB3 in the metastatic progression of PCa.

Methods: Immunohistochemistry was performed on human PCa specimens from men with androgen-dependent primary tumors, lymph node metastasis, bone metastasis, and androgen-independent primary tumors. The bone-derived PCa cell lines, MDA PCa2b and PC-3, were injected subcutaneously or intrafemorally into SCID mice to investigate the effect of tumor microenvironment on cellular localization of ErbB3. SCID mice injected with androgen-responsive MDA PCa2b cell line subcutaneously followed with castration were used to examine the effect of androgen on cellular localization of ErbB3.

Results: We found that ErbB3 expression was upregulated in PCa cells within lymph node and bone metastases. Interestingly, ErbB3 was also detected in the nuclei of the PCa cells in the metastatic specimens. These findings suggest that ErbB3, a plasma membrane receptor protein, has unique nuclear localization, and they also raise the possibility that ErbB3 in the nucleus may have unappreciated functions in the metastatic progression of PCa. Because most metastatic specimens were from men who had undergone androgen ablation, we examined the primary tumors from patients who had undergone hormone deprivation therapy. We found that a significant fraction of these specimens also showed nuclear localization of ErbB3, suggesting that androgen may be one of the factors that regulate ErbB3 cellular localization. We thus assessed the effect of androgens and the bone microenvironment on the nuclear translocation of ErbB3 by using tumor models generated from bone-derived PCa cell lines, MDA PCa2b and PC-3. In subcutaneous tumors, ErbB3 was predominantly in the membrane/cytoplasm; however, it was present in the nuclei of the tumor cells in femur. Castration of mice bearing subcutaneous MDA PCa2b tumors induced a transient nuclear translocation of ErbB3, with relocalization to the membrane/cytoplasm upon tumor recurrence.

Conclusions: These findings suggest that the bone microenvironment and androgen status, two major elements involved in the metastatic progression of PCa, influence the localization of ErbB3 in PCa cells. We speculate that nuclear localization of ErbB3 may aid PCa cell survival during androgen ablation and androgen-independent progression in bone.

Keywords: bone; metastasis; prostate

100 Novel Prognosis Prediction Model for Human Breast Cancer Patients Based on a Heterologous Tissue Culture Gene Expression Signature

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Patients with cancer have highly variable clinical outcomes owing to many factors, among which are genes that determine the likelihood of invasion and metastasis. This genetic predisposition may be reflected in the gene expression pattern of the primary tumor, which has the potential to predict outcomes and guide the choice of treatment better than traditional clinical predictors. We developed an mRNA expression-based model that can predict prognosis/outcomes of human breast cancer patients regardless of microarray platform and patient group. Our model was developed using genes differentially expressed in mouse plasma cell tumors growing in vivo versus those growing in vitro. The prediction system was validated using published data from

three cohorts of patients for whom microarray and clinical data had been compiled. The model stratified patients into four independent survival groups (BEST, GOOD, BAD, and WORST: log-rank test $p = 1.7 \times 10^{-8}$). Our model significantly improved the survival prediction over other expression-based models and permitted recognition of patients with different prognoses within the estrogen receptor-positive group and within a single pathological tumor class. Basing our predictor on a dataset that originated in a different species and a different cell type may have rendered it less sensitive to proliferation differences and endowed it with wide applicability. It also avoids overfitting, which has made it successful in analyzing patients from three different institutions. It is hoped that this predictor can be developed into a useful element in clinicians' choice of treatment for individual cancer patients.

Keywords: expression profile; genomics; survival

101 Evaluation of EGFR Gene Amplification Status, EGFR Protein, and Phosphoprotein Levels in Head and Neck Squamous Cell Carcinoma Surgical Patient Cohort Tumors

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Purpose: To evaluate EGFR gene amplification status and EGFR protein/phosphoprotein levels in tumors from patients with head and neck squamous cell carcinoma (HNSCC) surgically treated with curative intent from 2000 to 2004 in order to define relationships between EGFR gene amplification, EGFR protein, and EGFR site-specific phosphorylation levels and to assess the prognostic values of these biomarkers.

Background: EGFR has been reported to be overexpressed in head and neck cancers by us and others, and high levels of EGFR protein and EGFR gene amplification have been independently reported to be associated with poorer prognosis. Phosphorylation of EGFR at Y992, Y1068, and Y1086 has been reported to occur in the activated EGFR receptor. To our knowledge, tumor levels of site-specific phosphorylated EGFR have not been reported as prognostic indicators for head and neck cancers.

Methods: A total of 58 tumor specimens and 50 paired adjacent mucosal tissues from patients surgically treated for HNSCC were collected through an Early Detection Research Network (EDRN) study. Tissue microarrays (TMAs) were constructed using these paired patient samples and 30 oral mucosal specimens from cancer-free controls. Three cores from each specimen were arrayed on two TMAs and evaluated for EGFR gene amplification by dual-color fluorescence in situ hybridization (FISH) and for EGFR by immunohistochemistry (IHC). Corresponding fresh-frozen tumor specimens were evaluated by reverse-phase protein array (RPPA) for total EGFR, EGFR P-Y992, and P-Y1068 levels. Disease-free survival (DFS) was calculated using the University of Pittsburgh Head and Neck Cancer Registry data as months from date of surgery to date of first cancer recurrence, new primary, or death. Hazard ratios were determined using Cox proportional hazards models.

Results: High tumor levels of EGFR P-Y1068 as assessed by RPPA were significantly associated with reduced DFS in Cox proportional hazards models (HR = 3.32; p = .008). However, elevated tumor levels of EGFR P-Y992 were not associated with DFS. High EGFR protein levels as determined by RPPA tended to be associated with reduced DFS, but this association was not significant (HR = 1.69; p = .163). High EGFR protein levels determined by IHC were significantly associated with reduced DFS (HR= 3.04; p = .016). EGFR gene amplification was present in 23% of tumors while EGFR gene amplification was not detected in any of the paired adjacent mucosal specimens or cancer-free controls. Tumors with high-level EGFR gene amplification expressed significantly higher levels of EGFR protein as assessed by IHC than tumors without EGFR gene amplification ($p<.001$). Increased EGFR levels were also detected in 11.4% of tumors without EGFR gene amplification. EGFR gene amplification tended to be associated with poorer survival, but the association was not significant (HR = 1.99; p = .074).

Conclusions: Consistent with previous reports, we find EGFR tumor protein levels as assessed by IHC to be an indicator of poor prognosis. Similar to previous findings, we find that patients with tumor EGFR gene amplification tend to have shorter DFS than do patients without EGFR gene amplification. We find that tumors with EGFR gene amplification have significantly higher EGFR protein levels as assessed by IHC. Though increased EGFR protein levels in tumors are not always associated with gene amplification, this may be an important mechanism for increased EGFR expression. Similar though not identical survival trends were noted for EGFR protein levels evaluated by RPPA and IHC. Our findings suggest that while EGFR P-Y992 levels are not associated with DFS, high tumor levels of EGFR P-Y1068 may be an indicator of reduced DFS.

Keywords: arrays; recurrence; survival

102 The Prognostic Significance of *PTEN* Promoter Hypermethylation in Low-Grade Gliomas

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Purpose: Methylation of DNA promoter sequences can result in the transcriptional silencing of tumor suppressor genes and thus promote tumorigenesis. Recent data have demonstrated that the putative 5′-promoter region of the tumor suppressor *PTEN* is hypermethylated in approximately 50% of low-grade (grade 2) gliomas (LGGs). We sought to define the incidence of *PTEN* promoter methylation and to determine its prognostic significance in LGGs in order to determine the potential usefulness of rapamycin for the treatment of patients with LGGs.

Patients and Methods: Forty-three LGG (grade 2) tumor specimens from newly diagnosed patients were analyzed for methylation of the putative 5′-promoter region of the *PTEN* promoter using gel-based methylation-specific PCR (MS-PCR). Genomic DNA was isolated from frozen tissue samples, and then bisulfite treated using the CpGenome DNA Modification Kit (Chemicon). To assess *PTEN* promoter methylation, we used methylation-specific primers. Female-to-male ratio was 21:22. A total of 21 patients had astrocytomas (49%), 15 oligodendrogliomas (35%), and 7 mixed histology (16%). The median age at diagnosis was 37 years (range, 20-64). Overall survival (OS) and progression-free survival (PFS) were assessed using Kaplan-Meier methods and log-rank test. P-values are two-tailed, unless otherwise specified.

Results: Median followup for surviving patients in the unmethylated group was 5.1 years (range, 0.01-11.5), and 6.2 years (range, 0.01-11.69) in the methylated group. Of the 43 patients in the study, 26 had methylation of the *PTEN* promoter (60%). There was no statistically significant relationship between *PTEN* methylation status and PFS ($p = .32$). Nine patients progressed in each of the methylated and unmethylated groups. There was a trend toward a relationship between *PTEN* promoter methylation and overall survival (OS). Relative to the unmethylated group, patients with *PTEN* promoter methylation had shorter survival times that approached statistical significance ($p = .11$, one-tailed). Estimated 5-year survival was 93% for patients without methylation vs. 83% for patients with methylation. Estimated 6-year survival in the unmethylated group remained 93%, but dropped to 79% for the methylated group. Of 26 patients in the methylated group, 7 died versus 1 of 17 patients in the unmethylated group.

Conclusions: Patients with *PTEN* tumor methylation show a trend toward decreased OS relative to those without methylation. Continued followup of the current cohort and evaluation of additional specimens will provide improved estimates. *PTEN* promoter methylation did not correlate with PFS, which is likely because of ambiguities in conclusively establishing the progression of LGGs and a lack of stringent schedules for followup imaging in this cohort. Correlating *PTEN* promoter methylation with both PTEN expression and the activity of downstream effectors such as PKB/Akt, $p70^{S6K}$, 4E-BP1, and S6 will help ascertain the biological significance of the observed trend.

Keywords: glioma; methylation; *PTEN*

103 Cyclin B1 Overexpression and Anti-Cyclin B1 Antibodies as Prognostic Markers in Lung Cancer

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We previously identified the aberrantly expressed cell cycle regulator cyclin B1 (CB1) as a tumor antigen that can elicit both humoral and cellular immune responses in cancer patients. While CB1 is only transiently expressed in normal cells, tumors with deregulated CB1 constitutively overexpress the cell cyclin in the cytoplasm, and this correlates with poorer patient prognosis. We propose that this tumor-specific overabundance and cytoplasmic location of CB1 lead to the presentation of high—and therefore immunogenic—concentrations of CB1 peptides to the immune system. Further, work in our laboratory has demonstrated that vaccination against CB1 can prevent the growth of transplantable as well as spontaneous CB1-positive tumors. Together, these results indicate that studies of human anti-CB1 autoimmunity will yield clinically useful data in the fields of cancer diagnosis, prognosis, and prevention.

Our hypothesis is that CB1 overexpression as a negative prognostic factor in lung cancer may be tempered by the presence or absence of the immune response against this antigen. We have studied 103 lung cancer patients for CB1 expression in their primary tumors, serum anti-CB1 antibody at the time of surgery, and clinical outcome.

Over 90% of non-small cell lung cancers overexpress cyclin B1, with highest overexpression found in squamous cell tumors; 79% of patients had tumors with very high cyclin B1 expression. Additionally, we performed serum ELISA to determine the relative level of anti-cyclin B1 IgG in each patient: 21% of lung cancer patients have high levels of antibody while an additional 40% have at least detectable levels.

We are currently evaluating cyclin B1 overexpression and anti-cyclin B1 immune response, alone or in combination, as predictors of clinical outcome.

Keywords: autoantibody; immunology; overexpression

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

104 Early Assessment of Response to Targeted Therapy in Breast Cancer

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Rationale: The ability to identify molecular targets (ER, HER2/neu) in order to tailor treatment to tumor phenotype provides a major advance in breast cancer treatment. Potent targeted agents with low toxicity, aromatase inhibitors (AI) and/or trastuzumab, significantly improve outcomes. However, patients are usually subjected to months of treatment before efficacy can be adequately assessed. Early measurement of response is therefore a clinically important need for targeted therapy. Studies assaying material from serial biopsy have shown that a drop in proliferative index as early as 2 weeks predicts a favorable outcome. We have complemented biopsy-based measures of response using positron emission tomography (PET) imaging to measure response after 2 weeks of targeted therapy. Early assessment of response of the tumor may help refine targeting strategies and avoid prolonged futile therapy.

Preliminary Data: Breast cancer imaging with PET [F18]-fluorodeoxyglucose (FDG) 2 months into neoadjuvant therapy correlates with pathologic response and survival. Early PET FDG imaging of small-cell lung cancer, sarcoma, and GIST tumors has been shown to predict response. In addition, our group and others have used imaging to measure therapeutic target expression using molecular imaging more specific than FDG PET: we used [F18]-fluoroestradiol (FES) PET to measure ER function and observed that whole-body FES PET predicts response to hormone therapy. Preliminary data from our center and others support the ability of a combination of FES and FDG PET to measure early response to targeted therapy.

Methods: Patients initiating treatment on either AI or trastuzumab will undergo standard baseline staging imaging with FDG PET. Following 2 weeks of single-agent treatment, repeat core biopsy and FDG PET will assess early response. Patients on single-agent trastuzumab may then have additional hormonal therapy or chemotherapy added to their treatment. Core biopsies will be assayed for proliferation index (Ki-67 or MIB-1), hormone receptor expression, HER2 level expression, and quantitative hormone receptor levels by RTPCR. Quantitative changes in regional tracer uptake from imaging at 2 weeks will be compared to biopsy results and to subsequent standard measures of response. Patients with ER-positive tumors co-enrolled in FES imaging trials also have serial FES imaging performed.

Preliminary Results: Patients have found the procedures tolerable and agreed to participate in the study undergoing neoadjuvant and palliative therapy on and off clinical protocols. We have seen a dramatic drop in proliferation rate, which correlates with PET FDG findings and response in some patients, but long-term followup is limited. One patient with anthracycline refractory HER2-positive inflammatory breast cancer, undergoing single-agent trastuzumab followed by trastuzumab plus paclitaxel, showed a decline in proliferative index from 40% to 20% and an SUV drop from 7.9 to 3.4, and a robust clinical response. Serial PET FES imaging shows a drop in FES uptake in patients early on AI therapy, but this observation has been variable.

Discussion: At least in some patients, early dramatic changes are apparent by biologic imaging and tissue assay to denote a response to targeted therapy. Further analysis and patient numbers are needed to determine the significance of our early observations. Results from this work would establish a role for early PET to identify tumors destined to fail to respond to therapy and would indicate a need to change treatment for tumors lacking initial signs of response.

Keywords: breast cancer; PET FDG; targeted

105 Association of Megalin Genetic Polymorphisms With Prostate Cancer Risk and Prognosis

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Megalin, an endocytic receptor expressed by prostate epithelial cells, can internalize biologically active androgens bound to sex hormone binding globulin. Genetic variation of this gene could potentially influence levels of steroid hormone uptake. We carried out a population-based case-control study of 553 prostate cancer cases and 534 controls comprising Caucasian men ages 40-64 from the Seattle-Puget Sound region. Forty haplotype-tagging single nucleotide polymorphisms (htSNPs), which included three nonsynonymous SNPs, were selected from the HapMap European white population using Haploview Tagger software. We estimated prostate cancer risk using adjusted unconditional logistic regression for both individual SNPs and haplotypes. Risk of disease recurrence/progression and prostate cancer mortality were estimated using Cox proportional hazards (PH) regression.

There were 86 cases with physician-diagnosed recurrence/progression and 33 cases who died of prostate cancer in the average 10 years of followup. We found no strong evidence of altered risk of developing prostate cancer for any of the htSNPs when they were assessed individually or in haplotypes. However, four htSNPs were significantly associated with both disease recurrence/progression and prostate cancer mortality. Interestingly, three of these htSNPs show evidence of risk for both recurrence/progression and mortality being significantly modified by primary androgen deprivation therapy (ADT). An additional 13 htSNPs were significantly associated with altered rates of recurrence/progression, 9 of which showed evidence of modification by primary ADT. Finally, three htSNPs were significantly associated with altered risk of death from prostate cancer, with no evidence of interaction with ADT.

Preliminary results suggest that genetic variation in the *Megalin* gene could alter both risk of death and recurrence/progression for men with prostate cancer. In addition, ADT effectiveness may be modified by the activity of this gene. This is the first study that has examined *Megalin* polymorphisms and associations with prostate cancer risk and outcomes. Future work needs to be done to determine the biologic consequence of these SNPs and replicate these findings.

Keywords: outcomes; polymorphisms; prostate cancer

106 Quantifying Gene Expression in Heterogeneous Pancreatic Adenocarcinoma: Implications for the Molecular Analysis of Clinically Available Biopsies

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Introduction: Molecular analysis has been successfully employed in other cancers to enable the identification of specific targets, individualized treatments, and prognostic indicators, as well as to elucidate molecular mechanisms involved in tumor etiology. However, similar analysis of pancreatic adenocarcinoma (PAC) remains difficult due to specimen heterogeneity.

Objective: The goals of this study are (1) to validate the molecular analysis of clinically available PAC specimens in matched clinical PAC biopsies, including snap-frozen, formalin-fixed paraffin-embedded (FFPE), endoscopic ultrasound-guided fine-needle aspirate (EUS-FNA) and macro-dissected FFPE, (2) to identify potential prognostic indicators associated with survival, and (3) to determine whether treatment modulates gene expression in an independent set of synchronous EUS-FNA samples obtained prior to and 2 weeks following chemoradiation.

Methods: Total RNA was isolated from tissue specimens and mRNA levels were evaluated using real-time quantitative PCR formatted in a TaqMan Low Density Array (TLDA). A total of 610 genes involved in apoptosis, drug metabolism, cell cycle, DNA repair, inflammation, the ubiquitin-proteasome system, and the hedgehog pathway were examined. Gene expression profiles were compared using linear regression and Pearson correlation analysis. Differential gene expression between long-term survivors (LTS >24 months post-surgery) and those who died from the disease (DOD <12 months post-surgery) was determined by Student's t-test. Significance was defined as $p<.05$.

Results: A significant concordance (p<.0001) was observed in mRNA levels between matched surgically resected (both snap-frozen and FFPE) and EUS-FNA PAC biopsies. Enrichment of tumor cell population by macro-dissection improved concordance with immunohistochemistry. A total of 21 genes were significantly (p<.05) different between LTS and DOD patient survival groups. EUS-FNA biopsies obtained prior to and during chemoradiation demonstrated no significant change in gene expression.

Conclusion: Collectively these studies demonstrate that specific molecular profiling may be possible from heterogeneous PAC tissues. This represents a significant advancement in the ability to identify potential therapeutic targets, prognostic indicators, or pathways associated with tumor etiology and may facilitate stratification of patients toward more effective treatments.

Keywords: gene expression; pancreas; prognostic indicators

107 Immunohistochemical Biomarkers of Phospho-ERK and Ki67 Expression Are Better Prognostic Factors Than Thickness and Mitotic Rate in Patients with Stage 1/2 Primary Cutaneous Melanomas

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Objective: Thickness and mitotic rate in the vertical growth phase (VGP) are the most important prognostic factors based on morphology for metastasis and survival for patients diagnosed with cutaneous melanoma. Antibodies for genes related to proliferation are available and the expression of these biomarkers can be used to identify improved prognostic factors. Ki67 protein is expressed in all phases of the cell cycle except G0 and is a measure of cellular proliferation. Elevated phospho-ERK (pERK) expression is a characteristic of melanomas with abnormal signaling through the MAP kinase pathway. We hypothesized that the expression of these biomarkers in the primary tumor would be associated with poor prognosis and more accurately predict those who would have a 10-year metastasis (MET).

Methods: We evaluated Ki67 and pERK expression by immunohistochemistry using a retrospective nested cohort study of 61 patients with stages 1/2 vertical growth phase melanomas seen at the University of Pennsylvania's Pigmented Lesion Clinic (1972-1991) with survivors followed for 10-30 years. The percentage of melanoma cells positive for Ki67 in the VGP was determined using the Mib-1 antibody, and nuclear pERK expression by melanoma cells in the tumor's invasive edge (pERK/IE) was quantified using a modified H-score. A cut-point for each score was determined that maximized sensitivity and specificity to predict 10-year MET. The log-rank test was used to compare survival curves. Fisher's exact test (0.05, one-sided) has 75% power to detect an odds ratio (OR) of 4.3 assuming 19 and 42 patients with and without MET.

Results: The odds of 10-year MET was 10.6-fold, and 4.3-fold higher in patients having high Ki67 and high pERK/IE expression, respectively, compared to those with low expression (p =. 013 and p<.001, respectively). Thickness (OR = 1.7; p = .004) and mitotic rate (OR = 1.3; p = .002) were also associated with 10-year MET. In the multivariate analysis, the adjusted odds ratios for Ki67 and pERK were strong, 4.0 (p = .061) and 5.9 (p = .030); however, the association of thickness and mitotic rate with 10-year metastasis diminished and these factors were no longer significant independent prognostic factors.

Conclusion: Expression of Ki67 in melanoma cells in the VGP and expression of pERK by invading melanoma cells are strongly associated with prognosis and may be more informative than thickness and mitotic rate in predicting metastases. Their role as independent biomarkers of prognosis requires study by multivariable analysis in a larger cohort.

Keywords: MAP kinase pathway; metastasis; proliferation

108 Elevated Kallikrein 6 in Primary Glioblastoma Multiforme Is Associated With Poor Patient Survival

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While several protease families have been implicated in glioma progression, the potential contribution of tissue kallikreins has not been addressed in detail. Tissue kallikreins are a family of 15 structurally and functionally related serine proteases clustered on chromosome 19q13.4. The "classical kallikreins" K1 (tissue/renal/pancreatic kallikrein), K2 (prostate-specific glandular kallikrein), and K3 (prostate-specific antigen) were first to be identified. K2 and K3 are widely used biomarkers for prostate cancer

prognosis. Potential roles for the newly identified "neo-kallikreins" (K4-K15), as biomarkers in steroid hormone-related cancers, are emerging.

To determine the possible activity of kallikreins in mediating tumor progression in glioblastoma multiforme (GBM) we evaluated immunoreactivity (IR) of all 15 kallikreins in 3 to 4 tumor core samples taken from 38 primary grade 4 astrocytomas arrayed on glass slides. Kallikrein levels were quantified by determining (a) percent core stained: 0, 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), 4 (76%-100%); (b) staining intensity: 1 (low), 2 (moderate), 3 (high); and (c) immunoreactivity score reflecting (1) percent stained × (2) intensity. Cumulative survival probabilities were estimated using the Kaplan-Meier method. Recursive partitioning was used to create patient groups based on kallikrein levels. A Cox proportional hazards regression model was used to compare survival among kallikrein patient groups both univariately and adjusting for age at surgery and gender.

Several kallikreins showed either a positive or a negative association with patient survival, but this association remained statistically significant only for kallikrein 6 (K6) after controlling for age and gender. Survival differed significantly among GBM patients with different percent tumor core stained with K6 ($\leq$3, 532 days; >3, 256 days; hazard ratio = 3.55; 95% CI, 1.38-9.10; p = .009), and with different K6-IR scores ($\leq$6, 761.5 days; >6, 277 days; hazard ratio = 2.65; 95% CI, 1.07-6.58; p = .036). Findings were similar after controlling for Eastern Cooperative Oncology Group (ECOG) performance status, extent of resection, and concurrent radiation or chemotherapy. These data suggest that elevations in GBM K6 are negatively associated with patient survival.

An additional array containing grade 3 (n = 10) and grade 4 (n = 29) astrocytomas was examined for K6-IR. Grade 3 tumors were associated with significantly less K6-IR relative to grade 4 tumors (p<.001, Mann-Whitney Rank Sum Test). These findings suggest K6 expression correlates with tumor grade and support the hypothesis that K6 participates in tumor progression. To determine whether elevations in K6 within astrocytoma reflect bystander expression or play an integral role, the effect of K6 on glioma migration, proliferation, and intracellular signaling events is currently being examined.

Keywords: glioblastoma multiforme; progression; survival

109 Smad4 Expression in Colorectal Cancer Promotes Tumor Suppression and Enhances Survival

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Introduction: The transcriptional regulator Smad4 is a central downstream mediator of the TGF-β pathway. Smad4 expression may be decreased by mutation of the Smad4 gene or through epigenetic mechanisms. Restoration of Smad4 expression in Smad4-deficient SW480 human colon cancer cells results in decreased cell invasiveness and tumorigenicity. Immunohistochemical analysis of Smad4 in colorectal cancers (CRCs) suggests that decreased expression is associated with poor prognosis. The purpose of this study was to determine whether low Smad4 expression levels were associated with poor outcome and to determine the gene expression signatures for Smad4 abundance or deficiency that may be associated with altered survival.

Methods: 65 CRC patient samples available through our collaboration in the Gastrointestinal SPORE were obtained. RNA was isolated and submitted for hybridization on the Affymetrix U133 Plus 2.0 gene chip microarray platform. The raw expression data were obtained and converted to expression values using the Affy function in R (www.bioconductor.org). The Affy function allows for common options to convert probe level data into expression values. High and low expressors of Smad4 were determined in relation to a median level of Smad4 expression, and Kaplan-Meier survival curves were generated for high and low expressors. Tests for the difference between survival curves for the high and low Smad4 expressers were done via the log-rank test statistic for each Smad4 probe. We examined the association between these high and low expressors and other genes by performing gene level comparisons for each Smad4 probe using a permutation test approach. Candidate gene lists were generated for each Smad4 probe by ranking the permutational p-value (for those <.05) and fold-change. Each of the Smad4 candidate gene lists was then compared and those probes included in both Smad4 lists were evaluated (n = 1,159 of 54,676).

Results: Increased expression of Smad4 based on two separate oligonucleotide probes (DPC4 and Smad4) was correlated with better overall survival (p<.0112 and p<.0265). Low expression of Smad4/DPC4 resulted in a shorter median time to recurrence (p<.0087). Analysis of genes that were overexpressed in high Smad4 and high DPC4 expression resulted in 549 differentially expressed genes of the 1,159. Analysis of genes overexpressed in the setting of low Smad4 and low DPC4 expression levels revealed 241 differentially expressed genes of the 1,159.

Conclusion: Colorectal cancers that exhibit high levels of Smad4/DPC4 have a significantly lower rate of recurrent cancer and better overall survival in comparison with those with lower than median levels of Smad4/DPC4 expression. Smad4 levels and the genes that are significantly differentially expressed with high or low expression of Smad4 could be utilized as a potential genetic signature to provide valuable prognostic information to patients with colorectal cancer and should be investigated for utility in guiding systemic treatment decisions.

Keywords: colorectal cancer; Smad4; survival

110 Disseminated Tumor Cells in Prostate Cancer: Implications for Systemic Progression and Tumor Dormancy

Please see Poster Discussion Session Biomarkers II.

111 Epigenetic-Based Nomograms in Prostate Cancer

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Extensive scientific literature data point to reciprocal interactions between prostate stromal cells and prostate cancer (PCa) cells. We have defined reactive stroma in prostate cancer both functionally and biologically. To investigate whether these intratumoral reactive stromal cells in human PCa are predictive of survival, reactive tumor stroma volume was quantitated on TMAs. The relative volume of intratumor stroma (5% stroma = grade 0; 5% to 15% = 1; 15% to 50% = 2; >50% = 3) was quantitated and analyzed. Tumors with no stroma (RSG 0) and large amounts of reactive stroma (RSG 3) were associated with adverse prognosis, independent of other biomarkers used today. Subsequently reactive stroma was then analyzed on H&E sections of the radical prostatectomy specimens (869 patients). The percentage of the tumor composed of RSG 0/3 was quantified and analyzed. Results show it is an independent predictor of biochemical survival even as a continuous variable ($p = .000$; HR = 1.124). Finally, 224 cases of prostatic needle biopsies diagnosed as prostatic carcinoma from 1988 to 1998 were used to quantify stroma on H&E stains. By Cox proportional hazard analysis, RSG was an independent predictor of recurrence (HR = 1.953; $p = .0174$) in the overall population. In the Gleason 7 subset, it displaces 3+4 vs. 4+3 from the model.

To identify interactions between stromal elements in the prediction of PCa, we tested a multivariate model with interaction that showed how PNI diameter adds predictive information to RSG and whether these can be used coordinately for better prediction even in the presence of all other currently used clinico-pathologic parameters. This is proof of concept that stromal microenvironment elements have biologic interactions, as demonstrated biologically between nerves and stroma, and that these interactions also translate into the predictive realm ($p = .0062$). PNI diameter adds predictive information to RSG and can be used in combination, particularly in prostatectomies.

On the basis of the biochemical recurrence-free survival models described above, we developed a preoperative and postoperative nomogram and an electronic calculator for the risk of biochemical recurrence, based on the usual clinical/pathological parameters such as preoperative PSA, lymph node status, margins, ECE, SVI, and Gleason grade, including RSG. We have produced two nomogram models demonstrating that RSG grading provides significant and different information that carries weight similar to Gleason grading, both in the pre- and post-operative scenarios. Input of the required values into a user-friendly program screen displays a probability of staying biochemical recurrence-free at 2- and 5-year marks. The calculator is presented in the form of an Access program; it will be transformed into a Web-based tool by the Medical Bioinformatics Core.

This is the first study to demonstrate that nonepithelial reactive stroma elements in prostate cancer tumors can be used as prognostic indicators. These data also add to the concept that tumors are not purely epithelial and the epigenetic tumoral and host events must be considered an important biological component of the cancer.

Keywords: nomogram; prognosis; stroma

112 Protein Expression Profiling To Determine Potential Markers of Therapeutic Response in Breast Cancer

Please see Poster Discussion Session Biomarkers II.

113 Dual-Labeled Trastuzumab-Based Imaging Agent for the Detection of Human Epidermal Growth Factor Receptor-2 (HER2) Overexpression in Breast Cancer

Please see Poster Discussion Session New Technologies and Methodologies II.

114 A Mayo SPORE in Brain Cancer (P50 CA108961) and University of Iowa/Mayo Clinic Lymphoma SPORE (P50 CA97274) Inter-SPORE Project: Specific Chromosomal Abnormalities in Primary Central Nervous System Lymphoma Differ From Those of Systemic Diffuse Large B Cell Lymphoma, Suggesting a Distinct Pathogenesis

Please see Poster Discussion Session Biomarkers I.

115 Molecular Abnormalities in Histologically Normal Endometrium of Obese Women

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Objectives: Obesity is a well-known risk factor for endometrial cancer. The purpose of this study was to evaluate histologically normal endometrium of obese women for candidate molecular markers associated with the estrogenization of endometrium and endometrial cancer.

Methods: A prospective study was carried out to evaluate the endometrium of patients with BMI >30 kg/m^2. Premenopausal patients between 30 and 55 years of age were enrolled from September 2004 to July 2006. An endometrial biopsy was performed between days 7 and 10 of the proliferative phase of the menstrual cycle. Thirty-five endometrial tissue samples were evaluated for PTEN by immunohistochemical (IHC) staining. Previously, we demonstrated that genes for IGF-1R, EIG121, RALDH2, and SFRP4 are induced by estrogen. IGF-1R increases endometrial proliferation, while RALDH2 and SFRP4 have antiproliferative effects. Quantitative, real-time PCR analysis was performed to evaluate the endometrial samples for these transcripts. Endometrial cancer tissue of 43 patients with BMI >30 kg/m^2 was obtained from the tumor bank and evaluated for the same molecular markers. Statistical analysis was performed using the Kruskal-Wallis and Fisher's exact tests.

Results: Thirty-eight patients with BMI >30 kg/m^2 have been enrolled thus far, of which 35 had adequate tissue for analysis. Median age was 40 years and median BMI was 37.5 kg/m^2. No cases of hyperplasia or cancer were seen. Of the 43 obese cancer samples, 37 were available for IHC analysis of PTEN. Loss of PTEN by immunohistochemistry was evident in 5 of 35 (14.2%) obese normal endometrial samples and in 15 of 37 (40.5%) obese endometrial cancer samples ($p<.02$). Endometrial cancer tissue from obese patients exhibited higher levels of EIG121 and IGF-1R than benign endometrium of obese patients ($p = .0068$; $p<.0001$). Levels of RALDH2 and SPFR4 transcripts were significantly lower in obese endometrial cancers compared to levels in obese normal endometrium ($p<.0001$).

Conclusions: In obese women, loss of PTEN was evident in 14.2% of histologically normal endometrial samples, demonstrating premalignant molecular changes. In the obese normal endometrium and obese cancers, genes known to be associated with estrogenization were elevated. Evaluation of these markers in a series of thin women is ongoing. These molecular alterations may represent markers of risk in obese women.

Keywords: endometrial (endometrium); obesity; preclinical

116 Proteomics Analyses of Tyrosine-Phosphorylated and Associated Proteins That Regulate Activation and Death of T Lymphoma Cells

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Syk is a nonreceptor protein tyrosine kinase that plays a critical role in signaling events leading to activation, proliferation, and differentiation of hematopoietic cells such as T cells. We have previously shown that expression of Syk in a mouse T lymphoma cell line negatively regulates their activation and activation-induced cell death. However, the molecular mechanisms underlying the suppressive effect on these tumor cells remain elusive. Our results have demonstrated that expression of kinase-activated Syk, but not ZAP-70, in T lymphoma cells induces a unique set of tyrosine-phosphorylated proteins following the stimulation of these cells through their T cell receptor (TCR). We hypothesize that the expression and/or phosphorylation of these unique downstream proteins or their associated proteins play a critical role in the regulatory function of Syk that controls the activation and cell death of T lymphoma cells. In order to test the hypothesis and to find out which downstream molecules are responsible

for this novel regulatory effect on T lymphoma cells, we have used state-of-the-art differential proteomics approaches to identify a panel of proteins that are uniquely or highly expressed in Syk-expressing but not in Syk-negative T lymphoma cells.

Following activation of the T lymphoma cells through their TCR, tyrosine phosphorylated proteins and their associated proteins are immunoprecipitated from cell lysates and processed for mass spectrometry analyses. An LC/MS/MS spectrometer is used for generating sequence-specific peptide fragment ion spectra, which are used in a bioinformatics database search to confirm the protein identity. Using this proteomics approach, we have identified hundreds of proteins in the phosphotyrosine immunoprecipitates that highlighted some dramatic differences between Syk-expressing T lymphoma cells and Syk-negative T lymphoma cells. More than 60 of these proteins are uniquely or highly expressed in Syk-expressing T lymphoma cells. These proteins can be grouped into different categories on the basis of their functions, such as those involved in the regulation of cell growth, apoptosis, cell cycle, or gene transcription of T lymphoma cells. We are in the process of validating the proteomics data using conventional methods such as Western blotting analyses. Several of the identified proteins share high sequence and functional homology between mouse and human. Therefore, we will be able to perform functional studies to examine the effect of these proteins on not only mouse T lymphoma cells but also human T lymphoma cells. Ultimately, these studies will evaluate whether the results obtained from using mouse models may be translated into studies in humans and examine whether these studies can help us explore novel therapeutics targeting these proteins as molecular targets for treating T lymphomas.

Keywords: phosphorylation; proteomics; T lymphoma

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117 Overweight and Obese Perimenopausal and Postmenopausal Women Exhibit Increased Abnormal Mammary Epithelial Cytology

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Background: It is estimated that roughly 10 million U.S. women are at high risk for breast cancer. Excessive body weight, as reflected by a body mass index (BMI) ≥25 kg/m^2, is consistently associated with postmenopausal breast cancer, and increases the risk of dying from this disease. While previous studies have explored associations between body weight and indirect biomarkers associated with disease risk, such as circulating hormonal levels or mammographic density, few have explored associations between body weight status and direct measures on target tissue.

Methods: Random periareolar fine needle aspiration (RPFNA) is a research technique developed to sample mammary cells from the whole breast of asymptomatic women at high risk for developing breast cancer, to assess both (1) breast cancer risk and (2) response to chemoprevention. RPFNA has been used successfully to predict short-term breast cancer risk; the presence of cellular atypia in a breast RPFNA specimen has been prospectively validated to confer a 5.6-fold increase in breast cancer risk in high-risk women (see reference).

Results: Epithelial cytology was assessed in 62 high-risk perimenopausal and postmenopausal women using RPFNA. Masood Cytology Index scores were significantly higher among women with BMIs ≥25 than in women with BMIs <25 (13.9 ± 0.42 vs.12.7 ± 0.29; p = .017). Overweight or obese women also had significantly higher RPFNA epithelial cell counts compared to those who were of normal weight (1,230 ± 272 vs. 521 ± 185; p = .028).

Conclusions: These data suggest that overweight in perimenopausal and postmenopausal women is associated with direct cytological abnormalities within the breast. Further research is needed to confirm these findings and to determine whether this potential biomarker is responsive to changes in body weight resulting from diet and/or exercise interventions.

This work is supported by NIH/NCI grants CA68438-AV13 (Avon/NCI Partners in Progress), 2P30 CA14236, R01 CA088799 (V.L.S.), R01 CA98441 (V.L.S.), R01 CA114068 (V.L.S.); Susan G. Komen Breast Cancer Award BCTR0402720 (V.L.S.), a V-Foundation Award (V.L.S.), and American Institute for Cancer Research (W.D.-W.).

Reference

Fabian et al. *JNCI*, 2000.

Keywords: biomarkers; breast cancer risk; obesity

118 Polymorphisms of *Caspase 8* and *Caspase 10* Genes and Risk of Cutaneous Melanoma

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The *caspase 8* (*CASP8*) and *caspase 10* (*CASP10*) genes play key roles in regulating apoptosis, and their functional polymorphisms may alter gene functions, resulting in dysregulated apoptosis and carcinogenesis. However, no reported study has investigated association between polymorphisms of these genes and risk of cutaneous melanoma (CM). In a hospital-based case-control study of 805 non-Hispanic white CM patients and 835 cancer-free age- and sex-matched control subjects, we genotyped two reported functional polymorphisms, *CASP8 D302H* and *CASP10 I522L,* and determined whether they are associated with CM risk and interacting with the known risk factors. We found that a significantly lower CM risk was associated with variant *CASP8 HH* genotypes (OR = 0.73; 95% CI, 0.56-0.96) and *DH+HH* (OR = 0.73; 95% CI, 0.57-0.95) compared with the *DD* genotype, whereas no significantly altered CM risk was associated with the *CASP10* variant genotypes. However, a significantly lower risk was associated with the *HI* haplotype (adjusted OR = 0.12; 95% CI, 0.04-0.38) and combined *CASP8DD+DH/CASP10II* genotypes (OR = 0.56; 95% CI, 0.32-0.99) compared with the *DL* haplotype and *CASP8 DD/CASP10 IL+LL* genotypes, respectively. Furthermore, we found evidence of interactions between combined *CASP8/CASP10* genotypes and tanning ability (p = .010), freckling (p = .020), and lifetime sunburns with blistering (p = .043). Therefore, these variant *CASP8* and *CASP10* genotypes might serve as biomarkers for susceptibility to CM. Larger, preferably population-based studies are needed to validate and confirm our findings.

This research was supported in part by National Institutes of Health, National Cancer Institute, grants R01 CA100264 (Q.W.) and P50 CA093459 (E.A.G.).

Keywords: apoptosis; genotypes; polymorphisms

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

119 QM-MSP, Ki-67, and Cytomorphology of Random Periareolar Fine-Needle Aspiration Specimens From Women at High Risk for Developing Breast Cancer

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Promoter regions of genes known to be hypermethylated in breast cancer have been examined by quantitative multiplexed-methylation-specific PCR (QM-MSP) for use in breast cancer detection and prediction. Prior to initiating a prospective cancer prediction study that would incorporate QM-MSP, cytomorphology, and proliferation as risk biomarkers, it was necessary to validate certain aspects of the biomarkers. We have previously demonstrated (see reference) that cytomorphology, Ki-67, and the cumulative score for QM-MSP is adequately consistent between replicate slides prepared from the same specimen of benign breast epithelial cells acquired by random periareolar fine-needle aspiration (RPFNA). We have also demonstrated that laser capture microdissection (LCM) of ~500 of the most abnormal epithelial cells did not improve detection of methylation, with the possible exception of RASSF1A. We now examine possible relationships between cytologic characteristics and the results obtained by QM-MSP.

Women at high risk for developing breast cancer underwent RPFNA at the University of Kansas Medical Center Breast Cancer Prevention Center and 82 specimens were processed to slides. A slide was assessed for cytomorphology using a categorical descriptor as well as the Masood cytology index score, by both a cytotechnologist and a cytopathologist. A slide was assessed for Ki-67 by the percentage of cells with immunohistochemical expression. A slide was sent to Johns Hopkins for QM-MSP of the entire cellular content and a cumulative QM-MSP score for 6-10 genes was calculated.

Cumulative QM-MSP scores for 6-10 genes (RASSF1A, TWIST, Cyclin D2, HIN1, RAR-Beta, APC1, ER-Alpha, BRCA1, BRCA2, CDH1) ranged from 0.13 to 21.57 (median = 3.65). The QM-MSP score was strongly correlated with the number of cells estimated to be on the slide (p = .001) and weakly associated with Masood score assessed by the cytotechnologist (p = .047). There was no apparent correlation between QM-MSP score and cytology category, presence of hyperplasia, Masood score assessed by the cytopathologist, or percent Ki-67 expression.

In conclusion, QM-MSP can be reliably performed in specimens obtained by RPFNA of high-risk women and shows promise as a prospective risk biomarker along with cytomorphology and proliferation.

Reference

Kimler et al. AACR 2007 Meeting, abstract 1683.

Keywords: aspiration; methylation; prevention

120 Bronchial Epithelial Proliferation Measured by Ki-67 Is Related to Current Smoking and Sex but Not to Lung Cancer or Chronic Obstructive Pulmonary Disease

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Background: Bronchial epithelial proliferation as measured by Ki-67 immunostaining is a biologically plausible intermediate endpoint biomarker of lung cancer risk, but has not been validated. We designed a study to determine factors associated with increased bronchial epithelial proliferation and whether increased epithelial proliferation is associated with endobronchial dysplasia, chronic obstructive pulmonary disease (COPD), or lung cancer.

Methods: This is a cross-sectional study of 113 subjects undergoing white light and autofluorescence bronchoscopy; 27 never-smokers, 27 current or ex-smokers with normal spirometry, 31 current or ex-smokers with COPD, and 28 current, ex-smokers, or never-smokers with lung cancer. Ki-67 expresssion was determined by immunohistochemistry on all evaluable biopsy sites without carcinoma. A Ki-67 index was defined as the percentage of cells expressing Ki-67. Relationships between Ki-67 index and demographic variables, smoking, histology, and presence of COPD and/or lung cancer were determined.

Results: Results for both maximal and mean Ki-67 index are similar, so only the former are reported. Average maximal Ki-67 index was higher in current smokers than in either ex-smokers or never-smokers (48.0% vs. 30.6% vs. 22.6%; $p<.001$). Males had higher Ki-67 index than females, (39.9% vs. 23.6%; $p<.001$). Compared to subjects without disease (Ki67 index = 30.0%), maximal Ki-67 index was not significantly elevated ($p = .44$) in subjects with either lung cancer (Ki67 = 39.1%) or COPD (Ki67 = 38.9%)

Conclusions: Current smoking and gender are major determinants of Ki-67 index. No increase in Ki-67 index was found in the nonmalignant epithelium of patients with lung cancer or COPD. Although Ki-67 index may provide insight into the short-term effects of chemoprevention agents on cell proliferation, its lack of association with lung cancer or COPD raises a question about its utility as a surrogate endpoint in clinical trials.

Keywords: lung; preneoplasia; proliferation

121 Functional Proteomic Profiling of AML Predicts Response, Relapse, and Clinical Outcome

Please see Poster Discussion Session Biomarkers II.

122 NBS1 Polymorphisms and Haplotypes and Lung Cancer Development: A Population-Based Case-Control Study

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Tobacco smoke is a major known cause of lung cancer; however, it is not attributable to all lung cancer cases. Variations in our DNA damage repair mechanisms, such as the double-strand DNA break repair pathway, may be a contributing factor in lung cancer development. In a population-based case-control study in Los Angeles County with 611 lung cancer cases and 1,040

population controls, we explored possible associations between three polymorphisms of the *NBS1* gene: Ex16+1785 T>C (rs9995), Ex 16+572C>G (rs2735383), and Pro672Pro (rs1061302), its reconstructed haplotypes, and lung cancer. The *NBS1* polymorphisms were measured using the SNPlex method from Applied Biosystems. Unconditional logistic regression was employed to analyze associations under study. The results show a negative association between the heterozygote Pro672Pro and lung cancer (OR = 0.71; 95% CL, 0.55-0.90). We also observed weak positive associations between the homozygous recessive variant of each polymorphism and lung cancer among nonsmokers (OR = 2.05; 95% CL, 0.86-4.87; OR = 1.82; 95% CL, 0.83-3.99; OR = 1.71; 95% CL, 0.80-3.65, respectively). These results were also replicated in the haplotype analysis; a positive association was seen in the recessive haplotype of CGG and lung cancer among nonsmokers (OR = 1.47; 95% CL, 0.95-2.27). In conclusion, our study suggests that there might be possible associations between these *NBS1* polymorphisms and lung cancer.

Keywords: lung cancer; NBS1 polymorphisms; population-based case-control study

123 Association Between Breast Density and Subsequent Breast Cancer Events Following Treatment for Ductal Carcinoma In Situ

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Background: Risk of invasive tumor events following treatment for ductal carcinoma in situ (DCIS) is associated with both treatment- and tumor-related factors. However, it is unknown whether stromal factors may influence subsequent invasive breast events. This study investigates whether breast density is an independent predictor of subsequent breast events among women treated for DCIS.

Population: This is a prospective cohort study of 3,274 women ages 30-93 included in the Breast Cancer Surveillance Consortium (BCSC) diagnosed with DCIS. All women underwent lumpectomy between 1993 and 2005 and had an American College of Radiology Breast Imaging Reporting and Data System (BI-RADS) breast density measure recorded prior to diagnosis as part of mammography screening.

Methods: Ipsilateral and contralateral breast cancer events treated with lumpectomy with or without radiation were ascertained through a State tumor registry, regional Surveillance Epidemiology and End Results (SEER) program, or pathology databases. A Cox proportional hazard model was used to compare adjusted risk of breast cancer among women with high (BI-RADS 3 or 4) versus low (BI-RADS 1 or 2) breast density.

Results: During a median followup period of 39 months (0-132 months), 133 women developed invasive breast cancer: 83 of these were ipsilateral events; 30 women developed DCIS in the contralateral breast. After adjusting for age and radiation treatment, high breast density was associated with increased hazard for contralateral (HR 3.1; 95% CI, 1.6-6.1) but not ipsilateral (HR 1.0; 95% CI, 0.6-1.6) invasive breast events. High breast density was not significantly associated with increased hazard of contralateral DCIS (HR 1.5; 95% CI, 0.6-3.3).

Conclusion: High breast density is an independent predictor of contralateral but not ipsilateral invasive breast cancer following lumpectomy for DCIS. These findings suggest that women with high breast density undergoing treatment for DCIS may especially benefit from antiestrogenic therapy to reduce risk of contralateral invasive disease.

Keywords: breast density; DCIS; risk stratification

124 Characterization of Gene Expression in Normal Breast of Women Enrolled in a Gonadotropin-Releasing Hormone Agonist-Based Chemoprevention Regimen

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Initial results of a recently completed Phase Ib chemoprevention trial of a novel regimen using an intranasally administered drug combination of the gonadotropin-releasing hormone agonist (GnRHA) deslorelin with partial replacement of 17β-estradiol (E2) and testosterone (T) in women at high genetic risk of breast cancer (most of whom had a *BRCA1* mutation) indicated a significant decrease in mammographic density, an intermediate marker of breast cancer risk. The goal of the current study was to determine the cellular, histological, and biochemical effects of a 10-month GnRHA intervention in premenopausal *BRCA* carriers and to explore possible changes in breast MRI characteristics.

We have conducted a collaborative Phase II biomarker trial of the GnRHA intervention regimen in unaffected premenopausal *BRCA* positive women (n = 9 of 10 accrued to date). Mammogram, breast MRI, and breast biopsy were obtained at baseline and after at least 6 months administration of the GnRHA regimen. The resulting imaging studies will be correlated with changes in biopsy histology as well as a panel of immunohistochemical and molecular genetic (e.g., expression microarray) studies of the tissues.

It is predicted that such a chemoprevention regimen will reduce lifetime breast cancer risk by one-third if used for 5 years and by more than 70% if used for 15 years. The cellular, histological, biochemical, and gene expression effects of GnRHA intervention in *BRCA* carriers are expected to provide valuable information about this intervention.

Keywords: chemoprevention; gonadotropin-releasing hormone agonist; normal breast

125 The Role of MicroRNAs in Human Acute Myeloid Leukemia Samples With Different Cytogenetic Risk Profiles

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Acute myeloid leukemia (AML) is a heterogeneous disease characterized by highly divergent gene expression patterns and substantially differential treatment outcome in individual patient groups. The differential course of the disease is associated with cytogenetic alterations that distinguish individual AML subpopulations. The pathogenesis of AML is the consequence of multiple different molecular alterations targeting the physiological processes of differentiation and proliferation of hematopoietic precursor cells. There is now growing evidence that miRNAs can affect various key cellular functions such as differentiation and proliferation control; therefore, we hypothesized that miRNAs play important roles in the pathogenesis of AMLs. The current study was designed to investigate three subsets of leukemia patients (inv(16), normal karyotype, and monosomy-7) with precisely selected cytogenetic features to reduce the genetic complexity inherent to unselected AML patient cohorts. Principal component analysis (PCA) of miRNA microarray data of AML samples from patients with inv(16) showed minor differences relative to normal bone marrow (BM). In contrast AML patients with standard risk cytogenetic classification (SR) are clearly divided in two subgroups. Comparison of SR patient samples with BM using ANOVA tests revealed nine differentially expressed miRNAs. AML patients with aberrations of chromosome 7 showed highly a distinct PCA pattern as compared to BM. qRT-PCR analysis confirmed differential expression of miRNAs in patient samples between the three patient groups and normal samples. Furthermore, the expression of certain miRNAs correlated with other known markers, as well as differentiation and prognosis. These data support the importance of miRNAs in a variety of functions related to AML pathogenesis or progression.

Keywords: leukemia; microRNA; risk stratification

126 Correlation Between Circulating and Prostate Tissue Nutrient Levels and a Marker of Oxidative Stress

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Background: Epidemiologic studies support the role of certain dietary components, especially fish (rich in marine omega-3 polyunsaturated fatty acids) and tomatoes (rich in the antioxidant lycopene), in prostate cancer prevention. It is hypothesized that these foods/nutrients may also delay the progression of prostate cancer through anti-inflammatory and antioxidant effects, respectively. Some studies have related circulating levels of specific nutrients to the risk of prostate cancer incidence; however, there are extremely limited data on how well circulating measures of nutrition correlate with prostate tissue levels and whether the benefit of nutritional agents is via an overall systemic or tissue-specific effect.

Methods: To further study how nutrients may influence prostate cancer, we conducted a pilot study examining the correlation between selected circulating and prostate tissue nutrient levels among 23 men with prostate cancer who underwent radical prostatectomy as primary therapy. Antioxidants were measured using high-performance liquid chromatography and included lycopene, beta-carotene, alpha-carotene, beta-cryptoxanthin, lutein+zeaxanthin, alpha-tocopherol, and gamma-tocopherol. Gas chromatography was used to assess several fatty acids (FAs) including arachidonic (AA, 20:4n-6), linoleic (LA, 18:2n-6), alpha-linolenic (ALA, 18:3n-3), eicosapentaenoic (EPA, 20:5n-3), and docosahexaenoic (DHA, 22:6n-3). We also measured urinary 8-isoprostane via ELISA methods as a marker of oxidative stress and examined its correlation with the nutrients.

Results: There were moderate to strong positive correlations between prostate tissue and circulating levels of antioxidants and fatty acids (mean = 0.66 for antioxidants, range, 0.55-0.86; mean = 0.46, range = 0.21-0.82 for selected FAs). 8-Isoprostane tended to be inversely correlated with several antioxidant measures and was statistically significantly positively correlated with pathologic Gleason score (p-value = .03).

Conclusion: In conclusion, these pilot data suggest that circulating levels of carotenoids, vitamin E, and omega-3 and omega-6 fatty acids may reflect prostate tissue specific levels and provide preliminary support for the hypothesis that higher oxidative stress is related to worse Gleason grade.

Keywords: antioxidants; fatty acid; nutrients

127 Immunologic Scars in Patients With Hodgkin's Lymphoma

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Purpose: Near complete absence of interleukin-15 receptor alpha (IL-15Rα) expression on T lymphocytes in peripheral blood has been reported following EBV-associated acute infectious mononucleosis (AIM). We sought to determine whether a similar deficit might also be characteristic of EBV-related Hodgkin's lymphoma [EBV(+)HL].

Patients and Methods: Lymphocytes from donors with AIM, EBV-seropositive and EBV-seronegative healthy controls, EBV(+)HL and EBV(-)HL, were assayed to determine IL-15Rα and IL-7Rα expression.

Results: The frequencies of IL-15Rα $CD3^+/CD8^+$ expressing lymphocytes in EBV(+)HL patients were lower than in EBV(-)HL patients and lower than in EBV-seropositive healthy controls. Only healthy controls with a history of AIM showed lower frequencies. The frequencies of IL-7Rα $CD3^+/CD8^+$ T expressing lymphocytes in HL patients were lower than in any group of healthy controls. The frequencies did not differ between EBV(-) and EBV(+) HL patients. Both the IL-15Rα and IL-7Rα deficits were similar in untreated HL patients and in those more than a year in remission.

Discussion: This report extends the observation that EBV AIM is associated with markedly decreased frequencies of $CD3^+CD8^+$ IL-15Rα lymphocytes to patients with EBV(+)HL. Patients with history of HL[EBV(-) or (+)] also show reduced frequencies of $CD3^+CD8^+$ IL-7Rα. These deficits in IL-15Rα and IL-7Rα do not resolve in months or with remission induction but seem to persist indefinitely. The functional consequences of these deficits are largely unknown, but emerging data suggest that these IL-15Rα

and IL-7Rα pathways may be important in establishing and maintaining innate and acquired cellular immunity. It seems possible that these abnormalities may predispose to the development of HL or may be a consequence of the lymphoma and that these abnormalities may be important in explaining long-recognized cellular immune deficits in association with HL.

Keywords: Epstein-Barr; Hodgkin's; IL-15

128 Genomewide and Promoter-Specific Analyses Identify Novel Targets of Aberrant DNA Methylation in Adult Acute Lymphocytic Leukemias

Please see Poster Discussion Session Biomarkers I.

129 Identification of Pharmacodynamic Markers of Biologic Efficacy of PI3K Pathway Inhibition by Perifosine

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Perifosine is a novel ether alkylphospholipid compound. Recent studies suggest that perifosine inhibits information transfer through the phosphatidylinositol 3′ kinase (PI3K) pathway by preventing recruitment of the pleckstrin homology (PH) domain of Akt to the cell membrane. Herein we confirm that perifosine selectively prevents recruitment of Akt and blocks phosphorylation of Akt and its downstream effectors. Perifosine inhibited the growth of a variety of in vitro and in vivo models of breast, ovarian, and prostate cancers, and this was demonstrated to reflect apoptosis in two ovarian cancer cell lines. We demonstrated a clear correlation between perifosine inhibitory effects on cell line growth in vitro and basal Akt activation at Serine 473 as a result of activating genomic anomalies. In both cell line and xenograft models, there was a strong, statistically significant correlation between quantified perifosine-induced downregulation of phosphorylation of Akt at Serine 473 and of its downstream targets, particularly S6, and the antitumor efficacy of different doses of the drug. This correlation suggests that modulation of PI3K pathway activation may be particularly useful as an early pharmacodynamic marker, demonstrating delivery of a biologically relevant perifosine dose in human cancer clinical trials. Further, the activation status of the PI3K pathway may provide a method of selecting patients most likely to respond to perifosine alone or in combination with other drugs.

Keywords: biomarkers; perifosine; PI3K

130 High-Throughput Analysis of DNA Methylation in Leukemia Cell Lines Reveals Distinct Myeloid, B Cell and T Cell Signatures

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An abnormal pattern of DNA methylation occurs at specific genes in almost all neoplasms. The lack of high-throughput methods with high specificity and sensitivity to detect changes in DNA methylation has limited its application for clinical profiling. We overcame this limitation by introducing an improved method to identify methylated genes genomewide by hybridizing a CpG island microarray with amplicons obtained by the methylated CpG island amplification technique (MCAM). We previously validated this method in 3 cancer cell lines and 15 primary colorectal tumors, resulting in the discovery of hundreds of new methylated genes in cancer. The sensitivity and specificity of the method to detect hypermethylated loci were 76% and 98%, respectively, according to validation by bisulfite-PCR. Here, we extended this genomewide analysis of CpG island methylation to 10 leukemia cells with different cellular origin (myeloid cell lines KG1, KG1a, HEL, K562, and TF1; T lymphoid cell lines CEM and JTAG; and B lymphoid cell lines ALL1, BJAB, and Raji). On average, 206 genomic loci were found to be hypermethylated in these cell lines, ranging from 69 (KG1) to 415 loci (Raji), reinforcing the existence of extensive epigenome alteration in leukemia. Unsupervised hierarchical clustering perfectly reclassified the cell lines according to their lineage origin, justifying the use of methylation markers for uncovering tumor-specific pathways of gene inactivation. Several candidate genes with a putative role in cancer were identified by this method, for example, MKL2 (megakaryoblastic leukemia 2), MN1 (meningioma 1), MTX2 (metaxin 2), and WIT1 (Wilms' tumor upstream neighbor 1), among others. Our future work will focus on studying these candidate genes in primary leukemia samples and also on genomewide analysis of patient samples to correlate their methylation profile with response to therapeutic agents. In conclusion, our results support the use of high-throughput, genomewide methylation analysis to classify hematopoietic diseases into biological subgroups.

Keywords: CpG island microarray; DNA methylation; leukemia

131 B2 Bradykinin Receptor Mediates Induction of Cyclooxygenase-2 and Is Overexpressed in Head and Neck Squamous Cell Carcinomas

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Bradykinin (BK) has been shown to promote growth and migration of head and neck squamous cell carcinoma (HNSCC) cells via epidermal growth factor receptor (EGFR) transactivation, where targeting the BK receptor in combination with EGFR blockade inhibits HNSCC growth. It has also been reported that BK can cause the induction of cyclooxygenase-2 (COX-2), a pro-tumorigenic enzyme, via the MAPK (mitogen-activated protein kinase) pathway in human airway cells. To determine whether COX-2 is upregulated by BK in HNSCC, the current study investigated BK-induced EGFR transactivation, MAPK activation, and cyclooxygenase-2 (COX-2) expression in human HNSCC cells. BK induced a concentration- and time-dependent induction of COX-2 protein in HNSCC, which was accompanied by phosphorylation of EGFR and MAPK. These effects were abolished by the B2 receptor (B2R) antagonist Hoe 140 but not the B1 receptor (B1R) antagonist, Lys-[Leu^8]des-Arg^9-BK. COX-2 induction was accompanied by increased release of PGE_2. No effect of a B1R agonist (des-Arg^9-BK) on p-MAPK or COX-2 expression was observed. B2R protein was found to be expressed in all four head and neck cell lines tested. Immunohistochemical analysis and Western blot analysis revealed that B2R, but not B1R, was significantly overexpressed in HNSCC tumors compared to levels in normal mucosa from the same patient. In HNSCC cells, the BK-induced expression of COX-2 was inhibited by EGFR kinase inhibitors (gefitinib or erlotinib), or mitogen-activated protein kinase (MEK) inhibitors (PD98059 or U0126). These results suggest that EGFR and MAPK are required for COX-2 induction by BK. Thus, BK, acting through the B2 bradykinin receptor, induces COX-2, a known cancer-promoting stimulus. Upregulation of the B2R in head and neck cancers suggests this pathway is involved in HNSCC tumorigenesis.

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Keywords: bradykinin; COX-2; HNSCC

132 Restoration of E-Cadherin in Colon Cancer Cells: Identification by High-Throughput Screening

Please see Poster Discussion Session New Technologies and Methodologies II.

133 Correlation and Comparison of Genetic and Genomic Changes in Metastatic Melanoma Cell Lines and Tumors

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To date, few wide-scale analyses have been conducted to detect potential distinctive patterns of genomic aberrations on a large set of metastatic melanoma lesions. In addition, while cell lines commonly are used as a model system for melanoma, because of the difficulty obtaining tumor tissue, no formal genetic and genomic comparisons have been done to determine how representative they are of tumors. We have performed BAC-based array-based comparative genomic hybridization (aCGH) on 83 metastatic melanomas (27 melanoma cell lines, 34 frozen and 22 formalin-fixed, paraffin-embedded clinical melanoma specimens). We used ACE (Analysis of Copy Number Errors) to determine copy number amplification and deletion and the CGHanalyzer to determine high-level amplification and homozygous deletions. We used the STAC algorithm (Significance Testing for Aberrant Copy number) to determine regions of significant change across all our samples.

We observed recurrent and characteristic chromosomal aberrations: losses in chromosomes 5q, 6q, 9, 10q, 11q, and 8q, and gains in chromosomes 1q, 6p, 8p, 17p, and 20q. Many of these changes previously have been observed in cell lines, but not in tumors. High-level amplifications were seen at chromosomes 11q13 (*CCND1*) and 3p14 (*MITF*). A homozygous deletion on chromosome 13q34 including *RASA3* was found in two melanoma samples. We screened all of our samples for mutations in *BRAF* and *NRAS*. Of the 27 cell lines, 19 (70%) contained a *BRAF* mutation, and in all but two, the V600E mutation. Five (20%) had *NRAS* mutations, all at codon 61. Two cell lines harbored neither *BRAF* nor *NRAS* mutations. Mutational screening also was completed in the 47 metastatic melanoma samples: 25 tumors (53%) carried *BRAF* mutations, all but three showing V600E

substitution, 11 (23%) samples had *NRAS* mutations, and 11 (23%) metastatic melanomas were *BRAF* and *NRAS* wild-type. None of the 27 melanoma cell lines and 25 metastatic melanoma samples with *BRAF* mutation contained a *NRAS* mutation; thus, *BRAF* and *RAS* alterations were mutually exclusive. A two-way ANOVA test was performed to examine two questions: (1) to distinguish whether there were differences between tissue types (melanoma cell lines, paraffin-embedded and frozen melanoma samples) and (2) to determine whether *BRAF* mutation status was associated with specific DNA copy number aberrations and, if so, whether those aberrations varied with sample type.

We did not observe any significant differences in copy number aberration between tumor samples extracted from paraffin-embedded and fresh-frozen tissues. However, we did observe significant differences between cell lines and tumor samples; specifically, the cell lines had amplifications of chromosomes 2q and 20 and deletions of chromosome 4 that were not observed in tumors. Within both melanoma cell lines and tumors, the presence of a *BRAF* mutation frequently was associated with amplification of chromosomes 7 and 20p12-11 and deletion of 10q24-25 ($p<.01$). The most significant region of amplification in *BRAF* mutant samples was around *BRAF* itself. Using these data, we are able to identify genomic differences in cell lines that differentiate them from melanoma tumors and in cell lines and tumors that are associated with mutant *BRAF*.

Keywords: genetics; genomics; metastasis

134 Automated Molecular Morphology for Biomarker Profiling in Brain Tumors

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The activities of specific cell signaling pathways in tumor tissue contribute to the effects of molecularly targeted therapy response for individual glioblastomas. Because of this, glioblastoma clinical trials that target specific signaling pathways need to include molecular morphologic techniques for assessing the complex and heterogeneous signal transduction states of tissue having undergone histological preservation. Two fundamental techniques for molecular morphologic applications are standardized immunohistology (immunofluorescence [IF] and immunohistochemistry [IHC]) and in situ hybridization. Although each technique may be performed manually, an individual, case-by-case approach often promotes a procedurally associated variability in results that confounds meaningful comparison of data between different tumors. To address this concern, computer-assisted, automated instrumentation approaches have been advanced that permit a more accurate quantitation of IHC and comparative inter-tumor scoring. In addition, fully automated instrumentation provides a more efficient platform for developing tests for novel biomarkers. All steps of specimen processing can now be computer-programmed for each slide, including drying, dewaxing, and counterstaining. Furthermore, antibody incubations and antigen retrieval techniques (acidic/basic chelation and/or proteolytic treatment) can be programmed for customized times and temperatures, allowing for method optimization and highly reproducible data acquisition. Bar-coded slide labels record detailed technical specifications from each staining run, thus facilitating multi-investigator data collection. A special "liquid coverslip" permits the use of only 100 µL for all reagents, conserving limited-quantity antibodies and eliminating "edge-artifact" on tissue sections. The application of these automated approaches for EGFR and PTEN IHC analysis of glioblastoma multiforme surgical specimens and their corresponding xenografts will be presented at the meeting.

Keywords: EGFR; glioblastoma; immunohistochemistry

135 Self-Report of Symptoms Associated With Ovarian Cancer as an Additional Means of Evaluating a Woman's Risk for Ovarian Cancer

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While currently available tests for early detection of ovarian cancer show promise as potential screening tools, additional tests may be needed to improve the overall diagnostic accuracy of a screening program. Symptoms specific to ovarian cancer have recently been identified as a possible "marker" of disease and may be useful for screening. This study sought to examine whether a symptom index used in combination with CA125 could improve on the sensitivity or specificity of CA125 alone as a method of screening for ovarian cancer.

A prospective case-control study design was used. Participants included 75 women with ovarian cancer, 254 controls at high risk for disease owing to family history of breast or ovarian cancer who are participating in a research screening program for early detection of ovarian cancer, and 131 controls undergoing surgery for benign pelvic masses. Women were invited to fill out a survey of symptoms over the past year and to contribute blood for assessment of CA125 levels. Logistic regression analysis was used to determine whether the symptom index independently predicted cancer after controlling for CA125 levels. The diagnostic accuracy of CA125, the symptom index, and the two tests used in combination was also evaluated.

A symptom index identified 64.0% of the cancer cases in our study population (45.2% of those with early-stage disease and 78.0% of those with later-stage disease). Approximately 12% (11.8%) of the women in the high-risk screening program without cancer also received a positive symptom index score. In logistic regression analyses, symptom index information made a statistically significant independent contribution to prediction of ovarian cancer after controlling for CA125 levels. An either/or combination of CA125 and the symptom index identified 90.8% of the women with cancer, 80.6% of the early-stage cancers, and 97.6% of the late-stage cancers. The symptom index identified cancer in 50% of the affected women who did not have elevated CA125 levels. Women with both elevated CA125 levels and a positive symptom index included 53% of the cancer cases and none of the unaffected women in the screening program, suggesting that a multimodal screening program with symptoms as an initial screen might have good specificity.

Symptoms may be useful in identifying women with ovarian cancer. The addition of a symptom index to CA125 may significantly improve the sensitivity or specificity for detection of ovarian cancer associated with using CA125. The two tests combined identified over 80% of women with early-stage disease.

Keywords: early-stage disease; screening; symptoms index

136 Chemosensitization of Head and Neck Cancer Cells by PUMA

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Head and neck squamous cell carcinoma (HNSCC) is the eighth most common cancer worldwide. Patients often present with advanced disease, which responds poorly to chemotherapy and radiation therapy. PUMA is a BH3-only Bcl-2 family protein and a p53 target that is required for apoptosis induced by p53 and various chemotherapeutic agents. In this study, we found that PUMA induction by chemotherapeutic agents is abrogated in most HNSCC cell lines. Adenoviral gene delivery of PUMA induced apoptosis and chemosensitization more potently than did adenoviral delivery of p53 in HNSCC cells. Finally, we showed that PUMA suppressed the growth of HNSCC xenograft tumors and sensitized them to cisplatin through induction of apoptosis. Our data suggest that absence of PUMA activation in HNSCC cells contributes to chemoresistance and that gene therapy with PUMA might be an efficient substitute for p53 to enhance the responses of HNSCC cells to chemotherapy.

Keywords: apoptosis; chemosensitization; HNSCC

137 Molecular Characterization of Human Breast Tumor Vascular Cells

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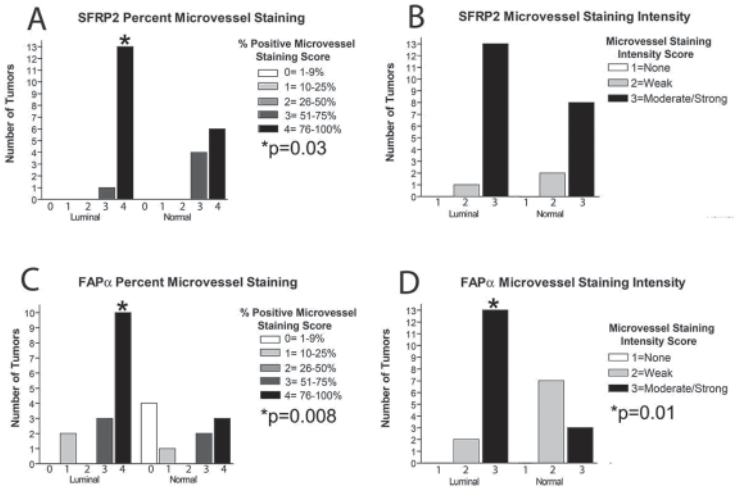
Introduction: Tumor and normal vessels are believed to possess different biological phenotypes. We hypothesized that these differences would be manifested in differences in gene expression that could be identified using gene expression analysis.

Methods: Immunohistochemistry (IHC) with factor VIII antibody was performed on sections from frozen human luminal breast tumors (n = 5) and from normal breast (n = 5), followed by laser capture microdissection of vascular cells. RNA was extracted, amplified, and fluorescently labeled cDNA was synthesized and hybridized to 44,000 feature DNA microarrays. Statistical analysis of microarrays was used to compare differences in gene expression between tumor and normal vascular cells. Protein expression of select genes was confirmed using IHC. A one-tailed t-test was used to evaluate differences in percent positive endothelial staining and intensity of endothelial staining between luminal breast tumors and normal breast tissue.

Results: 1,176 genes were differentially expressed between breast tumor and normal vascular cells, of which 55 had greater than fourfold differential expression. Of these 55 genes, we identified 7 genes that encode membrane proteins (FAP, FGFR3, IFI27, SLITRK6, CD19, T3JAM, and RAMP1) and 5 genes that encode secreted proteins (INHBA, SFRP2, KAZALD1, HTRA3, HIG2) that would offer particularly good drug targets because of their accessibility. SFRP2 and FAP protein expression in luminal, basal, and HER2/neu breast tumor endothelium was confirmed by IHC, and staining in luminal tumor endothelium was statistically significantly greater than in normal (see figure) endothelium.

Conclusions: Gene expression in breast tumor vascular cells is significantly different from that in normal cells, with SFRP2 and FAP also being validated on a second sample set using a different platform (IHC). These genes may serve as targets for developing angiogenesis inhibitors.

Keywords: angiogenesis; genomics; vascular disrupting agents



IHC for SFRP2 and FAP on luminal tumors and normal breast tissue.

138 An Association Between Variation in Genes Associated With Androgen Metabolism and the Efficacy of Primary Androgen Deprivation Therapy in Castration-Sensitive Prostate Cancer

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Background: In men with nonlocalized castration-sensitive prostate cancer (CSCaP), there is considerable heterogeneity in the time to PSA-TTP during treatment with primary ADT, with responses lasting from a few months to many years. To help explain this heterogeneity, we tested the hypothesis that host variation within genes important in androgen synthesis and metabolism may influence the length of response to ADT.

Methods: Using the Prostate Clinical Research Information Systems, we identified a cohort of patients from our institution for whom baseline and followup clinical information was available and who had submitted a blood sample for DNA analysis. We identified genes involved in androgen synthesis and metabolism and identified single nucleotide polymorphisms (SNPs) from http//:www.hapmap.org with a minor allele frequency of >.05 from a population of Northern and Western European ancestry (CEU) to interrogate each of these genes. SNP analysis was performed using the Sequenom platform.

Results: Researchers initially selected 220 SNPs in 23 genes. After filtering by Hardy-Weinberg criteria p<.01, genotyping call rate >80%, and minor allele frequency >5%, the resulting 127 SNPs in 20 genes involved in androgen synthesis and metabolism were genotyped in 530 patients. Median followup for these patients was 5.1 years, and median PSA-TTP was 23.4 months (95% CI, 19.0-26.3). 48.7% of these patients had metastatic disease at the time of ADT initiation, and 90.8% were treated with a luteinizing hormone-releasing hormone analogue as their primary means of ADT. 66% received an antiandrogen as part of their ADT. Median PSA at ADT initiation was 15 ng/mL (range, 0-15,090), and the median time between the diagnosis of CaP and ADT initiation was 2.21 years (range, 0-19 years). The presence of metastatic disease (MetCaP) at ADT initiation was strongly associated with PSA-TTP (15.8 months versus 31 months for presence versus absence of MetCaP at ADT initiation, respectively, p<.0001). Of the 127 SNPs tested, PSA-TTP was significantly associated with genotype in 3 in univariate and multivariate analyses. This effect was additive.

Conclusion: Germ line variation in genes involved in androgen synthesis and metabolism is associated with differences in PSA-TTP. Further analysis and validation of these data are needed.

Keywords: androgen deprivation therapy; castration; pharmacogenomics

139 Identification of Melanoma Cells With Resistance to BRAF and MEK Inhibitors

Please see Poster Discussion Session Therapy II.

140 Anti-CS1 Humanized Monoclonal Antibody HuLuc63 Inhibits Myeloma Cell Adhesion and Growth in the Bone Marrow Milieu and Targets the Cells for Killing via Antibody-Dependent Cellular Cytotoxicity

Please see Poster Discussion Session Therapy II.

141 Targeting MEK Induces Myeloma Cell Cytotoxicity and Inhibits Osteoclastogenesis

Yu-Tzu Tai[1], Mariateresa Fulciniti[1], Teru Hideshima[1], Weihua Song[1], Merav Leiba[1], Xian-Feng Li[1], Matthew Rumizen[1], Peter Burger[1], Aileen Morrison[1], Klaus Podar[1], Dharminder Chauhan[1], Paul Richardson[1], Nikhil C. Munshi[1,2], Irene Ghobrial[1], Paul Smith[3], Kenneth C. Anderson[1]

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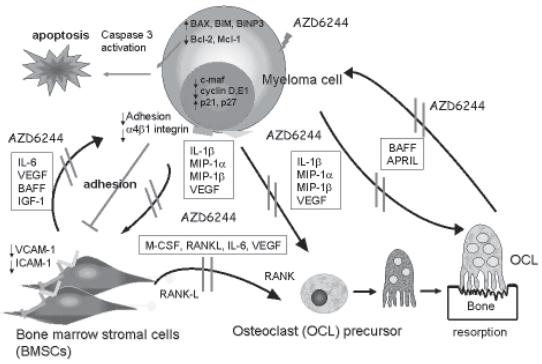
Activation of the extracellular signal-regulated kinase1/2 (ERK1/2) signaling cascade mediates human multiple myeloma (MM) growth and survival triggered by cytokines and adhesion to bone marrow stromal cells (BMSCs). Here, we examined the effect of AZD6244 (ARRY-142886), a novel and specific MEK1/2 inhibitor, on human MM cell growth in the bone marrow (BM) milieu. AZD6244 blocks constitutive and cytokine-stimulated ERK1/2 phosphorylation and inhibits proliferation and survival of human MM cell lines and patient MM cells, regardless of sensitivity to conventional chemotherapy. Importantly, AZD6244 (200 nM)

induces apoptosis in patient MM cells even in the presence of exogenous interleukin-6 or BMSCs associated with triggering of caspase 3 activity. AZD6244 sensitizes MM cells to both conventional (dexamethasone) and novel (perifosine, lenalidomide, and bortezomib) therapies. AZD6244 downregulates the expression/secretion of osteoclast (OC)-activating factors from MM cells and inhibits in vitro differentiation of MM patient PBMCs to OCs induced by ligand for receptor activator of NF-κB (RANKL) and macrophage colony stimulating factor (M-CSF). Finally, AZD6244 inhibits tumor growth and prolongs survival in vivo in a human plasmacytoma xenograft model. Taken together, these results show that AZD6244 targets both MM cells and OCs in the BM microenvironment, providing the preclinical framework for clinical trials to improve patient outcome in MM.



Keywords: ERK activation; microenvironment; osteoclastogenesis

142 Genetic Variations in Drug Metabolism and DNA Damage Response and Repair as Predictors for Clinical Outcomes of Pancreatic Adenocarcinoma

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Gemcitabine requires metabolic activation before exerting its tumor-killing activity. Previous studies have shown a clear association between metabolic enzyme activities and pharmacokinetics of gemcitabine. Repair of gemcitabine-induced DNA damage may require DNA mismatch repair (MMR) mechanisms and MMR-mediated signaling transduction. The overall goal of this study is to determine whether single nucleotide polymorphisms (SNPs), in genes involved in gemcitabine metabolism, DNA MMR, and DNA damage response influence response to treatment and overall survival of patients with pancreatic adenocarcinoma. We analyzed a total of 48 SNPs of 23 genes in 132 patients with potentially resectable pancreatic adenocarcinomas who were treated with preoperative gemcitabine and gemcitabine/cisplatin in combination with radiation from 1999 to 2006. The endpoints evaluated include toxicity data (according to the Common Terminology Criteria for Adverse Events v3.0 criteria), success rate of tumor resection, and overall survival time (from date of diagnosis to date of death or date of last followup). Ninety-one patients (68.9%) responded to therapy and had tumor resection. Ninety-two patients (69.7%) died by the end of March 2007. Genotyping used the Sequenom and TaqMan methods.

Among the genes examined, significant impact on overall survival was observed for the metabolic genes CDA (cytidine deaminase), MRP3 (ABCC3), and RRM1 (ribonucleotide reductase M1); DNA MMR genes MLH1, MSH2, and EXO1; and DNA damage response genes ATM, ATR, and CHK1. A strong and significant combined genotype effect was detected for genes involved in each of the three genetic pathways. For example, patients carrying 0, 1, 2, or ≥3 adverse alleles of the CDA, MRP2, RRM1, and hCNT3 genes had a median survival time (MST) of 54.8, 24.3, 18.4, and 14.5 months, respectively ($p = .03$, log rank test). Patients with 0, 1, 2, or ≥3 adverse alleles of the ATM, ATR, CHK1, and CHK2 genes had MST of 58.1, 35.7, 16.5, 10.5 months, respectively ($p = .007$). The MLH1 IVS1 C-169T SNP showed significant impact on the success rate of tumor resection, i.e., 76.5% for the CT/TT genotype vs. 48.8% for the CC genotype ($p = .003$, χ^2 test). Patients carrying the low-activity alleles of the CDA gene showed a higher frequency (74.4%) of ANC (<LLN – 1,500/mm^3 or <LLN – 1.5×10^9/L) compared to those with the high-activity alleles (53.2%) ($p = .027$). A significant difference in the frequency of reduced platelets (<LLN – 75,000/mm^3 or <LLN – 75.0×10^9/L) by MRP2 genotype was also observed. 78.0% of the patients carrying the protective MRP2 alleles for survival vs. 52.2% of the patients carrying the adverse alleles for survival had reduced platelets ($p = .004$).

These observations provide strong evidence supporting the hypothesis that genetic variations in drug metabolism, DNA damage response, and DNA repair affect clinical response to cancer therapy and, in turn, patient survival. Such information can be potentially used in the clinic in stratifying patients for clinical trials or in dose modifications. For example, patients with alleles coding for a higher activity of CDA and MRP2 or a lower activity of hCNTs may require a higher dose of gemcitabine. Patients with MMR deficiency may have a better response to gemcitabine- than to 5-FU-based chemoradiation.

Keywords: gene polymorphism; pharmacogenetics; survival

143 Upregulation of c-Jun Inhibits Proliferation and Induces Apoptosis via Caspase-Triggered c-Abl Cleavage in Human Multiple Myeloma

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Expression profiling of adaphostin-treated MM cells to identify its molecular targets was carried out. Surprisingly, c-Jun was the most upregulated gene even at the earliest point of analysis (2 hours). We also observed adaphostin-induced c-Abl cleavage in immunoblot analysis. Proteasome inhibitor bortezomib, but not melphalan or dexamethasone, induced similar effects, indicating unique agent-dependent mechanisms. Using caspase inhibitors as well as caspase-resistant mutants of c-Abl (TM-c-Abl and D565A-Abl), we then showed that c-Abl cleavage in MM cells requires caspase activity. Importantly, both overexpression of the c-Abl fragment or c-Jun as well as knockdown of c-Abl and c-Jun expression by siRNA confirmed that adaphostin-induced c-Jun upregulation triggers downstream caspase-mediated c-Abl cleavage, inhibition of MM cell growth, and induction of apoptosis. Finally, our data suggest that this mechanism may not be restricted to MM but may also be important in a broad range of malignancies including erythroleukemia and solid tumors.

Keywords: adaphostin; c-Abl; c-Jun

144 Targeting PKC in Multiple Myeloma: In Vitro and In Vivo Effects of the Novel, Orally Available Small-Molecule Inhibitor Enzastaurin (LY317615.HCl)

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In multiple myeloma (MM), protein kinase C (PKC) signaling pathways have been implicated in cell proliferation, survival, and migration. Here we investigated the novel, orally available PKC-inhibitor Enzastaurin for its anti-MM activity. Enzastaurin specifically inhibits phorbolester-induced activation of PKC isoforms, as well as phosphorylation of downstream signaling molecules MARCKS and PKCμ. Importantly, it also inhibits PKC activation triggered by growth factors and cytokines secreted by bone marrow stromal cells (BMSCs); co-stimulation with fibronectin, vascular endothelial growth factor (VEGF), or interleukin-6 (IL-6); and MM patient serum. Consequently, Enzastaurin inhibits proliferation, survival, and migration of MM cell lines and MM cells isolated from multidrug-resistant patients and overcomes MM cell growth triggered by binding to BMSCs and endothelial cells. Importantly, strong synergistic cytotoxicity is observed when Enzastaurin is combined with bortezomib and moderate synergistic or additive effects are observed when it is combined with melphalan or lenalidomide. Finally, tumor growth, survival, and angiogenesis are abrogated by Enzastaurin in an in vivo xenograft model of human MM. Our results therefore demonstrate in vitro and in vivo efficacy of the orally available PKC inhibitor Enzastaurin in MM and strongly support its clinical evaluation, alone or in combination therapies, to improve patient outcome in MM.

Keywords: enzastaurin; myeloma; PKC

145 Recognition of Prostate Tumor Cells by STEAP-Specific Helper T Lymphocytes in an HLA Class II-Restricted Manner

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The six-transmembrane epithelial antigen of prostate (STEAP) protein is an attractive candidate for T cell-based immunotherapy because it is overexpressed in prostate cancer and various other tumor types. Several peptide epitopes capable of stimulating cytotoxic T lymphopcytes (CTLs) that killed STEAP-expressing tumor cells were described in the past. Our present goal was the identification of helper T lymphocyte (HTL) peptide epitopes of STEAP for the optimization of CTL-based immunotherapies against

STEAP-expressing prostate cancer. Candidate HTL epitopes for STEAP were predicted using in silico algorithms for HLA Class II-binding peptides and were tested for their ability to elicit HTL responses by in vitro peptide vaccination of CD4 T lymphocytes from healthy individuals and prostate cancer patients. Two peptides, $STEAP_{102-116}$ (HQQYFYKIPILVINK) and $STEAP_{192-206}$ (LLNWAY-QQVQQNKED), were shown to be effective in stimulating in vitro antitumor HTL responses using lymphocytes from normal individuals and prostate cancer patients. Notably, both STEAP HTL peptides behaved as promiscuous T cell epitopes since they stimulated HTL responses in the context of more than one MHC Class II allele. These newly described STEAP HTL epitopes could be of value in the design and optimization of T cell-based immunotherapy against STEAP-expressing prostate tumors.

Keywords: immunology; peptide vaccine development; T cell epitopes

146 Molecular Correlates of Response to Epidermal Growth Factor Receptor-Targeted Therapy in Bladder Cancer

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[1]University of Texas M.D. Anderson Cancer Center, Houston, Texas; [2]McGill University Health Centre, Montreal, Quebec, Canada; [3]University of Virginia Health System, Charlottesville, Virginia

Introduction and Objective: The epidermal growth factor receptor (EGFR) is widely expressed in bladder cancer and its expression is predictive of disease outcome. Clinical trials evaluating EGFR-directed therapy in bladder cancer have yielded disappointing results because of the difficulty of identifying the subset of patients likely to respond to treatment. Here we evaluated potential molecular markers of sensitivity to EGFR-targeted treatment in bladder cancer.

Methods: Cetuximab (C225), a selective antihuman EGFR antagonist monoclonal antibody, was generously provided by ImClone Systems, Inc. (New York, New York). Twelve bladder cancer cell lines were stratified as sensitive, intermediate, or resistant to C225 by ^{3}H-thymidine uptake (>50%, 50-75%, and <75% inhibition, respectively). We have previously shown that these cell lines contain only wild-type EGFR. EGFR ligand expression (TGF-α, PDGF isotypes, and IGF-1) was measured by ELISA. Growth factor receptors (EGFR, HER2, HER3, HER4, PDGFR-α and -β, IGFR, and FGFR3) and markers of epithelial and mesenchymal differentiation were evaluated by Western blot. FGFR3 mutation analysis was performed by PCR amplification of exon 7 and automated sequencing. Tumorigenicity was evaluated following orthotopic injection of 1×10^6 cells in nude mice, and invasive potential determined by Boyden chamber assay.

Results: All tested bladder cancer cell lines expressed EGFR and its ligands in varying amounts that were not predictive of response. Of the growth factor receptors, expression of PDGFR and nuclear HER4 correlated with C225-resistance, and membranous HER4 was seen only in sensitive and intermediate lines. Loss of E-cadherin expression similarly correlated with C225 resistance. A looser association was seen with loss of β-catenin and plakoglobin. FGFR3 mutations were identified only in intermediate and resistant cell lines. C225-resistant cell lines were the most invasive, and C225-sensitive cell lines were least invasive. Three of the four most sensitive cell lines (253J-P, UMUC5, and UMUC10) were nontumorigenic in the orthotopic model, but all others were highly tumorigenic.

Conclusion: Specific growth factor expression and loss of epithelial markers correlated with resistance to EGFR-targeted therapy and invasive phenotype. These findings will be used in the prospective evaluation of patient response to EGFR-targeted therapy in a neoadjuvant trial prior to radical cystectomy. At the same time, we are pursuing the molecular mechanisms of these relationships and the potential targeting of other growth factor receptors, especially PDGFR-β and FGFR3.

This research was supported by the SPORE in Genitourinary Cancer, CA91846; T32 training grant; and AUA Foundation grant.

Keywords: E-cadherin; EGFR; EMT

147 High Cancer-Specific Expression of an Overexpressed Biomarker Is Attributable to an Upstream Enhancer

Please see Poster Discussion Session New Technologies and Methodologies I.

148 Targeting the Cancer Stem Cell Phenotype With Hedgehog Pathway Inhibitors

Craig D. Peacock, Qiuju Wang, Gregory S. Gesell, Ian M. Corcoran-Schwartz, Evan Jones, Jynho Kim, Wendy L. Devereux, Jonathan T. Rhodes, Carol A. Huff, Philip A. Beachy, William Matsui, D. Neil Watkins

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We have previously described a role for the hedgehog (HH) pathway in lung repair and tumorigenesis and have demonstrated that inhibitors of this pathway may represent a novel cancer therapy. Since HH signaling regulates progenitor cell fate in development and homeostasis, the tumor stem cell hypothesis would predict that aberrant HH pathway activation would be required for the maintenance of such a population in cancer. To answer this question, we studied HH signaling in a variety of normal and malignant stem cell models. Here we show that normal bone marrow stem cells possess a unique HH expression profile, with prominent expression of SMOOTHENED, the molecular target of HH pathway inhibitors. This expression profile, and HH pathway activity, is restricted to stem cell populations in highly differentiated cancers such as multiple myeloma (MM). In MM, rare malignant progenitors capable of clonogenic growth resemble B cells, whereas the much larger tumor cell population manifests a differentiated plasma cell phenotype that pathologically defines the disease. Inhibition of HH signaling has little or no effect on malignant plasma cell growth, but markedly inhibits clonal expansion accompanied by terminal differentiation of highly purified MM stem cells. These data reveal that HH pathway activation in MM is a heterogeneous phenomenon involving complex relationships between tumor stem cells and their progeny. Investigation of this mode of HH pathway activation in lung cancer will have significant implications for the translation of HH inhibitors in patients.

Keywords: cells; hedgehog; stem

149 A Novel Approach to Overcoming Cisplatin Resistance by HSP90 Inhibition in Head and Neck Cancer Cell Lines

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We have recently discovered that EGFR is activated on exposure to chemotherapeutic agents such as cisplatin and gemcitabine in head and neck cancer cell lines. This activation of EGFR leads to ubiquitin-mediated degradation of the receptor itself, causing cellular toxicity and radiation sensitization [1,2]. We hypothesized that the receptor degradation is a critical event in drug-induced toxicity and radiation sensitization. In order to test our hypothesis, we screened several head and neck cancer cell lines that are either sensitive or resistant to cisplatin. We have found that EGFR is not degraded in cisplatin-resistant cell lines. We hypothesized that if we could promote EGFR degradation, we could then sensitize these cells to cisplatin-mediated cytotoxicity radiosensitization. For this purpose we chose a novel approach toward targeting EGFR using Hsp90 inhibition. We chose this approach because it is known that Hsp90 is a major chaperone for ErbB-2, which shares major sequence homology with EGFR at the Hsp90 binding site. Our preliminary data show that (1) Hsp90 is activated by cisplatin only in cisplatin-sensitive cells, (2) EGFR and Hsp90 interact and can be co-immunoprecipitated, and (3) geldanamycin, an inhibitor of Hsp90, accelerates the degradation of EGFR in cisplatin-resistant cells, leading to both cellular toxicity and radiosensitization. These findings demonstrate that EGFR degradation after chemotherapy depends on Hsp90. Furthermore, they suggest that the new generation of geldanamycin analogues that are entering the clinic may potentiate cisplatin-mediated cytotoxicity and radiosensitization via EGFR degradation.

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Keywords: cisplatin; EGFR; Hsp90

150 Construction of Probasin and DF3/MUC-1 Driven Conditionally Replicating Adenoviruses Specific for Prostate and Breast Cancer Cells

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To extend the use of NIS-mediated radioiodine therapy to nonthyroid cancer, we have successfully transferred and expressed the sodium iodide symporter (NIS) gene in prostate and breast cancer cells both in vivo and in vitro using adenoviral vectors. Our experience with adenovirus-mediated NIS transfer and radioiodine therapy culminated in a Phase I trial for prostate cancer that will commence in early August 2007. In order to improve virotherapy efficiency, we are currently developing three conditionally replicating adenoviruses in which the E1a gene is driven by the prostate-specific promoter probasin (ARR_2PB, a gift from Dr. Robert Matusik, Vanderbilt) or two different versions of the tumor-specific promoter MUC-1 (from Dr. Sandra Gendler, Mayo Clinic Scottsdale) in order to target breast cancer cells. The phenotypic stability of the rescued viruses was tested and demonstrated by four consecutive passages on their respective permissive cells. Transduction of nonpermissive cell lines resulted in an unproductive viral infection. Our results demonstrate that the probasin promoter restricted viral replication to prostate cells that express the androgen receptor, while the two versions of the MUC-1 promoter allowed replication only in MUC-1-positive cell lines. Currently we are also inserting the NIS gene under the control of the CMV promoter at the E3 region into this construct. Replicating virus expressing NIS will combine oncolysis with radioiodine, thus targeting the radiotherapy only to infected tumor. Finally, it is reasonable to assume that tumor regions that are nonaccessible to infection might be affected because of the very efficient bystander effect resulting from NIS-mediated radioiodine concentration.

This research was funded by Mayo Prostate (CA91965) and Breast (CA116201) SPOREs.

Keywords: adenovirus; gene therapy; sodium iodide symporter

151 Liposomase for Localized Liberation of Liposomal Loads

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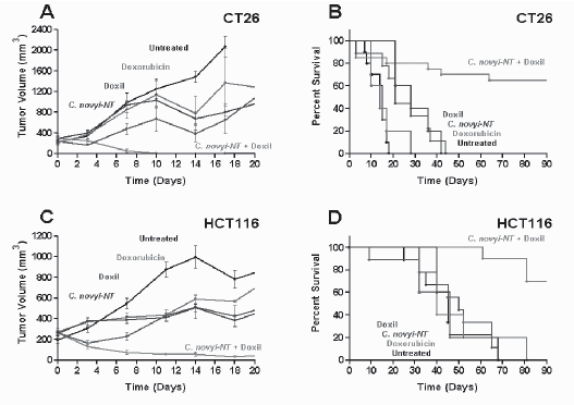

Figure 1. *C. novyi-NT* treatment enhances the antitumor activity of Doxil in mice. Mice bearing the indicated tumors were treated with various combinations of the indicated agents. Treatment with doxorubicin plus *C. novyi-NT* spores was toxic and resulted in deaths of all animals within 2 weeks (not shown). Means and standard errors of data collected from at least five mice per group are illustrated. The differences between *C. novyi-NT* plus Doxil and all other groups were significant (p<.0006, log-rank test) in the survival curves.

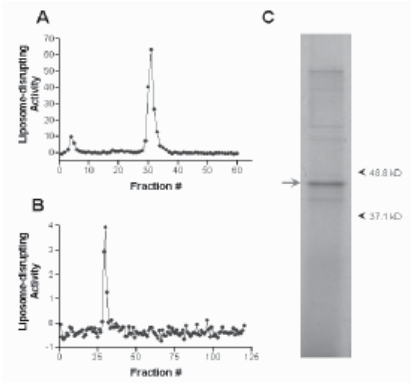

Figure 2. Biochemical purification and identification of liposome-disrupting activity from *C. novyi-NT*. (A) *C. novyi-NT* was grown until late log-phase and the medium cleared of cells by centrifugation. Following precipitation with 40% saturated ammonium sulfate, ion exchange chromatography was performed and fractions evaluated for liposome-disrupting activity. (B) The peak fractions (30-31) from (A) were pooled and further fractionated by gel filtration chromatography. (C) The peak fractions (29-30) from (B) were subjected to SDS-polyacrylamide gel electrophoresis. Silver staining of the gel revealed a predominant band migrating between protein standards with masses 48.8 kD and 37.1 kD.

Clostridium novyi-NT is a strictly anaerobic bacterium that can infect hypoxic regions within experimental tumors. Because *C. novyi-NT* is hemolytic, we hypothesized that its membrane-disrupting properties could be exploited to trigger the release of liposome-encapsulated drugs within tumors and thus enhance efficacy. Here, we show that treatment of mice bearing large, established tumors with *C. novyi-NT* plus a single dose of liposomal doxorubicin often led to eradication of the tumors (Figure 1). The bacterial factor responsible for enhancing drug release was purified and identified as a previously unknown protein termed liposomase (Figure 2). This protein could potentially be incorporated into diverse experimental approaches for the targeted delivery of chemotherapeutic agents to tumors.

Keywords: bacteria; liposome; targeted therapy

152 Targeting the Estrogen Signaling Pathway in Non-Small Cell Lung Cancer

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We previously demonstrated that the steroid hormone 17β-estradiol (E2) promotes the growth of non-small cell lung cancer (NSCLC) cells and that growth stimulation is significantly inhibited in vitro and in vivo with the pure estrogen receptor antagonist ICI 182,780 (Faslodex, fulvestrant). According to the classical model for estrogen action, estrogenic compounds bind to and activate estrogen receptors ERα or ERβ. The ligand-bound receptor functions as a transcription factor and regulates the expression of genes involved in cell differentiation, proliferation, and survival. In an immunohistochemical analysis of primary lung tumors (n = 106), we found that ERα was expressed in 73% of NSCLC cases with mainly an extra-nuclear localization. The ERα protein is likely a variant form, as it is detected using a C-terminal antibody but not an antibody directed against the N-terminus. ERβ was observed in 95% of cases and displayed nuclear and extra-nuclear localization. This differential localization suggests that ERα and ERβ may play distinct roles in regulating transcription in NSCLC. To test this hypothesis and provide an improved understanding of estrogen signaling in NSCLC cells, we determined the effect of the ERα selective agonist propyl pyrazole triol (PPT) and the ERβ selective agonist genistein (GEN) on gene expression. 201T NSCLC cells were transfected with either a CMV-ERα or a CMV-ERβ expression vector and an ERE-tk-luciferase reporter construct. The cells received no further treatment or were treated with E2, PPT, or GEN. In the ERα-transfected cells, a maximum twofold induction of reporter gene activity was observed following treatment with E2 or PPT. In the ERβ-transfected cells, E2 and GEN induced a sixfold and fourfold increase in reporter gene activity, respectively. Although ERα and ERβ displayed differential activity in the NSCLC cells, the receptors gave a comparable fourfold induction of reporter gene expression upon E2 treatment when transfected into control, ER-deficient CV-1 kidney cells. GEN but not PPT also mediated a significant increase in reporter gene activity in 201T cells transfected with the ERE-tk-luciferase vector alone, providing evidence that endogenous levels of ERβ but not those of ERα are sufficient to increase transcription. To confirm the role of ERβ, we evaluated its recruitment to the promoter of an endogenous estrogen target gene (progesterone receptor [PR]) by chromatin immunoprecipitation (ChIP). We have demonstrated that E2 treatment induced PR protein expression in nuclear extracts from NSCLC cell lines. In 201T cells, E2 and GEN specifically induced the recruitment of ERβ to the PR promoter. In parallel assays, neither E2 nor PPT was able to stimulate the recruitment of ERα. On the basis of the nuclear localization of ERβ and these transcription data, we conclude that ERβ mediates transcription responses to estrogen in NSCLC cells. In addition to examining the role of ERβ in lung cancer biology, we have also demonstrated that the estrogen synthesis pathway is active in NSCLC cells and aromatase is expressed in 48% of primary lung tumors and 95% of NSCLC cell lines examined. The aromatase inhibitor anastrozole (20 μg daily by oral gavage) decreased A549 lung tumor xenograft growth 25% in intact female mice, while, in male mice, there was no decrease in A549 xenograft growth. To maximize effects in females, mice were ovarectomized and supplemented with the steroid precursor androstiendione. Anastrozole under these conditions decreased A549 tumor growth by 40%. We are in the process of combining anastrozole with fulvestrant in this animal model system to examine enhanced benefits of inhibiting estrogen synthesis as well as downregulating ER protein. This novel combination could have clinical potential in selected NSCLC patients.

These studies were supported by P50 CA090440 (Siegfried).

Keywords: aromatase; estrogen receptor; fulvestrant

153 Cytotoxic T Lymphocyte Therapy for Hodgkin's Disease and Non-Hodgkin's Lymphoma

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EBV-associated Hodgkin's disease (HD) and some non-Hodgkins lymphoma (NHL) show type 2 latency expressing the subdominant EBV antigens EBNA1, LMP1, and LMP2, which may serve as targets for immunotherapy approaches. In previous studies, we used polyclonal EBV-specific cytotoxic T lymphocyte (CTL) in patients with relapsed EBV +ve HD and saw 2 complete and 1 partial response in 11 patients. Analyses of EBV-CTL lines showed that small populations of T cells reactive against the tumor-associated antigen LMP2 were present in the majority of the infused lines, with some expansion in the peripheral blood following infusion. We therefore hypothesized that CTL specifically targeting LMP2 might have greater efficacy in these patients. LMP2-CTLs were generated from 19 patients using dendritic cells for initial stimulations and then lymphoblastoid cell lines (LCL), both of which had been genetically modified to overexpress LMP2 by transduction with an Ad5f35LMP2 vector. Polyclonal LMP2-CTL lines recognized 1-7 (median 2) LMP2 epitopes, as determined using multimer and overlapping LMP2 peptide pools in ELISPOT assays. Eighteen patients have been treated on this dose escalation study. No immediate toxicity was observed. After CTL infusion, increased LMP2-specific T cells were detected in the blood of 10/11 evaluable patients (range, threefold to seventyfold) persisting for up to 3 months. Nine of ten patients who received LMP2-CTL as adjuvant treatment after chemotherapy remain in remission for up to 41 months after LMP2-CTL. Eight patients had detectable disease at the time of CTL, of whom three had progressive disease by 8 weeks and five had clinical responses (one very good partial and four complete responses). One of these four complete responders was biopsied 7 weeks after receiving CTLs, which were predominantly $CD4^+$(91.6%). Increased $CD4^+$ T cells were seen compared to pre-CTL biopsy specimens, and imaging studies confirmed remission. One other patient who had a complete radiological response had a supraclavicular lymph node resection, which showed selective accumulation of LMP2-tetramer positive cells (0.3% compared to 0.01% in the peripheral blood). Immunotherapy with LMP2-CTL is well tolerated in patients with relapsed lymphoma, and infused LMP2-CTL can accumulate at tumor sites and induce clinical responses.

Keywords: EBV; immunotherapy; recurrence

154 Targeted Expression of BikDD Eradicates Pancreatic Tumors Using Noninvasive Imaging Models of Pancreatic Cancer

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Pancreatic cancer is an aggressive malignancy with morbidity rates almost equal to mortality rates because of the current lack of effective treatment options. Recently, gene therapy has shown some promise in the treatment of human malignancies. In contrast with its use in other cancers, gene therapy of pancreatic cancer has remained less effective, mainly because of the lack of a useful control element to drive the therapeutic gene. Therefore, our goal was to develop a novel targeted approach to treating pancreatic cancer with potential therapeutic efficacy and safety in noninvasive imaging models of pancreatic cancer. On the basis of findings in the Serial Analysis of Gene Expression database and the literature, we identified the cholecystokinin A receptor (CCKAR), orphan G protein-coupled receptor (RDC1), urokinase-type plasminogen activator receptor (uPAR), and chymotrypsinogen B1 (CTRB1) as potential promoter candidates, screened them, and found that the human CCKAR promoter was the most active in pancreatic cancer cells. We then developed a versatile expression vector "VISA" (VP16-GAL4-WPRE integrated systemic amplifier) and used the CCKAR(C)-VISA composite to target transgene expression in pancreatic tumors. C-VISA dramatically increased the luciferase activity by 557-, 152-, and 1.5-fold as compared with CCKAR(C), CCKAR-WPRE (C-P), and CCKAR-TSTA (C-T), respectively. In all 13 pancreatic cancer cell lines, the C-VISA activity was as high as 85.5 ? 21.7% of CMV promoter activity. More importantly, the C-VISA promoter was also robust in primary cultured pancreatic cancer cells, with 42.5% of CMV promoter activity, while retaining stringent pancreatic cancer specificity. C-VISA was also shown to target expression of firefly luciferase to AsPC-1 xenografts in an orthotopic mouse model, confirmed by noninvasive imaging with an IVIS™ imaging system and measured with a luminometer. The cancer-specific index was 47.7 for C-VISA-Luc and 0.005 for CMV-

Luc in vivo. Furthermore, transgene expression of a Bik mutant (BikDD) driven by C-VISA (C-VISA-BikDD) induced death in all 13 human pancreatic cancer cell lines, a mouse pancreatic cancer cell line, and the primary cultured human pancreatic adenocarcinoma cells, comparable to CMV-BikDD. Targeted expression of *BikDD*, a potent proapoptotic gene, driven by CCKAR-VISA in liposomal complexes exhibited significant antitumor effects on pancreatic cancer and prolonged survival in multiple xenograft and syngeneic orthotopic mouse models of pancreatic tumors with virtually no toxicity. Taken together, the molecularly engineered C-VISA is robust and pancreatic cancer-specific. C-VISA-BikDD may prove to be a safe and effective pancreatic cancer gene therapy.

Keywords: BikDD; gene therapy; VISA system

155 Effect of Low-Fat Diet on Development of Prostate Cancer and Akt Phosphorylation in Myc-Transgenic Mouse Model

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Epidemiological studies suggest a correlation between fat consumption and the risk of developing prostate cancer (PCa). In previous studies, we reported that decreasing dietary fat intake slowed growth of human prostate cancer xenografts in SCID mice and modulated the circulating IGF-axis. In this report, the Myc transgenic mouse prostate cancer model was used to evaluate the effect of altering the quantity of dietary fat on the development of PCa. Myc mice develop murine prostatic intraepithelial neoplasia (PIN) at ages 3-6 months followed by invasive adenocarcinoma from 6 to 9 months due to the overexpression of human c-Myc in the mouse prostate.

Three-week-old Myc mice were placed on high-fat (42% Kcal) or low-fat (12% Kcal) diets and equal caloric intake was maintained until sacrifice at 7 months. The number of mice that developed invasive adenocarcinoma at 7 months was 28% less in the low-fat diet group (12/29) compared to the high-fat diet group (23/33, p = .04). Myc mice in the low-fat group had significantly higher serum IGFBP-1 levels than the high-fat group (6.23 + 1.02 ng/mL vs. 2.00 + 0.51 ng/mL; p = .002). Fasting serum IGF-1, IGFBP-2, and IGFBP-3 levels were similar in the low-fat and high-fat groups, while wild-type mice had lower levels of serum IGF-1 in response to a low-fat diet. The low-fat mouse serum reduced cell proliferation of LNCaP cells in vitro by 32% relative to serum from the high-fat group (p = .01). We also examined Akt phosphorylation, the hallmark of activation by the IGF-1-PI3 kinase signaling pathway, in Myc mouse prostates with Western blot analysis and detected 62% reduction (p = .016) of phospho-Akt (Ser 473) in mice in the low-fat diet group compared to the high-fat group. Furthermore, the prostate extract from the low-fat group had decreased Akt kinase activity when examined by in vitro kinase assay using GSK-3 α/β as a substrate.

We conclude that a low-fat diet delays transition from PIN to cancer in this Myc-driven transgenic mouse model, which mimics human prostate cancer development. The increased serum levels of IGFBP-1 in response to low-fat diets may lead to suppression of IGF-1 stimulation and subsequent Akt phosphorylation. Thus, dietary fat reduction may play an important role in preventing the development of prostate cancer.

Keywords: low-fat diet; prevention; prostate cancer

156 Ex Vivo Analysis of Prostate Cancer Tumor-Infiltrating Lymphocytes Demonstrates Immunosuppressive CD4+CD25+FOXP3+ and CD4+ICOS+IL-10 Producing Regulatory T Cells

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Regulatory T cells are important to maintaining immune homeostasis; however, in the setting of neoplasia, regulatory T cells may play a critical role in inhibiting effective immune responses against cancer cells. To investigate the status of regulatory T cells in prostate cancer, we enrolled patients onto an IRB-approved protocol and performed ex vivo FACS analyses to assess prostatectomy samples for the presence of regulatory T cells within the tumor microenvironment. Tumor infiltrating lymphocyte data were compared to information obtained from normal prostate tissues. Circulating lymphocyte data were also obtained from blood

samples of prostate cancer patients and healthy donors. Our data demonstrated comparable lymphocyte populations in the circulating blood between prostate cancer patients (n = 30) and normal healthy donors (n = 10). The tumor microenvironment of prostate cancer samples (n = 30) demonstrated increased numbers of regulatory T cells consisting of CD4+CD25+FOXP3+ICOS^{hi} IL-10-producing T cells as compared to normal prostate tissues (n = 10). The immunosuppressive effects of the regulatory T cells found within the tumor microenvironment were confirmed in co-culture suppression assays. In vitro experiments demonstrated that anti-IL-10 antibody was capable of interfering with the suppressive activity of regulatory T cells, indicating potential therapeutic benefit with this treatment in prostate cancer patients. To our knowledge, this is the first ex vivo demonstration of two distinct populations of regulatory T cells within the tumor microenvironment that can be potentially targeted for treatment of prostate cancer patients.

Keywords: immunobiology; regulatory T cells; tumor microenvironment

157 Therapeutic Inhibition of Sp1 Expression in Growing Tumors by Mithramycin A Correlates Directly With a Potent Antiangiogenic Effect on Human Pancreatic Cancer

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Purpose: Human pancreatic cancer overexpresses the transcription factor Sp1. However, the role of Sp1 in pancreatic cancer angiogenesis and its use as a target for antiangiogenic therapy remain unexplored.

Experimental Design: Archived human pancreatic cancer specimens were used to assess gene expression and microvessel density (MVD) status by immunohistochemistry; siRNA was used to determine the impact of altered Sp1 expression on tumor growth and angiogenesis; and mithramycin A (MIT) was used to evaluate Sp1-targeted antiangiogenic treatment of human pancreatic cancer in animal models.

Results: The expression level of Sp1 was directly correlated with the MVD status ($p<.001$) and the expression level of VEGF ($p<.05$). Knockdown of Sp1 expression did not affect the growth of pancreatic cancer cells in vitro but inhibited their growth and metastasis in mouse models. This antitumor activity was consistent with the in vitro and in vivo antiangiogenic activity resulting from Sp1 knockdown. Subcutaneous and intraperitoneal injection of MIT significantly suppressed the growth of human pancreatic cancer in mouse models. This robust tumor suppression correlated with drastic suppression of Sp1 expression in growing tumors but not in normal tissues. Moreover, treatment with MIT reduced tumor MVD, which was consistent with downregulation of VEGF, PDGF, and EGFR.

Conclusion: Clinical and experimental evidence indicates that Sp1 is a critical regulator of human pancreatic cancer angiogenesis and the antitumor activity of MIT is a result, at least in part, of suppression of Sp1 expression and consequent downregulation of Sp1's downstream targets key to angiogenesis.

Keywords: mithramycin; Sp1; targeted therapy

158 A Phase II Trial of Letrozole Plus Erlotinib in Postmenopausal Women With Hormone-Sensitive Metastatic Breast Cancer: Preliminary Results of Toxicities and Correlative Studies

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Background: EGFR overexpression is associated with breast cancer (BC) endocrine therapy failure, advanced disease, and shortened DFS and OS. Signaling through the EGFR pathway may promote ligand-independent ER gene transcription and stimulate growth factor signaling. We hypothesized that hormone-sensitive (ER and/or PR-positive) BC with EGFR and/or HER2/neu overexpression would exhibit resistance to aromatase inhibitors (AI). Hence, the combination of an AI and an EGFR tyrosine kinase inhibitor (TKI) may prevent or delay resistance development and provide greater growth inhibition than an AI alone in hormone-sensitive BC.

Methods: We initiated a Phase II clinical trial of letrozole (2.5 mg/d) and erlotinib (150 mg/d) as first-line (cohort 1) or second-line (cohort 2) endocrine therapy, until disease progression, for postmenopausal women with hormone-sensitive MBC. The main

endpoints of this clinical trial were to determine the clinical benefit rate (CR+PR+SD for 6 months) and safety of the letrozole (AI) and erlotinib (EGFR TKI) combination, as well as whether BC with EGFR and/or HER2/neu overexpression or ligand-independent activation of ER (ras-raf-MAPK pathway dependent activation of ER → Ser^{118} p-ERα) will exhibit longer TTP with the letrozole and erlotinib combination. We analyzed paraffin blocks from the primary or metastatic tumors for total ER and PR, EGFR (DAKO), Ser^{118} p-ERα (cell signaling), HER2/neu (HercepTest) immunohistochemistry (IHC), and HER2/neu fluorescent in situ hybridization (FISH, Vysis).

Results: Forty-two patients were enrolled to date, of which 38 were part of cohort 1. Six patients were not considered evaluable for responses (two patients withdrew consent after 2 weeks of therapy because of noncompliance, and four patients terminated treatment before 1 month of therapy because of toxicities). So far, 14/22 (76%) evaluable patients achieved clinical benefit for more than 6 months, with 1 CR, 8 PR, and 5 SD. Eight patients progressed before achieving clinical benefit and 14 other patients are still on treatment. To date, median TTP is 8 months. The most common grade 1 and 2 toxicities were rash (90%), diarrhea (73%), elevation of transaminases (60%), nausea (56%), and dry skin (56%). Grade 3 toxicities were uncommon, and consisted of rash (12%), elevation of transaminases (12%), vomiting (6%), diarrhea (3%), and hypermagnesemia (3%). There was one case of grade 4 rash. Paraffin blocks from 32 patients were available for IHC analysis. EGFR IHC was negative in all cases, and 5/32 patients were HER2/neu 3+ by IHC; all IHC 2+ cases were not HER2/neu-amplified by FISH. Total ER was positive in 31/32 and PR in 25/32 tumors. Ser^{118} P-ERα staining was positive (range, 1% to 100% positive tumor cells; mean, 64%) in 22/26 cases using conventional IHC. When retested using a catalyzed signal amplification kit (CSA), 18/21 cases stained positive (5% to 100% cells; median, 73%).

Discussion: EGFR (IHC DAKO) status did not correlate with clinical responses, although a technical limitation of this method for EGFR detection cannot be ruled out. Most cases stained positive for Ser118 p-ERα. Correlation between Ser^{118} p-ERα (MAPK dependence) and clinical outcome may provide further insights into the mechanisms of endocrine therapy resistance. The combination of letrozole and erlotinib is fairly well tolerated. So far, the clinical benefit rate is 76% and median TTP is 8 months. If 16 patients among the first 28 evaluable patients exhibit clinical benefit for more than 6 months, the trial will continue to accrue (two-stage optimal Simon design). If the final rate of clinical benefit is at least 65%, consideration for testing the combination in a Phase III study is warranted, where identification and validation of biomarkers would predict which patients are more likely to benefit from this combination.

Keywords: EGFR; endocrine; metastatic

159 Heterogeneity of Breast Cancer Metastases: A Rapid Autopsy Study Using Matched Primary-Metastases Tissue Microarrays

Please see Poster Discussion Session Biomarkers I.

160 Function of PIK3CA Mutations and Gene Copy Number in Human Lung Cancers

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Background: Somatic mutations mainly in the helical or kinase domains of *PIK3CA* have been reported in various human cancers including lung cancer, but the functional effects of these mutations in lung cancer are still unclear. Chromosome 3q, where *PIK3CA* is located, is known to be frequently amplified in lung cancer.

Methods: We analyzed the mutation status of exons 9 (helical domain) and 20 (kinase domain) of *PIK3CA* using 86 non-small cell lung cancer (NSCLC) cell lines, 47 small cell lung cancer (SCLC) cell lines and 691 resected NSCLC tumors from patients in Japan (n = 323), Taiwan (n = 148), United States (n = 150), and Australia (n = 70). We also searched for *PIK3CA* gene copy number in the same cell lines and 356 of 691 NSCLC tumors using quantitative real-time PCR. We also measured PI3-kinase activity in *PIK3CA* mutant and wild-type cell lines including one cell line with increased *PIK3CA* copy number to assess the effects of *PIK3CA* gene alteration. We investigated protein expression of PI3K/Akt pathway in *PIK3CA*-mutant and amplified cell lines by Western blotting. For functional analyses, we performed knockdown of PIK3CA using siRNA and verified the effects of inhibition of PIK3CA on cell proliferation status in *PIK3CA* mutant cell lines.

Results: We identified mutations in NSCLC cell lines (4 of 86, 4.7%), SCLC cell lines (3 of 47, 6.4%), and NSCLC tumors (11 of 691, 1.6%). The mutations in NSCLC were more frequent in exon 9 (3 of 4 in cell lines and 7 of 11 in tumors) than in exon 20, while all the mutations in SCLC cell lines were in exon 20. *PIK3CA* mutation status was not associated with patient age, gender, smoking history, clinical stage, or ethnicity in resected NSCLC tumors. We detected increased *PIK3CA* copy number in NSCLC cell lines (9 of 86, 10.5%), SCLC cell lines (2 of 47, 4.3%) and NSCLC tumors (61 of 356, 17.1%). Increased *PIK3CA* copy number was more frequent in squamous cell carcinoma (5 of 14 cell lines, 35.7%, and 46 of 139 tumors, 33.1%) than in adenocarcinoma (4 of 51 cell lines, 7.8%, and 12 of 195 tumors, 6.2%). Mutations and highly increased gene copy number in *PIK3CA* were mutually exclusive. While the mutation status of *EGFR*, *KRAS*, *BRAF,* and *HER2* genes were mutually exclusive, 1 cell line and 3 clinical samples with *PIK3CA* mutation also had *KRAS* or *EGFR* mutations. Therefore, mutation status of *PIK3CA* is not mutually exclusive to the other genes. All the *PIK3CA* mutant cell lines (4 NSCLC, 3 SCLC) had significantly increased activity of PI3-kinase compared to *PIK3CA* wild-type cell lines. One wild-type cell line with increased *PIK3CA* copy number also had increased PI3-kinase activity. Phospho-Akt was expressed in *PIK3CA*-altered cell lines indicating that mutant or amplified PIK3CA have increased kinase activity. Knockdown of PIK3CA by siRNA resulted in absent expression of phosphorylated Akt, which is downstream of PIK3CA, and inhibited cell growth of *PIK3CA* mutant cells.

Conclusions: *PIK3CA* mutations are present in a subset of NSCLC and SCLC cancers and amplifications target squamous cell carcinomas. Mutations and amplifications in *PIK3CA* are mutually exclusive whereas mutations in *PIK3CA* are not mutually exclusive of mutations in *KRAS* or *EGFR*. *PIK3CA* alterations are associated with increased PI3-kinase activity and knockdown of PIK3CA results in inhibition of cell growth of *PIK3CA* mutant cells. These findings suggest that PIK3CA may be a candidate for targeted therapy in lung cancer.

Keywords: amplification; mutation; PIK3CA

161 Characterization of Brain Tumor Exosomes: Biology, Biochemistry, Immunology

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Exosomes are 30 to 100 nm vesicles derived from the multivesicular bodies of late-stage endosomes that cells release into the extracellular milieu. The biological relevance of exosomes is unclear, but they may be involved in the removal of extraneous proteins and distal signaling. While many different cell types are documented to produce and release exosomes, there are few, if any, reports of exosomes from brain tumor cells. We have isolated these vesicles from the spent media of a number of different brain tumor-cultured cell lines, as well as from CD133+ sorted cells from tumor xenografts and from a patient tumor sample. Biochemical and proteomic analyses show that brain tumor exosomes contain proteins that are common to exosomes from many cell types, as well as proteins/potential antigens that are specific to brain tumors. Electron microscopic images of material from buoyant density gradient centrifugation reveal membrane vesicles of the appropriate size for exosomes, and FACS analyses demonstrate the outer membrane leaflet localization of certain proteins. As exosomes have been associated with immunostimulatory as well as immunosuppressive capacities, we are currently examining that aspect of exosome biology in a syngeneic mouse model of anaplastic astrocytoma.

Keywords: brain tumors; exosomes; immunology

162 Endostatin-Cytosine Deaminase Fusion Protein Suppresses Tumor Growth by Targeting Neovascular Endothelial Cells

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Endostatin, an angiogenesis inhibitor tested in multiple clinical trials, selectively targets neovascular endothelial cells, suppressing tumor growth. To enhance the therapeutic efficacy of endostatin, we fused endostatin with cytosine deaminase,

which converts a prodrug 5-flucytosine into a cytotoxic 5-fluorouracil. This therapeutic strategy was developed on the basis of the observation that the endostatin-green fluorescence protein gene and endostatin-luciferase gene selectively target to endothelial cells in vitro and to the tumor site in vivo, respectively. When we used the endostatin-cytosine deaminase (Endo-CD) fusion protein to treat s.c. grafted tumors or experimental metastasis tumors, our results showed that Endo-CD treatment provided stronger tumor growth suppression and increased mean survival time of the mice compared with the treatments of endostatin alone, cytosine deaminase alone, or endostatin plus cytosine deaminase. The Endo-CD protein significantly inhibited the growth of endothelial cells and preferentially induced tumor cell apoptosis.

Recently, the regulatory agency in China approved the use of endostatin as an anticancer agent for treating non-small cell lung cancer patients. We also have recently generated preliminary results that show an Endo-CD fusion protein exhibited therapeutic efficacy similar to Endo-CD gene therapy in cell culture and in animal models. Considering that multiple clinical trials have been performed for endostatin protein, and it has been approved as an anticancer drug in China, if the Endo-CD fusion protein shows more potent antitumor activity in animal models than endostatin or CD alone, it may be worthwhile to move it into clinical trials.

Keywords: angiogenesis; cytosine deaminase; endostatin

163 Analysis of the Interaction of Vorinostat (Suberoylanilide Hydroxamic Acid-SAHA) With Carboplatin and Paclitaxel in Non-Small Cell Lung Cancer

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Vorinostat, a novel histone deacetylase (HDAC) inhibitor, when combined with paclitaxel (ptx) and carboplatin (carbo), demonstrates provocative clinical activity in patients with advanced NSCLC (see reference). This was suggestive of enhancement of anticancer activity of the ptx/carbo regimen by vorinostat. To understand the mechanism(s) underlying this enhancement, we initiated preclinical studies described here. In MTT assays, vorinostat alone inhibited the growth of 128-88T, A549, 201T, and Calu-1 NSCLC cells in a dose-dependent manner. At a clinically achievable concentration (1μM), vorinostat alone suppressed cell growth by 16% to 40%. In 128-88T cells, combination of 1μM vorinostat with carbo/ptx resulted in synergistic inhibition of cell growth. This may be attributable to vorinostat enhancement of one or both agents. To separate the effects, MTT assays were utilized to measure growth inhibition by each of the two agents in the presence and absence of vorinostat. In A549 and 128-88T cells, the combination of vorinostat with carbo or with ptx resulted in greater than additive inhibition of cell growth. These data indicate that vorinostat increases the cytotoxicity of both carbo *and* ptx in NSCLC cells. To explain the favorable interactions between vorinostat and chemotherapeutic agents at the molecular level, we evaluated the expression of possible targets of therapy in A549 cells. In immunoblot assays, we found that tubulin acetylation was synergistically increased in cells treated with vorinostat plus ptx compared to either agent alone. The vorinostat-mediated increase in tubulin acetylation is expected to promote ptx binding and apoptosis induction. We also observed that both carbo and vorinostat induce expression of $p21^{Waf-1/Cip1}$ in A549 cells. However, p21 expression was greatest in cells treated with the combination of carbo plus vorinostat. Thus, vorinostat may augment carbo activity in NSCLC cells by increasing growth inhibitory signals generated downstream of p21 induction. Our preclinical and molecular studies support the notion that vorinostat increases the activity of both ptx *and* carbo, resulting in enhanced clinical efficacy in patients with advanced NSCLC.

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Reference

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Keywords: combination chemotherapy; histone deacetylase; molecular correlates

164 Sorafenib as a Novel Therapeutic Directly Targets on FLT3-ITD Mutant in Acute Myelogenous Leukemia

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Introduction: Fms-like tyrosine kinase 3 (FLT3) gene mutations, including internal tandem duplication (ITD) and missense point mutations (TKD mutations), are the most frequent molecular abnormalities (about 30%) in acute myelogenous leukemia (AML) [1]. FLT3 duplications in AML confer poor prognosis [2]. We have previously shown that ERK is constitutive phosphorylation of ERK has also been shown in the majority of primary AML samples [3] and is associated with poor prognosis in AML, even in the absence of FLT3 abnormalities [4]. These observations suggest that FLT3 mutations result in constitutive activation of the downstream Raf-MEK-ERK cascade and that activation of ERK through other mechanisms may also occur and contribute to the survival of AML blasts.

Sorafenib, initially designed as a small molecule inhibitor of *c-Raf*, has been shown to function as a multi-kinase inhibitor targeting tyrosine kinases such as FLT3, vascular endothelial growth factor receptor family, and the platelet-derived growth factor receptor family. Our previous data have already demonstrated that sorafenib dramatically induces growth arrest and apoptosis of FLT3-mutant AML cell lines at several 1,000-fold lower concentrations compared to AML cells with wild-type FLT3 [5]. We hypothesize that sorafenib directly targets FLT-ITD mutant AML cells.

Materials and Methods: The antileukemic activity of sorafenib was investigated in Ba/F3-FLT3 wild-type, Ba/F3-ITD mutant, and D835G, D835Y point mutant AML cell lines; primary AML cells; and a murine leukemia model by evaluating the profiles of cell signaling proteins, clonogenicity, apoptosis, and survival of leukemia-bearing mice. Furthermore, 11 refractory/relapsed patients with AML enrolled in a Phase I clinical trial were evaluated after different dose schedules of sorafenib treatment.

Results: Our results showed that sorafenib by virtue of direct targeting mutated FLT3-ITD induces cell growth arrest and apoptosis of Ba/F3-ITD cells, inhibition of clonogenicity of primary AML cells, major suppression of leukemia growth in an in vivo leukemia model, and dramatic reduction of circulating blasts in all FLT3-ITD mutant AML patients in a Phase I trial with sorafenib. Notably, the plasma concentrations of sorafenib achieved in clinical trials (7.1 mg/L at a dose of 400 mg bid p.o.) by far exceeded the growth-inhibitory concentrations in leukemic cells with FLT-3 mutations. Sorafenib was well tolerated clinically, and the MTD has not yet been reached.

Conclusion: The studies imply the therapeutic potential of sorafenib in patients with FLT3-ITD mutant AML, a form of AML that responds poorly to traditional chemotherapy.

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Keywords: acute myelogenous leukemia; FLT3-ITD mutant; sorafenib

165 Arsenic Trioxide Enhances the Antitumor Effects of Radiation Treatment While Protecting the Bone

Please see Poster Discussion Session Therapy I.

166 A Novel Model of Estrogen Receptor α-Positive Metastatic Breast Cancer

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Mortality from breast cancer results from the ability of some tumors to metastasize to distant sites. Primary tumor spread to regional lymph nodes at cancer diagnosis is the standard clinical approach to predicting distant spread of this disease because few biomarkers accurately predict metastasis. Unfortunately, we have only a few preclinical breast cancer models that metastasize to distant sites to study the complex process of metastasis for biomarker and target identification. These models are from estrogen receptor (ER) α-negative cell lines and thus not representative of the majority of patients, since ~70% of patients have ERα-positive tumors. We have discovered that a cytoskeletal regulatory gene, called Rho guanine disassociation inhibitor (Rho GDIα), is expressed at lower levels in metastatic, tamoxifen-resistant breast tumors compared to primary tamoxifen-sensitive tumors using expression microarray analysis. We have data suggesting that Rho GDIα may be a metastasis suppressor protein, because when we knock down its expression in ERα-positive MCF-7 cells to simulate our clinical tumor data, we generate a tamoxifen-resistant phenotype that metastasizes to the lung with a high frequency when grown as xenografts in athymic nude mice. The metastatic tumors from estrogen-treated animals were positive for the estrogen-induced progesterone receptor (PR), suggesting that they have maintained estrogen responsiveness. Preliminary experiments suggest that ERα is activated through PAK-1 phosphorylation of the receptor at serine 305 in Rho GDIα knockdown cells. We hypothesize that the loss of Rho GDIα function promotes distant metastatic spread of tamoxifen-resistant ERα-positive breast tumors by triggering downstream signaling molecules with metastasis-promoting activities, possibly mediating effects through ERα. These results are novel because there are currently no ERα-positive breast cancer models that reliably metastasize with a frequency useful for preclinical studies. Our overall goal is to determine whether Rho GDIα expression in the primary tumor identifies women at risk of recurrence.

Keywords: estrogen receptor alpha; metastasis; tamoxifen

167 Intrinsic Resistance of Tumorigenic Breast Cancer Cells to Chemotherapy

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Background: Tumorigenic breast cancer cells characterized by CD44 expression and low or undetectable CD24 levels ($CD44^+/CD24^{-/low}$) may be resistant to chemotherapy and therefore responsible for cancer relapse.

Methods: Paired breast cancer core biopsies before and after neoadjuvant chemotherapy or lapatinib were obtained and as single cell suspensions stained using antibodies against CD24, CD44, and lineage markers, and then analyzed by flow cytometry. Mammosphere (MS) formation in culture was compared before and after treatment. Global gene expression differences between cancer cells bearing $CD44^+/CD24^{-/low}$ cells and all other sorted cells, and between cancer MS and the primary bulk invasive cancers, were analyzed.

Results: We report that $CD44^+/CD24^{-/low}$ tumorigenic breast cancer cells obtained from primary human breast cancers were intrinsically chemoresistant. Chemotherapy led to increased $CD44^+/CD24^{-/low}$ cells, increased self-renewal capacity on MS assays, and increased tumorigenicity in immunocompromised SCID/beige mice. Conversely, in patients with HER2 overexpressing tumors, the EGFR/HER2 tyrosine kinase inhibitor lapatinib decreased tumorigenic $CD44^+/CD24^{-/low}$ cells, with the majority of these patients after conventional therapy achieving pathologic complete response, a validated surrogate marker for long-term survival. From the largest dataset to date, the gene transcription pathways that underlie chemoresistant, MS-forming $CD44^+/CD24^{-/low}$ cells involve genes belonging to stem cell self-renewal (polycomb group—PHC3, Notch pathway—mastermind-like 2, and NOTCH2N), FOXP1, epidermal growth factor/PI3K signaling (Sos, ITPR1, ITPR2, CK2, PI3K, Gab1/2, PTEN, PP2A), and early development pathways (JARID2, JMJD2C, and MBNL1).

Conclusion: This study provides the first clinical evidence that conventional chemotherapy failed to eradicate $CD44^+/CD24^{-/low}$ cells which were intrinsically chemotherapy resistant, and suggests that specific signaling inhibitors of the pathways identified may provide a therapeutic strategy for eliminating these tumorigenic cells.

Keywords: CD44+/CD24-/low; chemoresistance; tumorigenic breast cancer cells

168 Anchor-Modified and Glycosylated MUC1 Epitopes Show Enhanced Immunogenicity Compared to Native Epitope: Potential for Development of a More Robust Breast Cancer Vaccine

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Breast cancer remains a major health problem, especially in women with advanced-stage cancer who are at high risk of metastasis and relapse. In recent years, there has been great interest in cancer vaccines, which have the potential for controlling disease, prolonging time to recurrence, and ultimately even serving as a preventive measure. This project is focused on development of an optimal tumor vaccine designed to elicit cytotoxic T lymphocytes (CTLs) against carbohydrate antigens, using MUC1 as the core glycopeptide. MUC1 is a widely expressed tumor-associated antigen that is overexpressed and aberrantly glycosylated on greater than 90% of breast carcinomas and has been shown to elicit tumor-specific immunity. MUC1 occurs naturally as a heavily glycosylated protein that contains two well-known TACA: the disaccharide Thomsen-Friedenreich (TF) antigen (β-Gal-(1->3)-α-GalNAc-*O*-serine/threonine and its precursor, the monosaccharide Tn (GalNAc-*O*-serine/threonine). Our hypothesis is that eliciting immunity to glycosylated and/or anchor-improved MUC1 peptides will result in antitumor immunity and long-term immune memory.

Methods: We have developed several short (8 or 9-mer) glycosylated and/or anchor-modified peptides derived from the MUC1 extracellular tandem repeat (TR) and cytoplasmic tail (CT) sequence. Native MUC1-peptides were altered by introduction of the monosaccharide, GalNAc-*O*-threonine (Tn carbohydrate), at the threonine in fifth position, and/or modification of amino acids in the major histocompatibility complex (MHC)-Class I anchor positions. Preclinical studies were performed using the modified H-2K^b-restricted peptides in C57BL/6 mice transgenic for human MUC1 (MUC1.Tg mice). These mice are naturally tolerant to immunization with MUC1, making them an appropriate model for testing MUC1-based vaccines. The peptides for immunizations were selected on the basis of their binding ability in the RMA-S binding assay. Table 1 lists the peptides used for immunization in the mice. In addition to the mouse study, human CTLs were generated against the modified HLA-A2 restricted peptides using peripheral blood mononuclear cells (PBMCs) from normal HLA-A2 individuals. Table 2 lists the peptides tested for human CTLs.

Table 1. Mouse Peptides

Number	*Peptides*
1	SAPDTRPAP (native TR)
2	SAPD*T*(Tn)RPAP (strong)
3	SA*AA*T(Tn)R*A*A*M*
4	SA*AA*TR*A*A*M* (strong)
5	SAPD*T*(Tn)RP*L* (strong)
6	SAPDTRP*L* (strong)

Table 2. Human Peptides

Number	*Peptides*
1	STAPPVHNV (native TR)
2	STAP*T*(Tn)VHNV
3	SLAPPVHNV (strong)
4	S*L*AP*T*VHNV (strong)
5	S*L*AP*T*(Tn)VHNV (strong)
6	SLSYTNPAV (native CT)
7	ALGSTAPPV
8	ALGS*T*(Tn)APPV

Results: In the mouse, addition of Tn to the fifth position of the native peptide SAPDTRPAP showed stronger CTL induction compared to the native peptide. However, in the case of the anchor-modified peptide, SAAATRAAM, the unglycosylated peptide was stronger than its glycosylated counterpart. In the case of the anchor-modified 8-mer SAPDTRPL peptide, both the unglycosylated and its glycosylated counterpart showed high induction of CTLs. Future experiments will determine whether glycosylated MUC1 peptides (versus nonglycosylated peptides) will elicit a greater clinical response and long-term immunity in tumor-bearing MUC1.Tg mice. In the humans, the peptides most capable of generating CTLs against MCF-7 (HLA-A2$^+$ and MUC1$^+$) breast cancer cell line were all of the peptides (glycosylated and unglycosylated) that were anchor modified from the native STAPPVHNV to contain a leucine in the second position (see Table 2). The native STAPPVHNV peptide was ineffective in generating CTLs from normal individuals. Experiments are now under way using HLA-A2$^+$ breast cancer patient PBMCs.

Conclusion: We show that the use of anchor-modified MUC1 peptides containing carbohydrate resulted in CTLs of high affinity in both mouse and human studies. These preclinical studies will form the basis for a clinical trial using glycosylated MUC1 epitopes. This will be the first effort to use carbohydrate-induced immunogenicity to fulfill MUC1's potential as a therapeutic target.

Keywords: Class I peptide; immunotherapy; mucin

169 Aberrant Hedgehog Signaling Represents a Novel Therapeutic Target in B Cell Lymphomas

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Aberrant self-renewal is a hallmark of cancer and central to the initiation, maintenance, and relapse of clinical disease. The cellular processes responsible for self-renewal have not been delineated in most human cancers, but it is likely that conserved pathways required for the regulation of normal stem cells are involved. Notably, several highly conserved signaling pathways that regulate stem cell fate decisions during embryonic development, such as Notch, hedgehog (HH), and wingless (Wnt), are inappropriately activated in a wide variety of human cancers. We recently demonstrated that the HH signaling pathway is required for the maintenance of cancer stem cells in the plasma cell malignancy multiple myeloma. Since myeloma stem cells phenotypically resemble normal B cells, we hypothesized that aberrant HH signaling is a feature of other B cell malignancies. We studied established human cell lines derived from patients with classical Hodgkin's lymphoma (L428, KM-H2), diffuse large B cell NHL (HT, Pfeiffer, RL, and Hs 602), and mantle cell NHL (Granta 519, Jeko-1, Rec-1) and found that expression of the HH signaling pathway components *PATCHED (PTCH), SMOOTHENED (SMO),* and *GLI1, 2 or 3* by RT-PCR was markedly elevated compared to normal B cells in the majority of cell lines from each subtype of lymphoma. In order to examine the functional role of HH signaling on human lymphomas, cells were treated with recombinant human sonic HH ligand (SHh) or the naturally occurring inhibitor of *SMO*, cyclopamine, followed by evaluation of clonogenic growth in methylcellulose. Resulting colony formation was significantly increased in response to activating SHh ligand, whereas treatment with 5E1 or cyclopamine significantly inhibited clonogenic recovery. Similarly, treatment with the SHh-neutralizing monoclonal antibody 5E1 limited colony formation, suggesting that ligand binding by *PTCH* was required for pathway activation. This is similar to findings in other non-Gorlins tumors, such as small cell lung cancer, pancreatic carcinoma, and metastatic prostate cancer, that lack mutation of pathway components. Furthermore, we examined the activity of a novel synthetic cyclopamine analogue, IPI-609, and found that it also limited clonogenic lymphoma growth. The effects of IPI-609 were highly specific, since the clonogenic recovery of cell lines lacking expression of HH pathway components was not affected by treatment. Our previous studies in multiple myeloma have suggested that cancer stem cells can be identified by their relatively higher activity of the intracellular enzyme retinaldehyde dehydrogenase (ALDH) similar to normal hematopoietic and neural stem cells. We found that high ALDH activity could also identify rare cell populations with greater clonogenic growth potential compared to $ALDH^{low/neg}$ cells in the majority of lines, and treatment with cyclopamine or IPI-609 significantly reduced the relative proportion of $ALDH^{high}$ cells. Therefore, the HH signaling pathway may represent a novel therapeutic target in human lymphomas. Moreover, novel HH inhibitors, such as IPI-609, may inhibit highly clonogenic lymphoma cancer stem cells responsible for disease relapse.

Keywords: cancer stem cells; hedgehog; lymphoma

170 Conditioned Medium From Ad-IFNa-Treated Cells Is Cytotoxic to Cancer Cells Resistant to the Interferon Protein: Further Evidence for a Strong Bystander Effect

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We have previously shown that Ad-IFN is cytotoxic to bladder cancer cells that are resistant to high concentrations of the IFN protein and has a strong bystander effect [1]. More recently we have provided evidence for soluble factors producing this bystander effect using a co-culture system involving membrane inserts [2]. Conditioned medium (CM) from Ad-IFN-treated cancer cells resistant to the IFN protein has now been found to be similar in eliciting the bystander effect in various cancer types. The conditioned medium could be filtered, frozen, and thawed and diluted to at least 1 part conditioned medium to 10 parts control medium and still show a similar cytotoxicity as a 100% concentration of fresh conditioned medium. This was observed by both flow cytometry and MTT assays. Of interest, the cytotoxicity could not be blocked by the pan-caspase inhibitor, BOC-Asp(Ome)-fluoromethylketone. Since Ad-IFN is presently being evaluated in a Phase I trial for superficial bladder cancer, elucidating the identity of the soluble factor(s) causing this Ad-IFN-produced bystander effect in cell culture is presently a particularly important goal in our laboratory.

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Keywords: adenovirus; bladder cancer; gene therapy

171 Establishment and Molecular Characterization of Mouse Xenografts of Organ-Confined Human Prostate Tumors

Please see Poster Discussion Session New Technologies and Methodologies I.

172 Chemotherapy-Modulated Vaccination With Allogeneic GM-CSF-Secreting Breast Tumor Cells Can Induce Tumor Antigen-Specific Immunity

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Background: Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting cell-based tumor vaccines have shown promise for the treatment of solid tumors, but their activity against advanced disease is limited by immune tolerance. To test the hypothesis that sequencing vaccination with low doses of chemotherapy can abrogate immune tolerance to enhance vaccine activity, we are conducting a safety and chemotherapy dose-finding clinical trial of timed sequential therapy with cyclophosphamide (CY), doxorubicin (DOX), and an allogeneic, HER2/*neu*-expressing, GM-CSF-secreting breast tumor vaccine in patients with stable metastatic breast cancer.

Methods: The trial is designed as a three by three factorial testing a fixed dose of 5×10^8 vaccine cells with a range of low doses of CY and DOX in 24 women; 6 additional women received vaccine alone. Subjects are given three monthly vaccination cycles, with a boost given 6 months from trial entry. CY is given day 1, vaccination day 0, and DOX day +7. To date, 6 women received vaccination alone, and 18 women have received chemotherapy-modulated vaccination. Vaccine bioactivity was assessed by measuring serum GM-CSF levels, and vaccine site induration. Immune responses were evaluated by delayed type (DTH) hypersensitivity skin testing using validated HER2/*neu*-derived peptides and by ELISpot.

Results: Vaccination was bioactive, resulting in measurable levels of serum GM-CSF and induration at the vaccine site in all vaccinated patients. Vaccine-related toxicities were limited to local erythema, pruritus, and induration, with systemic fever and rash occurring less frequently. The addition of chemotherapy to vaccination did not exacerbate vaccine-related toxicities. Vaccination alone and combined with chemotherapy induced de novo HER2/*neu*-specific T cell immunity as determined by DTH and ELISpot in some patients.

Conclusions: These data suggest that vaccination with an allogeneic, GM-CSF-secreting breast tumor vaccine with or without low doses of CY and DOX is bioactive and safe in patients with metastatic breast cancer and can be associated with the induction of new tumor antigen-specific immunity.

Keywords: clinical trial; immunity; vaccine

173 Human Breast Cancer Cells Selected for Resistance to Trastuzumab In Vivo Overexpress EGFR and ErbB Ligands and Remain Dependent on the ErbB Receptor Network

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We have investigated mechanisms of acquired resistance to the HER2 antibody trastuzumab in BT-474 human breast cancer cells. BT-474 xenografts in athymic nude mice were eliminated by trastuzumab. Continuous cell lines (HR for Herceptin resistant) were generated from tumors that recurred in the presence of continuous antibody therapy. The isolated cells behaved resistant to trastuzumab in culture as well as when reinjected into nude mice. They retained HER2 gene amplification and trastuzumab binding and were exquisitely sensitive to peripheral blood mononuclear cells (PBMCs) ex vivo in the presence of the antibody. The HR cells exhibited higher levels of P-EGFR and EGFR/HER2 heterodimers. Phosphorylation of HER2 in HR cells was inhibited by the EGFR tyrosine kinase inhibitors erlotinib and gefitinib. Gefitinib also inhibited the basal association of p85 with P-HER3 in HR cells. Both inhibitors as well as the dual EGFR/HER2 inhibitor, lapatinib, induced apoptosis of the HR cells in culture. Growth

of established HR5 xenografts was inhibited by erlotinib in vivo. In addition, the HR cells overexpressed EGFR, TGFα, HB-EGF, and heregulin RNAs compared to the parental trastuzumab-sensitive cells. These results are consistent with the inability of trastuzumab to block the heterodimerization of HER2 and suggest that amplification of ligand-induced activation of ErbB receptors is a plausible mechanism of acquired resistance to trastuzumab that should be investigated in primary mammary cancers.

Keywords: HER2; resistance; trastuzumab

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

174 Antigen-Presenting Cell-Targeted hCGβ Vaccine for Cancer Therapy

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Background: The β subunit of human chorionic gonadotropin (hCGβ) is selectively overexpressed by a number of epithelial tumors and has been reported to correlate with stage of disease and prognosis. CDX-1307 is a novel vaccine approach in which this tumor-associated antigen is coupled to a human monoclonal antibody (B11) that targets the mannose receptor (MR) on human antigen presenting cells (APC)—dendritic cells and macrophages. Antigens bound to B11 are very efficiently processed for both TH1 and TH2 immune responses. CDX-1307 thus allows for systemic in vivo access of exponentially more autologous APC than do locally administered vaccines or in vitro APC preps. The activity of this approach has been demonstrated in a preclinical model of human MR transgenic mice implanted with syngeneic B16 tumor cells transfected to express a secreted form of hCGβ. Vaccination of these animals with CDX-1307 by either the intradermal or intraperitoneal route of administration, in combination with a variety of immunostimulatory molecules, resulted in a significant delay or complete protection against tumor growth and strong hCGβ-specific humoral and cellular responses. A clinical study of CDX-1307 in patients with advanced breast cancer utilizes systemic (intravenous) delivery to provide antigen targeting to a broad antigen-presenting cell population and investigates CDX-1307 in combination with immunostimulants to generate optimal immune effects.

Methods: In this Phase I, dose-escalating study, sequential cohorts of patients with advanced breast cancer receive four biweekly intravenous infusions of CDX-1307 at either 1, 3, 10, or 30 mg doses. In the 10 and 30 mg cohorts, the first three patients receive CDX-1307 alone, while the subsequent three to six patients receive CDX-1307 in combination with GM-CSF (100 mcg administered subcutaneously for four daily doses per vaccination). Study objectives include safety and tolerability, assessment of any dose-limiting toxicities, humoral immune responses to hCG-β, T cell responses measured by antigen-specific induction of INF-γ, serial pharmacokinetic profile, and clinical activity.

Results: Enrollment in the first two cohorts is complete (n = 8) with no DLTs. Enrolled patients had a median age of 52 years and time to diagnosis of 7.6 years, and ECOG performance status was either 0 (n = 6) or 1 (n = 2) at entry. Patients were heavily pretreated with prior chemotherapy (88%) and hormone therapy (88%), and sites of metastases included bone (6), lung (4), and liver (2). Seven patients completed therapy; one patient in the 3 mg cohort was discontinued after one dose because of progressive disease. Potential treatment-related toxicities were all mild to moderate in severity and were most commonly fatigue (25%), fever (25%), and myalgias (25%). Progressive disease was seen in all seven of these heavily pretreated patients who have completed the study.

Conclusions: Administration of CDX-1307 is well tolerated. Immune response, pharmacokinetics, and tumor impact are under evaluation. Administration in combination with an immune-stimulating adjuvant will be evaluated in the next two cohorts.

Keywords: antigen; metastases; vaccine

175 Compositional Differences in Peripheral Blood of Glioblastoma Patients and Its Relationship to Vaccine Development

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No effective therapy exists for patients with high-grade gliomas. Animal models and some human clinical trials indicate that high-grade gliomas may be susceptible to directed stimulation of the immune system. Specifically, autologous antigen-presenting cells known as dendritic cells (DCs) have been combined with tumor-derived antigens and used as a vaccine in patients with

recurrent glioblastoma (GBM). Cumulatively, these data suggest that GBM may be amenable to immunotherapy; however, additional work is needed to improve the efficacy of DC vaccines. A particularly acute area of deficiency in DC-based immunotherapy is the uniformity and quality control of cultured cells used for vaccination. In addition, DC vaccination regimens will be enhanced if used at times of maximum potency, that is, at times when patients have the best ability to respond to immune stimulation. To better understand these two influences, we have begun to characterize the immune competence of patients with GBM by peripheral blood immune phenotyping. First, we evaluated four culture conditions for generation of DCs in these patients. Using CD14+ cells from GBM patients, we have been able to produce numbers of high-viability DCs similar to those seen in normal volunteers. However, in all four culture conditions tested, the DCs generated from the blood of GBM patients were of lower purity and potency than cells from normal volunteers (65.5% ± 6.1 CD83+ in the best condition for GBM patients versus 95.2 ± CD83+ in normal volunteers; $p<.001$, $n = 13$). Second, the same patients were immune-phenotyped to look for abnormalities in immune suppression. GBM patients had more granulocytes as a percentage of white blood cells (76.5 ± 3.0% vs. 58.4 ± 3.0%; $p = .0005$) and more regulatory T cells (CD25+CD127- as a percentage of CD4; 7.8 ± 2.2% vs. 3.0 ± 0.7%; $p = .046$). GBM patients also showed reduced expression of HLA-DR on circulating lineage positive cells (47.7 ± 5.7% vs. 65 ± 2.5%; $p = .005$). We found that the ability to recover CD14+ cells during our isolation procedure was inversely proportional to our ability to culture pure DCs ($p = .014$; $n = 9$), suggesting a preexisting deficiency of monocytes in their ability to differentiate into DCs. We believe that this deficiency may represent a novel mechanism of immune suppression in patients with GBM.

This work was supported through a developmental research grant from the Mayo Clinic Brain Tumor SPORE CA108961-03.

Keywords: immunotherapy; leukocytes; vaccines

176 Apoptosis and EGFR Signaling Pathway-Targeted Molecular Therapy With FUS1-Nanoparticles and EGFR Inhibitors for Non-Small Cell Lung Cancer

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FUS1 is a novel tumor suppressor gene located in the human chromosome 3p21.3 region and plays an essential role in mitochondrial and Apaf-1-associated apoptosis. Deficiency in FUS1 protein expression was found in >80% of lung cancer cell lines, >70% of primary lung tumors, and premalignant lesions. Restoration of wt-FUS1 function in 3p-deficient lung cancer cells significantly suppressed tumor cell growth by induction of apoptosis in vitro and in vivo. EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib have been shown to be clinically effective in a subpopulation of non-small cell lung cancer (NSCLC) patients. Despite initial responses to these TKIs in some NSCLC patients, cancer eventually progresses by unknown mechanisms of intrinsic or acquired resistance. In this study, we explored the use of FUS1 by DOTAP: cholesterol nanoparticle-mediated gene transfer to enhance the chemotherapeutic potency of gefitinib and erlotinib and overcome TKI-induced resistance in NSCLC. We found that ectopic expression of wild-type FUS1 in FUS1-deficient and gefitinib/erlotinib-resistant NSCLC H1299, H322, H358, and H460 cells significantly ($p<.001$) increased their response to gefitinib or erlotinib treatment and synergistically induced apoptosis in vitro. The combination of intravenous injection of FUS1-nanoparticles and oral administration of gefitinib was more effective than either single agent in mediating tumor regression in an H322 orthotopic lung cancer mouse model. To understand the mechanism of gefitinib-induced resistance, we established a gefitinib-resistant HCC827GR NSCLC cell line (IC_{50} = 16 μM) by selecting against gefitinib from the parental HCC827 cells that contain an activating deletion mutation of the EGFR gene and are extremely sensitive to gefitinib treatment (IC_{50} = 0.016 μM). We found no secondary mutations in the EGFR gene in the HCC827GR cells, but these cells registered a significantly elevated level of phosphorylated Akt protein. Combination treatment with FUS1-nanoparticles and gefitinib or erlotinib at suboptimal dose levels of IC_{10} and IC_{20} significantly resensitized the cells to gefitinib and erlotinib, as demonstrated by synergistically enhanced growth inhibition and apoptosis. FUS1 nanoparticle treatment alone or with gefitinib/erlotinib markedly inactivated EGFR and Akt, as demonstrated by decreased phosphorylation levels of both proteins on Western blots, compared with either agent alone. Cleavage of caspase 3, caspase 9, and PARP was also significantly induced by the combination of FUS1 and gefitinib in HCC872GR and other gefitinib-resistant NSCLC cells. Our results suggest that a combination treatment of FUS1 nanoparticles and gefitinib/erlotinib could potentiate their antitumor efficacy and overcome drug-induced resistance by simultaneously inactivating the EGFR/Akt signaling pathway and by inducing apoptosis.

Keywords: EGFR inhibitors; NSCLC; tumor suppressor

177 CD45 Mediates Resistance to Galectin-1-Induced Apoptosis in Cutaneous T Cell Lymphoma

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Apoptosis resistance is an obstacle to development of new agents to treat non-Hodgkin's lymphoma (NHL). Several mechanisms of apoptosis resistance in lymphomas have been described. The galectins are a family of molecules expressed by a variety of cell types, including APCs and stromal cells, that regulate apoptosis. Resistance to galectin-mediated death has been described in NHL, including diffuse large B cell lymphoma (DLBCL) and cutaneous T cell lymphoma (CTCL). Our studies focus on novel strategies to overcome resistance to galectin-mediated death in NHL. We and others have found that CTCL cells are resistant to galectin-1-induced death; as galectin-1 is abundantly expressed in skin, resistance to galectin-1 death may contribute to the progression of CTCL. We have identified two distinct mechanisms for galectin-1 resistance by CTCL cells. The first is loss of CD7 expression, as CD7 is a galectin-1 receptor essential for galectin-1-induced death. The second is aberrant glycosylation of CD45; proper glycosylation of CD45 permits galectin-1 binding and inhibition of CD45 tyrosine phosphatase activity, as CD45 tyrosine phosphatase activity antagonizes galectin-1-induced apoptosis. We identified a CTCL cell line with a single point mutation in the gene encoding a glycosyltransferase that modifies CD45. The mutation dramatically reduces enzyme activity, so that the glycan ligands on CD45 recognized by galectin-1 are absent on these cells. Loss of these glycan ligands was also seen in primary $CD7^+$ MF lesions. Expression of wild-type enzyme in CTCL cells restored glycan expression and susceptibility to galectin-1. We also asked whether inhibition of CD45 phosphatase activity would directly sensitize CTCL cells lacking the glycan ligands to galectin-1 death. A specific CD45 inhibitor was investigated for the ability to sensitize CTCL cells to galectin-1 and overcome apoptosis resistance. Thus, glycosyltransferase haploinsufficiency resulting in altered cellular glycosylation and intracellular signaling is an apoptosis resistance mechanism in CTCL; overcoming resistance by inhibiting a downstream step in the cell survival pathway may provide a novel therapeutic approach for this disorder.

Keywords: galectin; lymphoma; signaling

178 Prostate Cancer Patients Exhibit High Levels of HLA-DR—Negative Peripheral Blood Monocytes

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We observed that dendritic cells (DCs) prepared from monocytes of prostate cancer (PCa) patients are less pure than DCs prepared from monocytes of normal donors. To determine the reasons underlying this difference, we analyzed isolated peripheral blood mononuclear cells (PBMCs) of 21 PCa patients (median age: 73, range: 63-86; median combined Gleason score: 7, range: 5-9; median PSA value: 7.8 ng/mL, range: 1.4-60.5 ng/mL) and compared them by flow cytometry with those from randomly selected blood donors (n = 6). We found no difference in the abundance of $CD4^+$ cells (PCa v. normal, mean ± SD: 59.8 ± 16.9% v. 67.1 ± 14.5% of T cells, p = .374), $CD8^+$ cells (38.1 ± 19.3% v. 27.0 ± 12.9%, p = .303), and $CD4^+CD127$ cells (4.0 ± 3.3% v. 4.4 ± 1.5%, p = .29). However, we observed unexpectedly high levels of $CD14^+HLA\text{-}DR^-$ cells in PCa patients relative to healthy controls (30.7 ± 15.0% v. 5.1 ± 4.1% of monocytes, p = .0007). To determine the ability of PCa patient monocytes to differentiate into mature DCs, we isolated $CD14^+$ cells and matured them into DCs with GM-CSF, IL-4, TNF-α, and PGE_2 in X-VIVO15 (DC_X) or RPMI-1640 (DC_R). The yields of DC_X and DC_R prepared from $CD14^+$ PCa cells were 25.1 ± 9.3% and 36.4 ± 13.7%, respectively (p = .0567). The content of $CD83^+$ cells was 62.3 ± 30.1% and 74.7 ± 9.9%, respectively, with the broader variance of the former (p<.0001). These data suggest either that $CD14^+HLA\text{-}DR^-$ cells cannot mature into DCs or that they inhibit maturation of those that can. Currently we are investigating the role of $CD14^+HLA\text{-}DR^-$ cells in immunity with reference to PCa patient age and androgen ablation.

This research was supported by National Institutes of Health grant P50 CA91956.

Keywords: immunosuppression; immunotherapy; vaccination

179 *Clostridium Perfringens* Enterotoxin as a Novel Targeted Therapeutic for Brain Metastasis

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Brain metastasis is the most commonly occurring intracranial tumor whose incidence appears to be increasing. With standard therapy, the average survival time of patients is approximately 8 months, and treatment often leads to neurological dysfunction in long-term survivors, emphasizing the need for novel therapeutics. *Clostridium perfringens* enterotoxin (CPE) has recently been shown to rapidly and specifically destroy cancer cells expressing CPE receptors, claudin (CLDN)-3 and -4. Unfortunately, the utility of CPE is precluded by systemic toxicity since its receptors are expressed in numerous organs. Here, we provide the first preclinical evidence that CPE may be uniquely suited to the local treatment of brain metastasis. By immunohistochemical (IHC) analysis, CLDN-3 and -4 were expressed frequently in metastases from breast (15/18), lung (15/20), and colon (12/14) carcinoma, and infrequently in metastases from renal cell carcinoma (2/16) and melanoma (2/16). In contrast, CLDN-3 and -4 expression was absent in adjacent normal brain tissue. Further examination of the central nervous system (CNS) revealed low or undetectable levels of CLDN-3 and -4 in all regions tested by Western and IHC analysis. Treatment of breast cancer cell lines (MDA-MB-468, NT2.5, MCF-7) and normal human astrocytes with CPE in vitro resulted in rapid and dose-dependent cytolysis exclusively in breast cancer cells, correlating with CLDN-3 and -4 expression. Moreover, intracranial CPE treatment of breast tumor xenografts in the brain of immunodeficient mice significantly inhibited tumor growth and increased survival, without any apparent local or systemic toxicity. These data suggest that CPE therapy may have efficacy against a wide variety of brain metastases without CNS toxicity.

Keywords: metastasis; therapeutics

180 Molecular Mechanisms of E1A-Mediated Sensitization to Paclitaxel

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Ovarian cancer is one of the most common cancers in women. The adenovirus type 5 E1A protein (E1A) has been shown to associate with multiple anticancer activities and to induce sensitization to different categories of anticancer drug-mediated apoptosis in different human cancers, including paclitaxel (Taxol) in breast and ovarian cancers. We have previously demonstrated the therapeutic efficacy of E1A in multiple animal models using gene therapy approaches. On the basis of these studies together with safety toxicity studies, multiple clinical trials using E1A/liposome have been completed. Currently, under the support of an Ovarian SPORE grant, a Phase I/II study entitled "A Phase I/II randomized study of intraperitoneal tgDCC-E1A and intravenous paclitaxel in women with platinum-resistant ovarian cancer" is ongoing at the M.D. Anderson Cancer Center. During the transition while the products are testing in clinical trials, it is imperative to understand the detailed molecular mechanisms regarding how the potential clinical products may exert their anticancer activities. Understanding the detailed molecular mechanisms that associate with E1A-mediated antitumor activity may also help to develop future clinical trials using the combination of chemotherapy with E1A gene therapy. To this end, we have recently found that Forkhead box O-class (FOXO) transcription factor FOXO3a is critical for E1A-mediated chemosensitization of paclitaxel. Knockdown FOXO3a expression by FOXO3a-specific siRNA dramatically abolished E1A-induced chemosensitization of paclitaxel both in vitro and in vivo. Furthermore, we found E1A stabilizes FOXO3a protein by preventing ubiquitin-dependent proteolysis. To explore which E3 ligase is involved in the stabilization of FOXO3a, we found that it is β-transducin repeat-containing proteins (βTrCP), and the binding of βTrCP to FOXO3a requires the phosphorylation of FOXO3a at Ser644 by IKKβ. E1A reduces βTrCP-mediated unbiquitination of FOXO3a by inhibiting IKKβ activity and its phosphorylation of FOXO3a at Ser644. Overexpression of βTrCP or constitutively active FOXO3a at Ser644 (FOXO3a/S644E) recovered βTrCP-mediated FOXO3a degradation in E1A-expressing cells. Further, the inhibition of IKKβ activity is due to E1A-induced PP2A expression which binds to TAK1, the upstream kinase of IKKβ, and inhibits TAK1 to activate IKK signaling. These results suggest that the combination of E1A and paclitaxel could be an effective treatment for cancer, especially in those paclitaxel-resistant patients whose basal FOXO3a expression is low.

Keywords: E1A; molecular mechanisms; ovarian cancer

181 Activation of the Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) Regulates Hypoxia-Induced Factor 2 alpha/c-maf Pathway in Human Multiple Myeloma in the Bone Marrow Milieu

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Activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) mediates multiple myeloma (MM) cell growth and survival induced by cytokines/growth factors and adhesion to bone marrow (BM) milieu. We recently demonstrated that a novel MEK1/2 inhibitor AZD6244 (ARRY-142886) specifically blocks ERK1/2 phosphorylation and induces growth arrest and apoptosis in MM cells, in the presence or absence of BM stromal cells (BMSCs) (see reference). To gain further insights into molecular mechanisms mediating AZD6244-induced cytotoxicity, expression profiling analysis was performed following treatment of MM1S and INA6 MM cells with this compound. Intriguingly, one of the most prominent features was the downregulation of the transcription factor c-maf that is frequently overexpressed in MM cell lines and patient MM cells regardless of chromosomal translocation. Inhibition of c-maf protein was next confirmed as early as 2 hours and sustained until 24 hours after drug treatment. Importantly, three identified c-maf target genes, cyclin D2, integrinβ7, and the C-C chemokines receptor-1 (CCR1), were subsequently downregulated by AZD6244, occurring at 4 hours after drug treatment. Analysis with the Ingenuity software further revealed that hypoxia-induced factor 2 alpha (HIF-2α), a gene directly regulating c-maf expression and a pro-angiogenic mediator, was significantly blocked in AZD6244-treated MM cells, suggesting a link between ERK1/2 inhibition and downregulation of HIF-2α and c-maf. Overexpression of HIF-2α by transfection with an HIF-2α expression vector or treatment with cobalt chloride was also blocked by AZD6244. Importantly, bortezomib, a novel anti-MM drug and proteasome inhibitor, upregulates HIF-2α expression, which was blocked by AZD6244 and associated with enhanced cytotoxicity when both compounds were combined at lower concentrations in MM1S cells. In addition, adhesion of MM cells to BMSCs induced ERK1/2 and STAT3 activation as well as upregulation of c-maf and its downstream targets (cyclin D2, integrin β7, and CCR1); conversely, AZD6244 specifically inhibited adhesion-augmented c-maf and its target protein expression but not phosphorylation of STAT3. Finally, AZD6244 blocked MM cell adhesion to BMSCs and inhibited adhesion-induced VEGF production. Taken together, our results further confirm a prominent role for MEK/ERK activation in MM cell adhesion to BMSCs and are the first to directly link ERK1/2 activation to the HIF-2α /c-maf pathway that not only stimulates cell cycle progression but also promotes pathological interactions between tumor cells and BMSCs. Moreover, this study strongly supports AZD6244 as a promising targeted therapeutic intervention in MM.

Reference

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Keywords: bone marrow stromal cells; c-maf; HIF-2α

182 Implication of Rac-Mediated Generation of Reactive Oxygen Species in Enhanced Sensitivity of Metastatic Head and Neck Cancer Cells to Induction of Apoptosis by the Synthetic Retinoid 4HPR Relative to Their Corresponding Primary Cancer Cells

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N-(4-hydroxyphenyl)retinamide (4HPR), a synthetic retinoid with lower toxicity than most natural retinoids, has demonstrated potential as a chemopreventive and therapeutic agent in vitro and in vivo as well as in a few clinical trials. 4HPR induces apoptosis by enhancing the production of reactive oxygen species (ROS) in a variety of cancer cells [1]. However, many gaps remain in understanding the mechanisms of increased ROS generation by 4HPR and its downstream effects. We surmised that the elucidation of such mechanisms might explain the sensitivity and resistance of cancer cells to this synthetic retinoid and will help to optimize its use for cancer therapy. Our preliminary attempts to determine how 4HPR increases ROS have shown that the small guanosine triphosphatase (GTPase) Rac, which is a regulatory subunit of the ROS, generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, is activated 5 minutes following 4HPR exposure. Moreover, inhibition of Rac activity using the specific inhibitor NSC-23766 abrogated ROS generation by 4HPR in human head and neck cancer cells. This correlated with almost complete abrogation of apoptosis induction by 4HPR as shown in the human head and neck squamous cell carcinoma (HNSCC) cells, UMSCC-22B. In addition, RNA interference-mediated knockdown of Rac1 expression decreased ROS generation.

Because metastatic cancer cells display enhanced motility and migration properties relative to the cells from the primary tumor as a result of higher levels and activities of Rho GTPase family members including Rac, we speculated that such cancer cells could be more sensitive to Rac-mediated ROS generation by 4HPR compared to their primary counterparts. Indeed, the metastatic cancer cell lines UMSCC22B and DM14 exhibited higher ROS generation and growth inhibition due to 4HPR exposure compared to their primary cancer cell counterparts, UMSCC-22A and Tu167, respectively. These findings support a novel therapeutic use of 4HPR against metastatic cancer cells through inhibition of Rac activation.

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Keywords: apoptosis; 4HPR; sensitivity

183 Combination of a Strategy to Prolong Dendritic Cell Survival and a Strategy to Bypass Antigen Processing Enhances DNA Vaccine Potency

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Intradermal vaccination via gene gun efficiently delivers DNA vaccines into dendritic cells (DCs) of the skin, resulting in the activation and priming of antigen-specific T cells in vivo. We have previously demonstrated that intradermal delivery of DNA vaccine encoding a single-chain trimer (SCT) composed of the most immunogenic epitope of the human papillomavirus type 16 (HPV-16) E6 protein (aa49-57), β2-microglobulin, and MHC Class I heavy chain (SCT-E6) can bypass antigen processing and lead to stable cell-surface presentation of E6 peptides. In addition, we have shown that co-administration of DNA vaccines with DNA encoding antiapoptotic proteins can prolong the survival of DNA-transduced DCs, resulting in significant enhancement of antigen-specific $CD8^+$ T cell immune responses. Thus, in the current study, we hypothesized that combining the SCT strategy and antiapoptotic strategy may further enhance DNA vaccine potency by augmenting antigen-specific $CD8^+$ T cell immune responses and antitumor effects in vaccinated mice. Here we show that C57BL/6 mice vaccinated with SCT-E6 DNA in combination with DNA encoding the antiapoptotic protein BCL-xL generated significantly higher E6-specific $CD8^+$ T cell responses compared to mice vaccinated with SCT-E6 DNA and a nonfunctional mutant BCL-xL (mBCL-xL). Furthermore, mice treated with SCT-E6 and BCL-xL DNAs generated a better antitumor effect against E6-expressing tumor cells (TC-1/Lucif) than mice treated with SCT-E6 and mBCL-xL DNAs. Our long-term bioluminescent kinetic observations also indicated significant reduction of tumor load in mice treated with SCT-E6 and BCL-xL DNA vaccines. We conclude that the combination of a strategy to bypass antigen processing and presentation mechanisms and a strategy to prolong the life of DCs can lead to significant enhancement of DNA vaccine potency.

Keywords: antiapoptosis; DNA vaccine; single-chain trimer

184 Antitumor Activity of the Insulin-like Growth Factor-I Receptor Tyrosine Kinase Inhibitor in Head and Neck Squamous Cell Carcinoma and Non-Small Cell Lung Cancer

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Background: The insulin-like growth factor receptor (IGFR) plays a key role in cell proliferation, survival, angiogenesis, invasion, and malignant transformation. Activation of IGFR is also associated with resistant mechanism of various cancer therapies, including those targeting the epidermal growth factor receptor (EGFR). In this study, we analyzed the antitumor activities of IGF-IR tyrosine kinase inhibitor (TKI), the EGFR TKI erlotinib, or the combination in head and neck squamous cell carcinoma (HNSCC) and non-small cell lung cancer (NSCLC).

Methods: The effects of IGF1-R TKI, erlotinib, and their combination on cell proliferation and apoptosis were assessed by MTT, Western blot, a flow cytometry-based TUNEL, and anchorage-independent colony formation assays in normoxic and hypoxic conditions. In vivo antitumor activity was evaluated by using an IGF-1 transgenic mouse model, in which IGF-1 is exclusively

overexpressed in the lung under the control of SPC promoter. A tobacco carcinogen was used to enhance lung cancer formation in this mouse model.

Results: The IGF-IR TKI effectively inhibited activation of IGF-IR and its downstream Akt but not MAPK. The IGF1-R TKI inhibited cell proliferation through G1 cell cycle arrest and apoptosis. Dual targeting of IGFR and EGFR signaling pathways by combined treatment of the IGFR TKI and erlotinib showed enhanced antiproliferative activities in both normoxic and hypoxic conditions and in vitro and in vivo.

Conclusion: The IGF-IR TKI showed antiproliferative activities in HNSCC and NSCLC cells in vitro and in vivo. Combination treatment of IGFR TKI and erlotinib showed enhanced antiproliferative activities compared to single treatment. These results suggest that the IGF-IR TKI is a promising target for HNSCC and NSCLC therapy. In addition, simultaneous treatment with inhibitors of IGF-1R and EGFR signaling pathways could be an effective strategy for these cancers.

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Keywords: EGFR; IGFR; TKI

185 Alphaviral Vector-Transduced Dendritic Cells Are Potent Therapeutic Cancer Vaccines

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While dendritic cell (DC) vaccines function to protect hosts from tumor challenge, there are little data to suggest efficacy at inhibiting the growth of established tumors. Previously, we have shown that human DCs transduced with Venezuelan equine encephalitis virus replicon particles (VRPs) produced significant levels of proinflammatory cytokines and were potent stimulators of antigen-specific T cells in vitro. Therefore, we have evaluated the ability of VRP-transduced DCs (VRP-DCs) to induce therapeutic immunity in vivo against tumors overexpressing the neu oncoprotein. Similar to human DCs, transduction of murine DCs with VRPs resulted in high-level transgene expression, DC maturation, and secretion of proinflammatory cytokines. Vaccination with VRP-DCs expressing a truncated neu oncoprotein induced robust neu-specific CD8+ T cell and anti-neu IgG responses. Most importantly, a single therapeutic vaccination with VRP-DCs induced the regression of large established tumors. Interestingly, depletion of CD4+, but not CD8+, T cells completely abrogated inhibition of tumor growth following vaccination. Taken together, our results demonstrate that VRP-DC vaccines induce potent immunity against established tumors, and emphasize the importance of the generation of both CD4+ T cell and B cell responses for efficient tumor inhibition. These findings provide the rationale for future evaluation of VRP-DC vaccines in the clinical setting.

Keywords: HER2/neu; vaccine; viral vector

186 The MEK Inhibitor AZD6244 Blocks Growth of Thyroid Cancer Cells With Mutations in the BRAF Gene

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Almost all cases of papillary thyroid carcinoma, and a substantial fraction of cases of anaplastic thyroid carcinoma, have aberrant activation of the RAF/MEK/ERK signal transduction pathway, due to mutations in the *BRAF* or *RAS* genes, rearrangement of the *RET* receptor tyrosine kinase gene, or other molecular abnormalities. This activation suggested that inhibition of the RAF/MEK/ERK pathway might be an effective therapeutic strategy for thyroid cancer. We have examined the effect of the MEK inhibitor AZD6244 (ARRY-142886) on the growth of a panel of thyroid cancer cell lines in culture and in an in vivo xenograft model. We show that AZD6244 efficiently inhibits the growth of thyroid cancer cell lines harboring a mutation in the *BRAF* gene, with a GI_{50} of less than 50 nM. Cell lines with wild-type *BRAF* were substantially less sensitive to AZD6244 treatment. In the sensitive cell lines, AZD6244 induced a G1 cell cycle arrest but did not induce apoptosis. In a xenograft model, using the *BRAF* mutant thyroid cancer cell line ARO, oral gavage of AZD6244 delayed tumor doubling time from 6.5 days in vehicle-treated mice to 11.5 days

(p<.005). Since the *BRAF* mutation is present in 29%-83% of papillary thyroid cancers, these results suggest that MEK is a promising therapeutic target for thyroid cancer.

This research was supported by the National Institutes of Health SPORE in Head and Neck Cancer.

Keywords: BRAF; therapy; thyroid

187 Design and Evaluation of Novel Small-Molecule Inhibitors of the MDM2-p53 Interaction as a New Anticancer Therapy

Please see Poster Discussion Session Therapy II.

188 Inhibition of EGFR Signaling Elevates 15-Hydroxyprostaglandin Dehydrogenase in Non-Small Cell Lung Cancer

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The induction of cyclooxygenase-2 (COX-2) and high prostaglandin E2 (PGE_2) levels contribute to the pathogenesis of non-small cell lung cancer (NSCLC). However, in addition to overproduction by COX-2, PGE_2 concentrations also depend on the levels of the PGE_2 catabolic enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH). We find a dramatic downregulation of PGDH protein in NSCLC cell lines and in resected human tumors when compared with matched normal lung. Affymetrix array analysis of 10 normal lung tissue samples and 49 resected lung tumors revealed a much lower expression of PGDH transcripts in all NSCLC histological groups. Treatment with the EGFR TKI erlotinib increased the expression of 15-PGDH in a subset of NSCLC cell lines. This effect may be due in part to an inhibition of the ERK pathway since treatment with MEK inhibitor, U0126, also caused an increase in 15-PGDH protein levels. Quantitative RT-PCR of a responsive cell line reveals that the transcript levels of ZEB1 and Slug transcriptional repressors are dramatically reduced in a responsive cell line upon EGFR and MEK/ERK inhibition. In addition, the Slug protein binds to the PGDH promoter and represses transcription. As these repressors function by recruiting histone deacetylases to promoters, it is likely that PGDH is repressed by an epigenetic mechanism involving histone deacetylation, resulting in increased PGE_2 activity in tumors. This effect is reversible in a subset of NSCLC upon treatment with an EGFR-TKI.

Keywords: COX; erlotinib; PGDH

189 Combined Caspase-3/mTOR Inhibition Enhances Radiosensitization of Non-Small Cell Lung Cancer via Upregulation of Autophagy

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Lung cancer remains the leading cause of mortality for cancer deaths despite the availability of various chemo- and radio-therapeutic regimens. One of the limitations is due to resistance of cancer cells to apoptotic stimuli of various antineoplastic agents. Autophagic cell death has recently been shown to be increased in the irradiated apoptosis-incompetent cells. Therefore, we tested whether inhibition of apoptosis and induction of autophagy can be utilized as an adjunctive to the radiation therapy, using cell culture and xenograft models of lung cancer. In order to test this hypothesis, we asked whether inhibitor of caspase 3, Z-DEVD, and an inducer of autophagy, RAD001, can enhance the cytotoxic effects of irradiation in human lung cancer cells, H460. We found that combination of Z-DVED and RAD001 markedly enhanced radiosensitization of H460 cells in comparison with individual treatment alone both in vitro as well as xenograft murine model. The enhanced radiosensitization was associated with upregulation of autophagosome formation. Furthermore, when we addressed the specific cellular behavior associated with combination treatment, we observed defects in angiogenesis, inhibition of proliferation, and reduction in apoptosis. We demonstrate that radiation therapy, combined with inhibition of caspase 3 and induction of autophagy, may be a novel therapeutic option for lung cancer patients.

Keywords: autophagy; lung cancer; mTOR

190 Gamma-Secretase Inhibitors Prevent Notch 3 Activation and Reduce Proliferation in Human Lung Cancers

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Notch signaling is key in regulating development by controlling cell-fate determination in multicellular organisms. Genes that are important in normal development play a role in cancer when they become dysregulated. Notch receptors have been demonstrated to promote and maintain tumor cell survival in many cancers. In lung cancer, we previously have shown that Notch 3 is overexpressed in a significant subset of lung cancers. Furthermore, we also have demonstrated that this receptor plays an important role in tumor survival. Thus, inhibition of Notch signaling may represent a promising treatment strategy. The gamma-secretase protein complex induces proteolytic cleavage of Notch and release of the NICD (Notch Intracellular Domain), which translocates to the nucleus and induces target gene transcription. A class of pharmacologic agents known as gamma-secretase-inhibitors prevents the release and activation of the NICD and has been shown to inhibit tumor growth and induce apoptosis in several tumor types. In this study, we determined the ability of MRK-003, a gamma-secretase inhibitor, to inhibit Notch 3 signaling, growth, and apoptosis in lung cancer cell lines (HCC2429, H460, A549) in vitro and in vivo using mouse xenograft models. We found that MRK003 inhibited Notch 3 signaling, reduced tumor cell proliferation, inhibited serum independence, and enhanced apoptosis. It has also been reported that the NICD interacts with CSL (CBF-1, Su(H), Lag-1) protein, resulting in transcription of *Hairy/Enhancer of split* genes (*HES*). We have demonstrated that Notch 3 inhibition by MRK-003 regulates *HES-1*, *Hairy-related transcription factor-1* (*Hey-1*), and their target gene human *Achaete-Scute Homolog* (hASH1). This drug had no effect when Notch 3 expression was knocked down using siRNA, suggesting that the observed effects were mediated by specific action on this receptor. In conclusion, these results support the hypothesis that inhibition of Notch activation using γ-secretase inhibitors represents a promising new approach for the treatment of lung cancer.

Keywords: gamma secretase; Notch

191 Targeting Histone Deacetylases in Non-Small Cell Lung Cancer

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Histone deacetylation is a crucial step in the loss of specific homeostatic molecular signals in the transition from normal lung epithelium to in situ carcinoma, and finally to invasive and metastatic cancers. The loss of TGF-β-induced tumor suppressor function in tumors plays a pivotal role in this process, and resistance to TGF-β in lung cancer occurs mostly through the loss of type 2 receptor (TβRII) expression. We have observed that the expression of TβRII is lost or reduced in 77% of lung tumors. As a proof of principle, we have determined that stable expression of TβRII in lung adenocarcinoma cell lines, lacking TβRII expression, restored TGF-β-mediated biological effects including Smad2/3 and Smad4 complex formation, TGF-β responsive reporter gene activation, inhibition of cell proliferation, increase in apoptosis, and decrease in tumorigenicity. Our initial experiments have resulted in an important observation that activation of MAPK/ERK pathway by either activated Ras or activated EGF signaling causes downregulation of TβRII through histone deacetylation. In order to understand the mechanism, we have identified a region of the TβRII promoter required for its activation by HDAC inhibitors (HDI) and proteins that are involved in the regulation of TβRII expression using proteomics and biochemical methods. TGF-β-induced tumor suppressor function is restored in TGF-β resistant lung cancer cell lines by the treatment of HDIs. In addition, the expression of other tumor suppressor genes including PGDH, E-cadherin, and $p21^{Cip1}$ is reduced in lung cancer cell lines because of histone deacetylation. Therefore, these results suggest that restoration of TGF-β signaling and the expression of tumor suppressor genes by HDIs, anticancer drugs currently in clinical trials, may be a potential alternative for therapeutic intervention of lung cancers and may be particularly effective for patients whose tumors demonstrate loss of TβRII expression.

Keywords: HDAC; TGF-Beta

192 Mechanisms of Silencing and Desilencing of BRCA2 Gene Expression in Human Breast Cells

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The tumor suppressor protein BRCA2 is essential for DNA recombination repair as well as the regulated and synchronized growth of human breast cells in the developing breast tissues. Its expression is strictly regulated depending on the growth stage of the breast cell. It is not expressed in the nondividing human breast cells and is expressed only in the dividing stages of the cells. The hypothesis is that balanced expression of BRCA2 in the rapidly dividing human breast cells is critical to preventing the malignant transformation of these cells. We describe here our findings of an epigenetic mechanism of human BRCA2 gene expression regulation through an Alu repeat and E2-box (CACCTG/CAGGTG)-containing transcriptional silencer located at the promoter of this gene. In this mechanism, the transcriptional repressor protein SLUG binds to the silencer in nondividing human breast cells to inhibit the expression of this gene by chromatin remodeling via the recruitments of CtBP1 and HDAC1 and histone deacetylation as was established by chromatin immunoprecipitation and reporter gene expression analyses. Although alanine-substitution mutagenesis revealed that the putative CtBP1 binding site (PSDTSSK) in human SLUG protein is essential for its suppressor activity, yeast-2-hybrid and co-IP analysis showed that it does not directly bind to CtBP1 protein. We are in the process of developing peptide aptamers against this SLUG-specific essential PSDTSSK site to test whether these aptamers can manipulate SLUG function and thus BRCA2 gene expression in human breast cells. In the dividing breast cells, the DNA binding nuclear isotype of peroxiredoxin 5 (PRDX5) apparently desilences BRCA2 gene expression by competing with the binding of SLUG to the silencer. Our data also suggest that, as estrogen and the members of the P53 family of proteins regulate SLUG gene expression, they may also cell cycle-dependently regulate the expression of BRCA2 gene in human breast cells, perhaps by regulating SLUG gene expression. Understanding the molecular mechanisms involved in the silencing/desilencing of the expression of the BRCA2 gene in the human breast cells is important because any perturbation or malfunction of these mechanisms will result in the dividing cells lacking appropriate levels of BRCA2 at "the time of need" during cell cycle and thus will predispose these cells to malignancy. If we understand the silencing/desilencing mechanisms in detail, we should be able to effectively intervene in the pathways and thus be able to efficiently combat the onset of human breast cell malignancy caused by these malfunctions.

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Keywords: BRCA2; peroxiredoxin 5; SLUG

193 Role of LDH-A in Hereditary Leiomyomatosis and Renal Cell Cancer

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Hereditary leiomyomatosis and renal cell cancer (HLRCC) patients develop leiomyomas of the skin and uterus as well as invasive kidney cancers. The genetic basis for HLRCC is believed to be a germline inactivating mutation in the gene for the TCA cycle enzyme FH. Since FH is a critical enzyme in the TCA cycle that converts fumarate to malate, inhibition of this process should necessitate that glycolysis followed by fermentation of pyruvate to lactate will be required to provide ATP as well as to regenerate NAD^+ required to sustain metabolic pathways. It has recently been shown that FH deficiency leads to upregulation of HIF1α transcript, and RNA for targets for HIF1α, such as lactate dehydrogenase-A (LDH-A), has been found to be increased in tumors of patients with FH. LDH-A is of particular interest because it is involved in pyruvate lactate interconversion.

Currently, there are no established HLRCC cell lines that can be used to study metabolic alterations and signaling events associated with FH deficiency. We have used the A549 lung cancer cell line (VHL positive) as a model system to generate FH-deficient cell lines and demonstrate that these cell lines have increased lactate accumulation compared to control cell line, enhanced VEGF production, and increased VEGF and Glut-1 message levels. These data suggest that glycolysis followed by pyruvate to lactate conversion is enhanced by FH deficiency. Collectively, these data indicate that our A549 FH deficient cells "mimic" tumors from HLRCC patients.

Moreover, we have shown that FH deficiency leads to enhanced LDH-A activity, which correlates with enhanced in vivo expression of LDH-A in an HLRCC tumor sample. We have also demonstrated that blocking LDH-A expression in RCC4, a renal cancer cell line, reduces proliferation and induces apoptosis.

Our goal is to identify small-molecule inhibitors that specifically target LDH-A and can be used in the clinic for HLRCC treatment and to study the causal role of LDH-A in HLRCC.

Keywords: genomics; kidney cancer; metabolism

194 Rapamycin Prevents Tobacco-Carcinogen-Induced Lung Tumorigenesis and Selectively Reduces Lung-Associated Foxp3 Positive Cells

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Inhibitors of the serine/threonine kinase mTOR can prevent tumorigenesis in many model systems, but the use of mTOR inhibitors for cancer prevention in humans is theoretically limited by the risk of secondary malignancies, which was observed in patients receiving rapamycin as part of multidrug immunosuppressive therapy. However, little is known about immune modulation by rapamycin as a single agent. Previously, we showed that physiologic concentrations of rapamycin inhibited lung tumorigenesis driven by a tobacco-specific carcinogen, NNK. Here we characterize immune modulation in this system. NNK alone acutely depleted multiple lineages of immune splenic cells without suppressing immune function. In contrast to these nominal systemic effects, NNK doubled the number of cells expressing the T_{reg} marker Foxp3 in lung tissues prior to tumor development and caused a dose-dependent increase in tumor-infiltrating Foxp3+ cells at 12 weeks. Rapamycin rapidly decreased splenic Foxp3+ cells, and prevented the NNK-induced increase in Foxp3+ cells in lung tissues. At 12 weeks, rapamycin inhibited tumorigenesis, decreased the number of tumor-associated Foxp3+ cells by 88%, modestly decreased systemic CD4+ and CD8+ cells, and did not induce an immunosuppressive Th2 phenotype. Thus, single-agent rapamycin prevents lung infiltration of Foxp3+ cells that occurs early in tumorigenesis without causing profound immunosuppression.

Keywords: mTOR; prevention; tobacco carcinogenesis

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195 Effect of Aspirin on Mammographic Breast Density in Postmenopausal Women

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Epidemiologic studies have found a reduced risk of breast cancer among women who regularly use nonsteroidal anti-inflammatory medications (NSAIDs), including aspirin. Mammographic breast density is an excellent candidate biomarker for testing chemoprevention effects on the breast because it predicts risk of breast cancer development, can be measured with a computer-assisted imaging program, is modifiable, can be obtained at relatively low risk to the woman, and is relevant to routine breast cancer screening. In a two-arm randomized placebo-controlled clinical trial, we evaluated the effects of 6 months of administration of 325 mg/d aspirin on total mammographic breast dense area and percentage of the mammographic breast image occupied by dense areas (% density) in 144 postmenopausal women. Eligible women were healthy, not taking oral contraceptives or other hormone therapy, and had elevated mammographic breast density (American College of Radiology Breast Imaging Reporting and Data System [BI-RADS®] category 2, 3, or 4) within 6 months before study enrollment. Mammograms were administered prerandomization and poststudy, using the same machine and the same technician. Mammographic films were scanned using the Kodak LS85 laser film digitizer and electronically stored. One technician measured total and percent mammographic breast density using the Cumulus program method (University of Toronto).

Women were recruited in the greater Seattle area through mass mailing, media, and physician referrals. A total of 2,661 women responded to mailing, media, or referral; 332 were eligible after screening interview, 211 attended an informational session,

192 attended a clinic visit, and 144 were randomized. At baseline, women were on average 59.5 (5.5 s.d.) years of age and had a mean BMI of 26.4 (5.4 s.d.) kg/m^2; 92% were non-Hispanic white, and 74% had a college degree or higher; 26% had a first-degree family history of breast cancer, and 14% had a second-degree family history. One-quarter had had a previous breast biopsy. The distribution of BI-RADS® mammographic breast density classification was category 2 (scattered fibroglandular) 41.7%; category 3 (heterogeneously dense tissue present) 50%; and category 4 (extremely dense tissue present) 7.6%. All 144 randomized women returned for a followup mammogram at 6 months, and 86.8% of study pills were taken (as determined by pill count at the 6-month clinic visit). Analyses of mammographic breast density changes in aspirin vs. placebo are under way and will be presented.

Future work will include genotyping women for polymorphisms in the UGTs that metabolize aspirin and assessing their role in any aspirin effect on mammographic breast density. We have also collected and stored blood specimens to explore other relevant biomarkers.

Keywords: chemoprevention; mammographic density; NSAIDs

196 Insulin-like Growth Factor Binding Protein-5 Is Overexpressed in Breast Cancer Cells Resistant to IGF-1R Inhibition

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The insulin-like growth factor 1 receptor (IGF-1R) is a receptor tyrosine kinase that demonstrates proliferative and pro-survival signaling in breast and other cancers. IGF-1 signaling through the IGF-1R has also been implicated in the resistance of existing breast cancer therapies, such as tamoxifen, trastuzumab, and cytotoxic chemotherapy. Thus, agents targeting the IGF-1R may have clinical utility in inhibiting breast cancer proliferation, increasing the effectiveness of current therapies, and reversing or preventing the development of resistance to current therapies. As IGF-1R-targeted agents enter clinical trials, it will become critical to identifying markers of sensitivity or resistance to this strategy that will aid in patient selection. To help understand the mechanism of resistance to IGF-1R inhibition, we have developed a breast cancer cell line, MCF-7R4, that is resistant to the small-molecule IGF-1R inhibitor BMS-554417. In proliferation assays, MCF-7R4 cells are more than fiftyfold more resistant to BMS-554417 compared to parental MCF-7 cells. They are cross-resistant to the structurally related IGF-1R inhibitor BMS-536924. To investigate the mechanism of resistance, we performed DNA microarray experiments using Affymetrix HG-U133 Plus 2.0 GeneChip arrays. Using five replicates of purified RNA from MCF-7 and MCF-7R4 cells, expression patterns were sorted by significance and fold changes. Based on the microarray data, insulin-like growth factor binding protein-5 (IGFBP-5) mRNA expression was one of the most highly upregulated transcripts (15.5-fold) in MCF-7R4 cells compared to MCF-7 cells, with a p value of 2.97×10^{-7}. IGFBP-5 upregulation represented the largest change in messages coding IGF-1-related proteins. IGFBP-6 expression was also increased in the resistant MCF-7R4 cells, albeit to a lesser degree (fivefold to sixfold). No significant differences in RNA expression were seen with other IGF binding proteins. Using Western blotting, IGFBP-5 was easily detectable in conditioned medium from MCF-7R4 cells. However, medium from MCF-7 cells in the presence or absence of serum or BMS-536924 (10 μM) had undetectable levels of IGFBP-5 by Western blotting of the conditioned medium. A model for how IGFBP-5 can contribute to IGF-1R kinase inhibition is proposed. As IGFBP-5 can be detected in serum, its presence may be useful as a determinant of sensitivity to therapies targeting the IGF-1R.

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Keywords: breast cancer; IGFBP-5; IGF-1R inhibition

197 Heterogeneous Activation of the Unfolded Protein Response Determines Sensitivity to Proteasome Inhibitor-Induced Apoptosis in Human Pancreatic Cancer Cells

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Proteasome inhibitors induce apoptosis in human pancreatic cancer cells, but cell lines and xenografts derived from them display marked heterogeneity in sensitivity to cell death. We recently demonstrated that the proteasome inhibitor bortezomib (Velcade) induced apoptosis in human L3.6pl pancreatic adenocarcinoma cells by causing an unorthodox form of endoplasmic reticular (ER) stress, characterized by defective activation of pancreatic ER kinase (PERK) and downstream phosphorylation of the translational initiation factor eIF2α. As a consequence, levels of global protein synthesis did not decline in the cells, and the continuous buildup of misfolded/aggregated proteins resulted in their sequestration within structures termed aggresomes. Histone deacetylase (HDAC) inhibitors such as TSA or SAHA disrupted aggresome formation, enhancing ER stress and apoptosis. Here we investigated the mechanisms underlying resistance to apoptosis induced by the combination of proteasome plus HDAC inhibitors in a subset of cell lines. Like L3.6pl, human BxPC-3 and Panc-1 cells displayed high basal phosphorylation of eIF2α, and exposure to bortezomib or NPI-0052 caused a time- and concentration-dependent decrease in phosphorylation. In contrast, human HS766T cells displayed much lower basal eIF2α phosphorylation and displayed time- and concentration-dependent increases in eIF2α phosphorylation that were similar to those observed in cells exposed to a positive control (thapsigargin or 2,5-dibutyryl-1,4 benzohydroquinone). Consequently, proteasome inhibitors inhibited global protein synthesis in the HS766T cells but had no effect on translation in the other cell lines. Proteasome inhibitors caused aggresome formation in L3.6pl, BxPC-3, and Panc-1 but not in HS766T cells. Finally, aggresome disruption following exposure to SAHA or a more HDAC6-selective agent (tubacin) augmented proteasome inhibitor-induced apoptosis in the L3.6pl, BxPC-3, and Panc-1 cells but had minimal effects in HS766T cells. Overall, our data strongly suggest that basal levels of UPR activation are heterogeneous in human pancreatic cancer cells. Specifically, cells with high basal eIF2a phosphorylation are more sensitive to cell death induced by proteasome plus HDAC inhibitors, probably because proteasome inhibitors do not efficiently block protein synthesis in the cells.

Keywords: aggresome; histone deacetylase inhibitors; unfolded protein response

198 Development of Novel Curcumin Analogs for the Treatment of Head and Neck Cancers

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Treatment options for head and neck cancer patients are very limited, necessitating innovative approaches to the development of efficacious therapeutic strategies. Our project aims at developing novel natural product analogs that target critical survival pathways in head and neck cancer cells, with a specific focus on curcumin-derived compounds. The wealth of safety information together with the promising preventive and therapeutic potential of curcumin begs for efforts to develop curcumin-based agents with improved bioavailability and enhanced anticancer efficacy. In an attempt to retain curcumin's medicinal qualities and safety profile, while increasing its potency, our studies have led to the synthesis of over 100 compounds, many of which are cytotoxic to a range of cancer cell lines. Studies with a panel of head and neck cancer cell lines have identified a number of analogs with tenfold to twentyfold greater potency than curcumin in inhibition of head and neck squamous cell carcinoma (HNSCC) cell growth. These compounds include EF24, a monoketone fluoroaromatic derivative, and EF31, a pyridine analog. In a variety of in vitro and cell-based head and neck cancer cell assays, both compounds perform equally well, and together they represent our leads for therapeutic development. In order to understand the mechanism of action of EF24 and to identify molecular biomarkers that indicate its therapeutic efficacy, we investigated a number of signaling pathways and identified the NF-κB signaling pathway as a major target of EF24. NF-κB is partially located in the nucleus in HNSCC cells. TNFα treatment rapidly drives NF-κB completely into the nucleus, while addition of EF24 before TNFα treatment effectively blocks the nuclear translocation of NF-κB in a dose-dependent manner. Interestingly, the effective dose of EF24 for the inhibition of NF-κB translocation is similar to its IC_{50} for inducing cytotoxicity in the same cancer cells. These data suggest that the anticancer activity induced by EF24 is in part

mediated by its inhibition of the NF-κB pathway. To improve the bioavailability of these compounds, we have generated glutathione (GSH)-conjugated EF24 and EF31 analogs, which turn out to be completely water soluble. These analogs behave very much like the parent compounds, showing potent activity against a variety of head and neck cancer cells. Interestingly, these four candidate compounds are highly active against the metastatic cell lines PCI37B and 686LN-M4e cells, raising the possibility that they may prove to be development candidates for the treatment of metastatic HNSCC. In sum, our study has set the stage for further detailed mechanistic, pharmacological, and clinical investigation of these novel curcumin analogs for the treatment of head and neck cancers.

This research was supported in part by National Cancer Institute grant P50 CA128613.

Keywords: curcumin analogs; metastasis; NF-κB

199 Passive Transfer of L2-Specific Cross-Type Neutralizing Antibody Protects Mice From Human Papillomavirus 16 Challenge

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Persistent oncogenic human papillomavirus (HPV) infection is a necessary cause of cervical cancer. Although there are at least 15 oncogenic HPV types (e.g., HPV18, HPV31, HPV45, HPV52, HPV58), one type, HPV16, is responsible for 50%-60% of all cases of cervical cancer. HPV6 and HPV11 cause benign genital warts, whereas HPV5 is associated with nonmelanoma skin cancer in patients with the rare hereditary condition epidermodysplasia verruciformis. Minor capsid antigen L2 is a possible alternative to multivalent L1 virus-like particle (VLP) vaccines (such as Gardasil and Cervarix) to obtain broad protection against oncogenic HPVs. Vaccination with L2 as full-length protein or polypeptides protects animals against homologous type viral challenges at both cutaneous and mucosal sites. Protection is not mediated by cellular immunity, suggesting the importance of neutralizing antibodies. L2 is subdominant in the context of L1/L2 VLPs, but antibodies elicited by recombinant L2 immunogens are able to neutralize a remarkably broad range of HPV genotypes. Studies of HPV vaccines have been hampered by difficulties in producing infectious virions, an absence of a quantitative in vitro infectivity assay, and no animal model for oncogenic HPV infection. Here we describe a new HPV16 challenge model in mice. Mice can be challenged at cutaneous or vaginal mucosal sites with HPV16 pseudovirions carrying luciferase or red fluorescent protein, respectively. Infection is determined by measurement of reporter expression using bioluminescence or fluorescence. Infection is not detected if the HPV pseudovirions lack L2 or the mice were previously vaccinated with the HPV16 L1 VLPs. We generated a monoclonal antibody, RG-1, that binds to highly conserved L2 residues 17-36 and neutralizes HPV16 and HPV18. This overlaps the 13-31 region of L2 that we have previously shown to bind to the cell surface and to contain residues critical for infection. This suggests that neutralizing epitopes within L2 may be conserved across HPV types as a result of some critical viral function. Passive immunotherapy with RG-1 was protective in mice, suggesting that induction of L2-specific neutralizing IgG is sufficient to mediate immunity. HPV16 L2 17-36 peptide antiserum neutralized pseudoviruses HPV5, HPV6, HPV16, HPV18, HPV31, HPV45, HPV52, HPV58, and HPV11 native virions. Depletion of HPV16 L2 17-36-reactive antibodies from cross-neutralizing rabbit and human L2-specific sera abolished cross-neutralization and drastically reduced neutralization of the cognate type. This cross-neutralization of diverse HPVs associated with cervical cancer, genital warts, and epidermodysplasia verruciformis suggests the possibility of a broadly protective, L2 peptide-based vaccine.

Keywords: papillomavirus; prevention; vaccine

200 Identification of the Src Tyrosine Kinase Fyn as a Novel Dasatinib-Sensitive Molecular Target in Glioblastoma

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Glioblastoma is the most common malignant primary brain tumor and among the most lethal of cancers. Recent clinical trial results indicate that glioblastoma patients can benefit significantly from molecularly targeted therapies, and consequently the identification of new pathway targets, particularly those for which safe and effective inhibitors are available, is a top priority. The dual Src/Abl kinase inhibitor dasatinib (BMS-354825), which has proven effective in treating chronic myeloid leukemia, has yet to be investigated for activity against solid tumors. Here, we have examined gene expression data from glioblastoma clinical samples using a comprehensive molecular interaction network approach in identifying the Src-family tyrosine kinase Fyn as a previously unrecognized target in glioblastoma. We show that Fyn is overexpressed and persistently phosphorylated in independent glioblastoma patient sample sets. Reduction of Fyn expression significantly inhibited tumor cell invasion and proliferation, whereas Fyn overexpression promoted these activities. Similar results were observed with the related Src-family kinase Lyn. Glioblastoma cells were highly sensitive to dasatinib in vitro, demonstrating dramatic inhibition of tumor cell invasion and proliferation concomitant with inhibition of Fyn phosphorylation. In an orthotopic, bioluminescence-enabled glioblastoma xenograft mouse model, dasatinib significantly prolonged survival and reduced tumor luminescence at clinically achievable doses. Collectively, these results identify the Src-family kinases, particularly Fyn and Lyn, as compelling targets for therapy and suggest that dasatinib may have promising clinical efficacy for the treatment of glioblastoma.

Keywords: biomarker; BLI; preclinical

201 Examining Expression of Folate Receptor in Squamous Cell Carcinoma of the Head and Neck as a Target for a Novel Nanotherapeutic Drug

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The development of novel drug delivery approaches using nanotechnology has been an important strategy to increase the therapeutic efficacy and decrease the side effects of chemotherapeutics. We have recently conjugated a novel ternary structure nanoparticle, heparin-folate-taxol (HFT), which demonstrated greater antitumor efficacy than the free taxol in vivo (see reference). To identify whether folate receptor (FR) is a good target for the newly synthesized nanotherapeutic drug (HFT), we investigated the FR expression status of squamous cell carcinoma of the head and neck (SCCHN) tissue. We retrospectively examined the clinical characteristics of patients with SCCHN treated at the Winship Cancer Institute (WCI), Emory University, and investigated correlations with FR expression in tissue specimens. FR expression in primary tumors and metastatic sites was detected by immunohistochemistry (IHC). The IHC staining was quantitated and graded on a 0 to +4 scale. We analyzed 143 specimens from a total of 95 patients; 47 patients (49%) had nonmetastatic (NM) disease and 48 (51%) had metastatic (M) disease. The median age was 62 years (22-88 years) and male/female ratio was 3/1. Tumor grade included moderately differentiated (MD) in 62 patients (65%), well differentiated (WD) in 17 (18%), and poorly differentiated (PD) in 16 (17%). The median overall survival (OS) for the whole group was 4.8 years. The 3- and 5-year OS were 68% and 43%, respectively. The median disease-free survival (DFS) was not reached, with a 3- and 5-year DFS of 65% and 57%, respectively. FR expression of +1 or more was detected in 43 (45%) primary tumors and 19 (40%) lymph node metastases (LNM). An expression level of +2 or more was detected in 24 (25%) primary tumors and 11 (23%) LNM. There was a strong correlation between FR expression in primary tumors of the M group and their corresponding LNM (p = .0002). FR expression was also correlated to DFS in the NM group (p = .0048) and the M group (p = .0127). Furthermore,

a strong correlation was noted between FR expression in LNM and OS (low expression associated with longer survival p<.0001), as well as DFS (high expression associated with earlier recurrence p<.001). No correlation was found between FR expression in primary tumors of the NM and M groups (p = .563). No correlation existed between FR expression in primary tumors of NM group and LNM of the M group (p = .81). None of the 10 bone marrows examined from cancer-free patients expressed FR. FR is expressed in a significant proportion of SCCHN tissues, in both primary tumors and LNM, and appears to be correlated with clinical outcome. These findings open the door for FR-mediated nanotherapeutic drug delivery with the potential advantages of reducing drug exposure to normal tissues, thereby reducing toxicities, and potentially enhancing drug uptake by tumor cells.

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Reference

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Keywords: chemotherapeutics; heparin-folate-taxol; nanotechnology

202 Anti-HGF Monoclonal Antibody Therapy Inhibits Lung Tumorigenesis in Transgenic Mice Overexpressing HGF

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The hepatocyte growth factor (HGF)/c-Met signaling pathway is involved in lung tumor development and progression and agents that target this pathway have clinical potential for lung cancer treatment. Recently, monoclonal antibodies (mAbs) that target specific growth factor signaling pathways have been successful as therapeutic cancer agents. A potent antihuman HGF neutralizing mAb, L2G7, demonstrated complete inhibition of human HGF-induced lung tumor cell proliferation, cell invasion, and P-MAPK induction in vitro. Transgenic mice that overexpress human HGF in the airways were utilized to study the efficacy of L2G7 for lung cancer prevention. These mice develop lung tumors that mimic aggressive human lung adenocarcinoma with high HGF levels. Mice were treated with 24 mg of a tobacco carcinogen, nitrosamine 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) over 4 weeks. Beginning at week 3, interperitoneal treatment with 100 µg L2G7 or isotype-matched antibody control, 5G8, was initiated and continued through week 15. The mean number of tumors per mouse in the L2G7 treated group was significantly smaller than in the control group (1.58 versus 3.19, p = .0005). The percentage of animals with tumors was 68% in the L2G7-treated group while it was 83% in the 5G8 treated group. Cell proliferation as measured by the number of Ki67 positive stained cells was decreased by an average 48% (p = .013) in tumors from L2G7-treated mice versus 5G8 treated mice while apoptosis was increased in these same tumors by fivefold (p = .0013) using ApopTag in situ hybridization. P-MAPK expression was also significantly decreased by 84% in tumors from L2G7-treated mice versus 5G8-treated mice (p = .0003). L2G7 treatment specifically inhibited the formation of wild-type K-ras tumors in this animal model, suggesting that targeting the HGF/c-Met pathway will not be as effective if downstream signaling is activated by a K-ras mutation. These results demonstrate that blocking the HGF/c-Met interaction with a single mAb delivered systemically can have profound inhibitory effects on lung tumor growth and supports the significance of HGF as an important mediator of lung tumorigenesis.

Keywords: hepatocyte growth factor; neutralizing antibody; transgenic mice

203 Bim Mediates Tyrosine Kinase Inhibitor-Induced Apoptosis in Lung Cancers With Activating EGFR Mutations and Its Upregulation Is Attenuated by Secondary Resistant Mutations, T790M and a Novel L747S

Please see Poster Discussion Session Therapy II.

204 GSK-3β Inhibition Enhances Sorafenib-Induced Apoptosis in Melanoma Cell Lines

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Glycogen synthase kinase-3β (GSK-3β) regulates virtually every aspect of cellular biology, including metabolism, proliferation, and cell survival. When activated, GSK-3β can induce apoptosis or, alternatively, provide a survival signal that minimizes cellular injury. We previously demonstrated that the multikinase inhibitor sorafenib induces apoptosis in melanoma cell lines. In this report, we show that sorafenib activates GSK-3β in multiple subcellular compartments and that this activation undermines the lethality of the drug. Pharmacologic inhibition and/or downmodulation of the kinase enhance sorafenib-induced apoptosis as determined by propidium iodide staining and by assessing the mitochondrial release of apoptosis-inducing factor (AIF) and Smac/DIABLO. Conversely, the forced expression of a constitutively active form of the enzyme GSK-3β$^{Ser\text{-}9\text{-}Ala}$ protects the cells from the apoptotic effects of the drug. This protective effect is associated with a marked increase in basal levels of Bcl-2, Bcl-XL, and survivin, and a diminution in the degree to which these antiapoptotic proteins are downmodulated by sorafenib exposure. Pharmacologic inhibition of GSK-3β downmodulates Mcl-1, and this effect is additive to the downmodulation induced by sorafenib. These data provide a strong rationale for the use of GSK-3β inhibitors as adjuncts to sorafenib treatment.

Keywords: apoptosis; GSK-3; sorafenib

205 Histone Deacetylase Inhibitors and Paclitaxel Cause Synergistic Effects on Microtubule Stabilization, Apoptosis, and Tumor Growth in a Mouse Xenograft

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Goal: Histone deacetylase (HDAC) inhibitors have proven efficacious in a variety of malignancies. In this investigation, we sought to determine the utility of these inhibitors in combination with traditional cytotoxic chemotherapy in endometrial cancer.

Methods: The HDAC inhibitor trichostatin A (TSA) was administered alone or in combination with traditional cytotoxic agents to ARK2 (serous) and KLE (endometrioid) endometrial cancer cell cultures as well as to a subcutaneous mouse xenograft. Effects on cell growth, morphology, and apoptosis were assayed using cell growth curves, cell cycle analysis, nuclear staining, and Western blot analysis for PARP and caspase 8 cleavage products. Effects on microtubule acetylation were measured using specific antibodies. Xenografts were weighed and subject to molecular analysis after six treatment cycles.

Results: Significant reductions in cell culture growth were observed following treatment with TSA, paclitaxel, doxorubicin, and carboplatin. However, the combination of TSA and paclitaxel showed the most synergistic effects on cell death and a dramatic activation of the apoptotic cascade. A clear synergistic increase in acetylated microtubules was observed following TSA and paclitaxel administration, suggesting the underlying mechanism of this combination. Using a mouse xenograft, the administration of TSA or paclitaxel alone resulted in little change in tumor weight compared to controls. However, the combination reduced tumor weight by 50% compared to controls or those mice treated with either agent alone ($p<.05$). Significant increases in acetylated microtubules were also observed in the xenograft treated with both TSA and paclitaxel.

Conclusions: The combined administration of TSA and paclitaxel results in a synergistic acetylation of tubulin causing alterations in microtubule reorganization, activation of apoptosis, and tumor reduction in a mouse xenograft greater than with either agent alone. This study provides strong in vitro and in vivo evidence that anticancer effects of HDAC inhibitors may be attributable to nonhistone protein acetylation. The combination of TSA and paclitaxel appears to hold promise for the treatment of poorly differentiated endometrial cancer.

Keywords: endometrial cancer; histone deacetylase inhibitors; tubulin

206 In Vitro and In Vivo Chemotherapy Sensitivity of Human Basal-Like Tumors

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Gene expression profiles have identified five major molecular subtypes of breast cancer (luminal A, luminal B, basal-like, HER2+/ER-, normal breast-like) that show significant differences in survival. Mounting evidence also suggests that these subtypes vary in their responsiveness to chemotherapeutics and biologic agents. In fact, expression of drug targets like HER1 and HER2 within different subtypes suggests that logical combinations of chemotherapeutics and biologic agents may be subtype-specific; nonetheless, criteria for selecting the right chemotherapeutic(s) and the right biologic agent combination for each subtype have yet to be experimentally or clinically determined.

The basal-like subtype of breast tumor is particularly challenging because of its lack of validated biological targets (i.e., they are ER-, PR-, and HER2-); therefore, we established a series of basal-like tumor model systems including human tumor-derived cell lines, primary tumor xenografts, and genetically engineered mice (GEM) and evaluated their sensitivity to chemotherapeutics. In cell lines, the SUM102 and SUM149 models were the most sensitive to carboplatin and the most resistant to 5FU when compared to HMEC and luminal lines. In two different human primary basal-like tumor xenografts grown in SCID mice, carboplatin was again the most effective single agent tested, and a carboplatin/paclitaxel combination was the most effective combination tested.

Interestingly, a carboplatin/erlotinib regimen was highly effective on one xenograft, but not the other, suggesting differential HER1-pathway sensitivity across basal-like tumors. Lastly, a GEM model that develops basal-like tumors (C3(1)-T-antigen) was also tested for sensitivity to six different chemotherapeutics. Carboplatin was the most effective single agent, and carboplatin/paclitaxel was the most effective combination. All of these results suggest that carboplatin is an effective chemotherapeutic for basal-like tumors. Current efforts are now focused on identifying the optimal biological agent-carboplatin regimen for human basal-like tumors

Keywords: basal-like tumors; carboplatin chemotherapy; molecular subtypes

207 TBCRC 001: EGFR Inhibition With Cetuximab in Metastatic Triple Negative (Basal-Like) Breast Cancer

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Background: Basal-like breast cancer (BBC) has low expression of ER, PR, and HER2 and is thus often called "triple negative" (TN) BrCa. Preclinical studies suggest that BBC is EGFR (HER1)-dependent for proliferation. TBCRC 001 is a multicenter randomized Phase II study of cetuximab with carboplatin in TN BrCa. In March 2007, Arm 1 (cetuximab alone) was closed according to prespecified response criteria. This report summarizes the interim analysis of cetuximab alone in stage 4 TN BrCa.

Methods: Eligible patients (pts) had measurable TN metastatic BrCa, ≤3 prior chemotherapies, and no prior platinum or EGFR inhibitor. Pts randomized to Arm 1 received cetuximab alone (400 mg/m^2, then 250 mg/m^2 weekly) with carboplatin (AUC 2 weekly, 3 of 4 weeks) added upon progression (PD). Pts on Arm 2 received cetuximab + carboplatin throughout. The primary endpoint is objective response (RR, CR+PR).

Results: Of 21 evaluable pts in Arm 1: mean age 48 (range 33-67), 28% African American, 10% Hispanic, 68% PS = 0, about 90% had received (neo)adjuvant chemotherapy, usually anthracycline/taxane-based; most also received at least one regimen for metastatic disease. Single-agent cetuximab was well tolerated; grade 3 drug-related AEs: rash (4 pts), fatigue (1), cellulitis (1), back pain (1), irregular menses (1), and elevated LFTs (1). One grade 4 anaphylaxis occurred. There were 1 PR (5%), 8 SD (38%), and 12 PD (57%). Most progressed quickly, 6 (29%) PD 4 weeks into therapy, and 6 (29%) by 8 weeks. 18 pts had carboplatin added to cetuximab after PD; 16 are currently evaluable, 4 (25%) have unconfirmed PR, and 5 (31%) have SD. Molecular subtyping

on 10 tumors from the overall study confirmed BBC in 9 (1 claudin subtype). Further subtyping and preliminary studies of biologic effects of EGFR inhibition will be presented.

Conclusion: Cetuximab alone was well tolerated but failed criteria for ongoing study. Rapid progression in this population may have limited evaluation of efficacy. Cetuximab + carboplatin is ongoing, with expected completion in mid-2007. Translational studies confirming molecular subtype, identifying biologic effects of EGFR inhibition and platinum, evaluating CTC, and studying BRCA1 pathway are ongoing.

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Keywords: basal-like; breast cancer; EGFR

208 Translation of a Peptidomimetic Targeted to the IL-11Rα Into Clinical Applications

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The bone marrow of the axial skeleton is a preferred site of metastases in most patients with prostate cancer. This striking selectivity is an example of site-specific cell-cell interactions that are essential to prostate cancer progression. We have developed an in vivo selection system in which targeted phage capable of homing to tumors is recovered from a phage-display peptide library after that library has been intravenously administered to patients. We have previously used this strategy to target a receptor for interleukin-11 (IL-11), which is present at low levels in the normal prostate gland but highly expressed in soft tissue and bone metastases. Studies from our laboratory and others suggest that IL-11 is a "metastasis gene" that is functionally relevant in the progression of prostate cancer. Here we have investigated the mechanisms by which IL-11 promotes prostate cancer progression in tumor microenvironment and have developed a targeted therapy based on delivery of pro-apoptotic peptides to IL-11Rα, with this drug termed "bone metastasis targeting peptide-11" (BMTP-11). A first-in-human clinical trial has been designed to provide evidence supporting selective therapeutic targeting by this peptidomimetic agent. The proposed study will be conducted in patients with androgen-independent and biopsy-confirmed metastatic prostate cancer to bone. This trial will provide the dose-escalation, PK/PD, and acute toxicity data necessary to establish feasibility for IL-11Rα targeting in patients. Good manufacturing practice (GMP)-grade BMTP-11 has been produced and is being evaluated for preclinical efficacy and toxicity in rodents, in preparation for a U.S. Food and Drug Administration investigational new drug (IND) application. This clinical study will serve as a basis for further developing ligand-directed therapies and imaging agents based on in vivo phage display combinatorial technology.

Keywords: IL-11Rα; metastases; peptidomimetic

209 Development of Second-Generation PSMA DNA Vaccines

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Our focus is on the development of new DNA vaccines that induce potent T cell responses to antigens expressed on prostate cancer. While self-antigens, and particularly differentiation antigens, are most frequently recognized on human cancers, because of tolerance, these self-antigens are poorly immunogenic. T cells with high avidity for self-peptides are deleted or, if they arise, inactivated by signals present on or secreted by the tumor. Lower avidity T cells remain, but in order to overcome tolerance and develop robust autoimmunity to a self-antigen expressed on prostate cancer, they must be activated, expanded, and maintained in the host. We previously showed that tolerance can be overcome by immunization with altered forms of antigen, including xenogeneic and randomly mutated DNA vaccines (see reference). These preclinical data led to a Phase I trial (IRB 00-133) using xenogeneic (altered self) PSMA DNA vaccines in patients with prostate cancer. Among 22 evaluable patients at the higher dose levels of 1,500 and 4,000 µg, we observed 2 cases of serum PSA declines of 45% and 59%, respectively. One of these patients was the only castrate metastatic patient at these higher doses. This suggests that an immune response to a PSMA DNA vaccine may influence clinical parameters, although the data do not reach significance. The vaccine was well tolerated with no adverse

effects. These encouraging preliminary results have prompted us to design a new PSMA DNA vaccine specifically engineered to overcome T cell tolerance. In our current preclinical studies, we are developing a rationally optimized PSMA DNA vaccine for use in a pivotal clinical trial.

We did not observe autoreactive cytotoxic T cell (CTL) responses in mice immunized with an unmodified xenogeneic vaccine, human PSMA. In order to overcome this tolerance to mouse PSMA, we introduced highly immunogenic heteroclitic epitopes into syngeneic and xenogeneic PSMA DNA and further modified the genes to decrease protein stability and increase antigen presentation. Using this strategy, CTLs that recognize mouse PSMA were generated. Furthermore, mice immunized with genetically optimized PSMA DNA vaccines were protected from challenge with tumors expressing mouse PSMA.

In other work, we have developed a method to quantify T cell responses to PSMA peptides in immunized patients using a library of overlapping PSMA peptides. In this way, we have identified two peptides recognized by CD4 T cells in a prostate cancer patient. This method will be used to describe the T cell responses to PSMA in patients receiving DNA vaccines.

Reference

Gregor et al. *Int J Imm, 2005.*

Keywords: DNA vaccine; T cell response; tumor immunology

210 Stimulation of Antitumor Immunity Using Dendritic Cell/Tumor Fusions and Anti-CD3/CD28

Please see Poster Discussion Session Therapy I.

211 Systemic Therapy With Tumor Suppressor FUS1-Nanoparticles for Stage 4 Lung Cancer

Please see Poster Discussion Session Therapy I.

212 Bioluminescence Imaging-Based Human Lung Cancer Orthotopic Xenograft Model

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Purpose: We have developed new models of human lung cancer and preneoplastic lesions to study the molecular pathogenesis of lung cancer and to test standard and novel targeted therapies of lung cancer. These include identification of lung cancer lines representing all histologic types of lung cancer and representing the full spectrum of genetic and epigenetic abnormalities and drug sensitivities and resistance found in lung cancer. We also need in vivo model systems to verify the in vitro data, which could mimic clinical settings of lung cancer, including orthotopic growth in the lung as well as in regional and distant metastatic sites, and could be subject to tumor detection, growth, and treatment followup. To meet these goals, we have established bioluminescence imaging (BLI) compatible human lung cancer orthotopic xenograft models.

Methods and Results: A CMV promoter-driven firefly luciferase lentiviral G418 selectable expression vector was prepared and stably introduced into 34 different human lung cancer lines. These lung cancer lines represented small cell and non-small cell lung cancer and had the full range of genetic and epigenetic changes found in lung cancer. For tumor formation, we injected 10^6 luciferase-containing cancer cells intrabronchially into female nude mice under general anesthesia. For monitoring tumor growth, we injected D-luciferin (450 mg/kg), subcutaneously into anesthetized (isoflurane) mice; 10 minutes later the amount of light production (for 5 minutes) was measured using a CCD camera; and the light intensity was analyzed with Igor software. With BLI, external monitoring of orthotopic lung tumors and quantitative serial followup of lung tumor growth was possible weekly over 8 weeks or more. We first asked whether gene expression profiles were changed because of the luciferase manipulation or with xenograft growth at both subcutaneous and lung sites. Gene expression profiles (Illumina WG6-v2) of four NSCLC cell lines (with and without luciferase) grown in tissue culture (TC) and subcutaneous (SC) and orthotopic (OT) xenografts were analyzed. These showed there is minimal change between lung cancer lines with or without firefly luciferase integration, or between xenografts grown SC or OT. Approximately 150 genes showed differential expression between TC and xenografts (twofold to sevenfold

variation), 5 of which were also validated by QRTPCR. Of note in unsupervised clustering analysis, each tumor line was more related to its xenograft expression pattern than to the other lines or xenografts. Tumor lines with vastly different in vitro chemosensitivity sensitivities to gemcitabine (>1,000-fold) were treated in vivo and monitored with BLI. We found the responses of orthotopic lung tumor xenografts in vivo maintained their sensitivity or resistance to gemcitabine.

Conclusions: Lung cancer lines can yield orthotopic (lung) xenografts with histology of the primary tumor that can have their growth monitored by BLI. The addition of luciferase does not change gene expression patterns, and similar patterns are seen in tumors grown SC or OT. While there are changes in gene expression between cells grown in TC compared to xenografts, the predominant signatures of the tumor line persist. These xenografts also retain the in vitro sensitivity and resistance phenotypes to chemotherapy in the in vivo xenograft setting.

Keywords: drug response phenotype; gene expression profiles; novel imaging

213 Investigation of Taxol Resistance Using a Folate Receptor-Targeted Ternary Therapeutic Nanoparticle

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The development of novel drug delivery approaches using nanotechnology is an important strategy to increase the therapeutic efficacy and decrease the side effects of chemotherapeutics. Moreover, conjugation of a drug to a ligand specifically targeted to a cancer cell surface receptor may not only help reduce dose-limiting toxicities but also enable the drug to bypass drug resistance mechanisms, especially those mediated by P-glycoprotein (P-gp), as the drug should be internalized via receptor-mediated endocytosis. We have recently conjugated a novel ternary structure nanoparticle, heparin-folate-taxol (HFT), which demonstrated greater antitumor efficacy than the free Taxol in vivo. In this study, we sought to investigate whether HFT could also minimize P-gp-mediated Taxol resistance using a xenograft model of KB-8-5 and KB-V1 Taxol-resistant cells. HFT is composed of heparin as the carrier, folate as the moiety targeting folate receptor (FR), and Taxol as the anticancer drug. Flow cytometry revealed high expression levels of FR in both parental (KB-3-1) and drug-resistant derivatives (KB-8-5, and KB-V1), while higher P-gp levels were observed in the drug-resistant cells (KB-8-5, KB-V1) compared with the parental cells, consistent with their Taxol-resistant phenotype. KB-8-5 and KB-V1 cells, which were known to be about 10 times and 1,000 times more resistant to Taxol than their parental KB-3-1 cells, respectively, were then injected subcutaneously into nude mice. The animals were treated with free Taxol, HT, HFT or Cremophor EL as a control. Each drug (80 mg/kg Taxol equivalent) was administered via tail vein injection once a week for 4 weeks (four injections total). Only HFT significantly inhibited growth of KB-8-5 and KB-V1 xenograft tumors in vivo as compared with the control (KB-8-5, $p = .03$; KB-V1, $p = .027$). Although the inhibitory effect of HFT on KB-8-5 tumor growth was not significantly better than that of free Taxol ($p = .33$), we observed a trend that HFT was superior to free Taxol in KB-V1 tumors ($p = .06$). Confocal microscopy analysis of freshly fixed KB-8-5 xenograft tumor sections revealed perturbations of the microtubule cytoskeleton in the HFT-treated animals, such as microtubule bundling, aberrant mitotic spindles, and cells with a lagging chromosome phenotype, consistent with the in vivo antitumor efficacy of HFT. No such effects were observed in the tumors treated with either free Taxol or HT. Furthermore, the weighted index of Ki-67 expression was lower in HFT-treated samples compared with the other three treatment groups, reflecting the in vivo antitumor efficacy of each treatment. These results indicate that a novel nanotherapeutic drug, HFT, can minimize or overcome the resistance of KB-8-5 and KB-V1 to Taxol and deserves further investigation in clinical trials.

This research was supported in part by National Cancer Institute grant P50 CA128613.

Keywords: nanoparticle; resistance; Taxol

214 Effect of Combination Treatment With Green Tea Epigallocatechin-3-gallate and an Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor (Erlotinib) on Squamous Cell Carcinoma of the Head and Neck

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Epigallocatechin gallate (EGCG) is the main polyphenol extracted from green tea. It has shown antitumor or chemopreventive activity in a variety of cancer types including squamous cell carcinoma of the head and neck (SCCHN). One of the mechanisms of the antitumor activity of EGCG may be associated with its effect on epidermal growth factor receptor (EGFR)-mediated signaling transduction pathways. Thus, combining EGCG with the EGFR-tyrosine kinase inhibitor (EGFR-TKI, erlotinib) may augment EGFR-TKI-induced inhibition of SCCHN cell growth. To test this hypothesis, five head and neck cancer cell lines were used to study the effects of the combination of EGCG and erlotinib on cell growth, cell cycle progression, and apoptosis. Our results showed that the combination of EGCG and erlotinib synergistically inhibited cell growth in SCCHN cell lines. Cell growth inhibition was associated with significant induction of cell cycle arrest at G0/G1 phase and apoptosis. Synergistic inhibition of pEGFR and pAKT by the combination of EGCG and erlotinib was associated with marked increases in activation of caspases 9, 3, and PARP compared with inhibition induced by EGCG or erlotinib alone. Further studies have elucidated a novel mechanism of inhibition of EGFR signaling pathways by the combined treatment. It was found that erlotinib inhibited phosphorylation of EGFR, thus stabilizing EGFR at the plasma membrane, while EGCG enhanced EGFR internalization and subsequent degradation by ubiquitination, thereby limiting EGFR recycling back to the plasma membrane and activation by its ligands. This leads to the depletion of cytoplasm EGFR and ultimately a decrease in the levels of both total and activated EGFR by combination treatment with erlotinib and EGCG. Finally, the hypothesis that EGCG and erlotinib synergistically inhibit tumor growth was tested in xenograft mice bearing SCCHN tumor cells. Twenty-five nude mice were orally gavaged with vehicle control (1% Tween 80), EGCG (125 mg/kg), erlotinib (50 mg/kg), or a combination of EGCG and erlotinib at the same doses for 7 days followed by subcutaneous injection with 2×10^6 SCCHN Tu212 cells. The animals were continuously administered the agents 5 days per week for a total of 7 weeks. The combined treatment significantly inhibited tumor growth and delayed tumor progression as compared with the control and the single agent treatment groups. Tumor growth inhibition was associated with increased apoptosis, decreased cell proliferation, and reduced pEGFR and pAKT. Our results strongly suggest a synergistic antitumor effect of combined treatment with EGCG and erlotinib and provide a promising regimen for future chemoprevention and treatment of SCCHN.

This research was supported in part by National Cancer Institute grant P50 CA128613.

Keywords: EGCG; erlotinib; synergism

215 PVT1 as a Therapeutic Target in Ovarian Cancer

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Genome copy number analyses using molecular inversion probe comparative genomic hybridization (CGH) have shown that amplification of a region at 8q24 is the most frequent copy number abnormality in ovarian cancers, occurring in approximately 60% of all cancers. Moreover, amplification of this region is significantly associated with reduced survival duration. These observations motivated studies to elucidate the roles of amplification at 8q24 in the pathophysiology of ovarian cancer. To accomplish this, we measured responses of ovarian and breast cancer cell lines to specific small interfering RNAs (siRNA) against the oncogene, *MYC,* and a putative noncoding RNA, *PVT1*, both of which map to 8q24. These analyses showed that siRNA-mediated reduction in either *PVT1* or *MYC* expression inhibited proliferation in ovarian cancer cell lines in which they were both amplified and overexpressed but not in lines in which they were not amplified/overexpressed. In addition, inhibition

of *PVT1* expression induced a strong apoptotic response in cell lines in which it was overexpressed, but not in lines in which it was not amplified/overexpressed. Inhibition of *MYC*, on the other hand, did not induce an apoptotic response in cell lines in which *MYC* was amplified and overexpressed. These results suggest that *MYC* and *PVT1* contribute independently to ovarian and breast pathogenesis when overexpressed because of genomic abnormalities. They also suggest that *PVT1* mediated inhibition of apoptosis may explain why amplification of 8q24 is associated with reduced survival duration in patients treated with agents that act through apoptotic mechanisms. We are now evaluating a siRNA against *PVT1* encapsulated in a neutral liposome as a therapeutic agent for treatment of ovarian cancer with high-level amplification at 8q24.

Keywords: apoptosis; ovarian cancer; *PVT1*

216 Endothelin (B) Receptor Mediates the Endothelial Barrier to T Cell Homing to Tumors, and Its Neutralization Enables Tumor Immune Therapy

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The success of immune therapy partly depends on the ability of effector cells to infiltrate tumors. The mechanisms governing homing of effector cells into tumors remain poorly understood. In particular, the role of tumor endothelium in tumor immunotherapy has not been investigated to date. Because of the clear dichotomy between presence and absence of intraepithelial TIL, we hypothesized that ovarian cancer is a suitable model to investigate the role of tumor endothelium in regulating T cell homing. Transcriptional profiling of microdissected tumor endothelial cells from human ovarian cancers revealed the molecular signature of endothelium associated with the absence of intraepithelial tumor-infiltrating lymphocytes (TIL). The endothelin B receptor (ET_BR) was associated with absence of TIL and short survival. ET_BR blockade by BQ-788 increased T cell adhesion to human endothelium, an effect countered by ICAM-1 blockade or NO donors in vitro. In the mouse, ET_BR neutralization by BQ-788 enabled tumor response to immune therapy in vivo, including vaccine therapy and adoptive lymphocyte therapy. This was mediated by increased homing of effector T cells to tumors, without changes in systemic antitumor immune response, and was attenuated by ICAM-1 blockade. These findings provide a novel molecular mechanism that can be pharmacologically manipulated to enhance the efficacy of tumor immunotherapy in humans. Because ET_BR inhibitors are available for immediate clinical testing, our findings provide a new immediate opportunity for cancer combinatorial therapy.

Keywords: effector cells; endothelial; immunotherapy

217 Alterations in Endothelial Cell Gene Expression in Human Ovarian Cancer

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Therapeutic strategies based on antiangiogenic approaches are beginning to show great promise in clinical studies. However, full realization of these approaches requires identification of key differences in gene expression between endothelial cells from tumors versus their normal counterparts. Here, we examined gene expression differences in purified endothelial cells from 10 invasive epithelial ovarian cancers and 5 normal ovaries using Affymetrix U133 Plus 2.0 microarrays. Over 400 differentially expressed genes were identified in tumor-associated endothelial cells. We selected and validated 23 genes that were overexpressed by 2.9-fold to 60-fold using real-time RT-PCR and/or immunohistochemistry. Among these, the polycomb group protein enhancer of *Zeste homolog 2(EZH2)*, protein tyrosine kinase 2 (PTK2, also known as Fak), and the Notch ligand *Jagged1* were elevated 2.9-, 3.0-, and 4.3-fold, respectively, in tumor-associated endothelial cells. Silencing *EZH2, Fak,* or *Jagged1* genes with short interfering RNA (siRNA) blocked endothelial cell migration and tube formation in vitro. The present study demonstrates that tumor endothelium and normal endothelium differ at the molecular level, which may have significant implications for the development of antiangiogenic therapies.

Keywords: antiangiogenesis; gene expression; ovarian cancer

218 Phase II Trial of Single-Agent Cetuximab in Patients With Persistent or Recurrent Epithelial Ovarian or Primary Peritoneal Carcinoma With the Potential for Dose Escalation to Rash

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Background: Data in other tumor types showed a correlation between severity of acneiform rash and cetuximab (Erbitux®) response. This study evaluated whether cetuximab dose escalation to cause grade 2 rash correlated with antitumor activity and whether sera-based markers could predict likelihood of response.

Methods: A 2-stage design was used with 50 patients planned. Criteria for eligibility were persistent/recurrent ovarian or primary peritoneal carcinoma; up to 2 prior regimens, including 1 platinum/taxane-based regimen; ECOG PS≤2; and measurable EGFR-positive tumors. Patients received an initial dose of cetuximab 400 mg/m^2, and then 250 mg/m^2 weekly for two 3-week cycles. Patients who had SD and <grade 2 rash were dose escalated in 75 mg/m^2 increments every 3 weeks until grade 2 rash or maximum 400 mg/m^2. Blood, pre- and/or post-treatment, was obtained from 14 of 25 patients with measurable response, and the sera evaluated for potential predictive markers of response.

Results: The study was stopped owing to an inadequate number of responders among the first 25 patients: median age: 58 years (range, 31-79); median number of cycles: 2 (range, 1-11). At first assessment, 4 patients remained at 250 mg/m^2; 8 patients were dose-escalated to 325 mg/m^2; of these, 4 were increased to 400 mg/m^2. One patient achieved PR; 9 patients had best response of SD. Median PFS: 2.1 months; 4-month PFS rate: 25%; 1-year survival rate: 54.8%. Rash (96%) was the most common drug-related adverse event (2 patients with grade 3); 1 patient had cetuximab-induced grade 4 hypersensitivity. Serologic markers including cytokines, chemokines, and growth and angiogenic factors (82 markers in all) were analyzed in sera from 10 patients exhibiting PD and 4 patients with SD. In pre-treatment samples, the average values of 9 of these markers were significantly reduced in patients exhibiting SD vs. PD (p = .043-.00037, two-sided t-test). Change in the level of 4 markers in sera from 4 patients exhibiting PD and 3 patients with SD from baseline to cycle 3 were further indicative of disease control (p = .10-.03, two-sided t-test).

Conclusions: Single-agent cetuximab showed minimal activity in these previously treated patients. However, the low number of dose-escalated patients limits complete evaluation of the hypothesis regarding response and dose escalation to rash. We did identify that patients with reduced levels of 9 or increased levels of 2 serologic markers at baseline were more likely to have disease control on cetuximab treatment. The use of multimarker combinations may further increase their predictive capacity. These results merit further investigation in future trials to determine their utility as biomarkers of cetuximab activity and clinical benefit in patients with ovarian cancer and other tumor types.

Keywords: cetuximab; dose-escalated rash; recurrent epithelial or peritoneal

219 In Vitro Effects of ZD6474, a Dual Inhibitor of EGFR and VEGFR, on Head and Neck Cancer Cell Lines

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Dual inhibition of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) tyrosine kinases has been suggested as an effective anticancer strategy. In several tumor types, the inhibition of EGFR has been shown to result in the decrease in VEGF production by the tumor cells. This effect, in turn, is expected to potentiate the inhibition of VEGFR on endothelial cells. Therefore, we studied the effects of ZD6474, a dual inhibitor of EGFR and VEGFR tyrosine kinases developed by AstraZeneca, on head and neck squamous cell carcinoma (HNSCC) cell lines. ZD6474 efficiently inhibited the activation of EGFR in HNSCC cell lines as determined by the decrease in EGFR autophosphorylation on Western blot. The decrease in phosphor-EGFR (pEGFR) was accompanied by a corresponding decrease in the activation of downstream signaling elements MAPK, Akt, and Stat3. More importantly, the downregulation of EGFR and Stat3 activities were accompanied by a decrease in Stat3 target genes including VEGF. ZD6474 also produced significant antiproliferative effects on HNSCC cell lines. The IC_{50} for antiproliferative effects ranged from 0.1 to 2 μM of ZD6474. Greater than 90% inhibition was achieved by 2-3 μM depending on the cell line. Lastly, ZD6474 potentiated the cytotoxic effects of cisplatin on HNSCC cell lines when both agents

were used together. In summary, ZD6474 inhibits the activation of EGFR and the associated downstream signaling pathways in HNSCC cell lines. These effects are associated with a decrease in tumor cell production of VEGF. HNSCC cell lines also appear to be sensitive to the antiproliferative effects of ZD6474. On the basis of these data, we expect ZD6474 to produce inhibition of tumor growth in murine xenograft models of HNSCC by targeting both tumor cell and endothelial compartments.

Keywords: EGFR; HNSCC; VEGF

220 Targeted Downregulation of c-Myc Through Disruption of the Transcription Factor/G-quadruplex DNA Complex

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Aberrant expression of the c-Myc oncogene has been identified in a number of human cancers, including prostate, breast, cervical, small-cell lung carcinomas, osteosarcomas, glioblastomas, pancreatic adenocarcinomas, myeloid leukemias, and colon. Colorectal cancers have been shown to express c-Myc protein at levels that range from fivefold to fortyfold over surrounding normal tissue owing to deregulation of endogenous c-Myc gene expression. The signals that trigger expression are terminally mediated by a large group of transcription factors (TF) that bind to elements in the regulatory regions of the c-Myc gene, including NFκB, YY1, SP1, E2F, hnRNP K, *Nucleolin,* and *NM23-H2*. We hypothesize that our recent identification of the critical involvement of the TF-G-quadruplex complex in the activation of c-Myc expression will enable us to identify lead molecules that target these complexes and that drugs derived from these leads can be advanced into Phase I clinical trials. Using fluorescent resonance emission topography (FRET), we have identified several small-molecule compounds that enhance the formation of the G-quadruplex, which was confirmed with circular dichroism (CD). These compounds demonstrate a functional disruption of the DNA-polymerase complex, generating shorter DNA product in the pol-stop assay. When taken into a cellular model of colorectal cancer, HCT-116, the small-molecule compounds cause a significant time- and dose-dependent downregulation of c-Myc mRNA and protein expression. EMSA studies demonstrated a disruption of TF binding. In particular, a dose-dependent displacement of both *Nucleolin* and *NM23-H2* binding to the c-Myc promoter was shown by lead small-molecule compounds. In summary, our studies have identified candidate compounds for drug development that are novel molecularly targeted agents downregulating a critical oncogene product. This downregulation apparently occurs via the disruption of specific TF binding to DNA secondary structures within the promoter region of c-Myc. This work represents a new paradigm for drug targeting of diseases in which oncogenes are overexpressed, and the identified compounds hold great promise for the treatment of colorectal cancer.

Keywords: GI cancer; Myc; small-molecule inhibitors

221 Developing Anti-Mullerian Inhibiting Substance Type II Receptor Immunoconjugates To Detect and Treat Ovarian Cancer

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The Mullerian inhibiting substance type II receptor (MISIIR) is a promising target antigen for immunotherapy of ovarian cancer. MISIIR is a member of the bone morphogenetic protein (BMP) type II serine/threonine receptor kinase family. In adult females, MISIIR expression is limited primarily to ovarian surface epithelium. Recently it has been observed that human MISIIR is expressed in a large percentage of both ovarian cancer cell lines and cells isolated from the ascites fluid of ovarian cancer patients and that the binding of the Mullerian inhibiting substance (MIS) to MISIIR on these cells can trigger apoptosis [1]. The highly selective expression pattern of MISIIR suggested to us that it could be an attractive target for the targeted treatment of ovarian cancer. Furthermore, the functional nature of this target suggests that anti-MISIIR antibodies that are specific for the ligand-binding site could trigger apoptotic effects. This is supported by the observation of Lefevre et al. that polyclonal anti-idiotypic antibodies to MIS bound to MISIIR and induced regression of Mullerian duct tissue in explant cultures [2].

We have produced recombinant human MISIIR extracellular domain (ECD) as a fusion protein with human IgG Fc domain and have stably expressed the MISIIR ECD on the surface of HEK 293 cells and have used these to isolate MISIIR-reactive single-chain Fv (scFv) molecules from a series of human scFv combinatorial phage display libraries. We have also modeled the MIS binding site on MISIIR, expressed the target peptide sequences, and isolated scFv molecules that specifically bind to these peptides. Using these techniques, over a dozen scFv different clones have been isolated which exhibit specific binding with low

to moderate affinity to MISIIR by ELISA, flow cytometry, and surface plasmon resonance on the BIAcore instrument. Six of the most promising clones were employed as the basis for larger divalent scFv-Fc constructs in order to increase functional affinity (avidity) and prolong half-life in circulation. One of these clones, GY4-Fc, was found to be capable of specifically targeting a catalytic toxin (saporin) to ovarian cancer cells and triggering cell death in vitro. We are currently preparing sufficient saporin-GY4-Fc conjugate to perform in vivo efficacy studies in mice bearing human ovarian cancer xenografts.

We have established a functional transient reporter gene cell line expressing a chimeric form of the MISIIR receptor that signals through luciferase on pGL3 under control of the TLX2 promotor for use in identifying antibodies that directly signal through the MISIIR receptor. A stable reporter gene system has also been developed and is currently under evaluation. These systems will be used to screen the hybridoma clones from a recent fusion. In conclusion, we believe that MISIIR offers an attractive target for treatment of ovarian cancer. Anti-MISIIR antibodies can be effective either as immunoconjugates or by directly triggering target cell death by altering signaling through the receptor.

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Keywords: antibodies; Mullerian inhibiting substance type II receptor; tumor-associated antigen

222 Targeting Aberrant Expression of NPM in Colorectal Tumors by ARF

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Cell growth and cell division are fundamental events in life. Studies have correlated deregulation of protein biosynthesis with cancer. NPM (B23/nucleophosmin) is a multifunctional nucleolar protein essential for normal embryonic development and ribosome biogenesis. NPM is frequently mutated in tumors through translocations or gain-of-function mutations, while increased levels of NPM have been noted in solid tumors including those of the gastrointestinal tract. The role of NPM in ribosome biogenesis and the fact that oncogenic c-Myc or Ras induces NPM overexpression suggest a possible link between cancer cell growth and increased NPM expression. Normal cells have much lower levels of NPM than tumor cells and association of a high level of NPM with cell transformation was also observed in primary tumors, including adenomas and carcinomas of the colorectum. NPM has been directly implicated in the p53 network by its well-documented interaction with the ARF tumor suppressor, a major upstream activator of p53.

We previously found that increased levels of ARF after oncogenic stress targets NPM for degradation, whereas others in addition also found that ARF blocks NPM nucleocytoplasmic shuttling and thereby cell growth. We could recently demonstrate that a highly conserved motif in the NPM core domain is essential for mediating ARF binding in vivo. Mutagenesis of conserved NPM core residues prevented NPM from interacting with ARF. We also built an in silico model of the NPM core, indicating that substitutions in loop motifs could trigger conformational changes, thereby obstructing ARF binding. We found that NPM core loop mutants were unstable, defective for oligomerization, and de-localized from the nucleolus to the nucleoplasm. NPM core mutants displayed increased ubiquitination and proteasomal degradation. The high level of NPM in tumor cells could make it a good prognostic factor and potential target for cancer therapy. Enforced expression of ARF, or an ARF-like molecule, might interfere with the proper structure and cellular function of NPM. Since NPM plays a critical role in ribosome biogenesis, targeting NPM by small ARF mimetic might specifically inhibit this process (growth) while initially maintaining cell cycle progression. Such "uncoupling" of cell growth and cell division might trigger apoptosis or senescence. Results from our continued studies on the NPM protein will be presented.

Keywords: biomarker; colorectal tumor; nucleophosmin

223 Therapeutic Potential of the Akt Inhibitor Perifosine in Combination With Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand for Metastatic Head and Neck Cancer

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Lymph node metastases remain a long-term problem in the treatment of head and neck squamous cell carcinoma (HNSCC). In an effort to understand the biology of metastasis of HNSCC and to develop effective therapeutic regimens against metastatic HNSCC, we have established highly metastatic HNSCC cell lines through in vivo selection (see reference). Using gene array and Western blot analysis, we found that highly metastatic HNSCC cell lines exhibit increased levels of death receptor 5 (DR5), Fas-associated death domain (FADD), caspase 8, caspase 9, and the short form of c-FLIP ($FLIP_S$). Moreover, these metastatic cell lines had decreased levels of the long form of c-FLIP ($FLIP_L$) and undetectable levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Accordingly, the metastatic HNSCC cell lines are relatively more sensitive than their parental nonmetastatic cell line to exogenous TRAIL treatment. These results suggest that metastatic HNSCC cells retain the capacity to undergo death receptor-mediated apoptosis. Perifosine is the first oral alkylphospholipid with Akt-inhibitory activity and is being tested in Phase II cancer clinical trials. We found that perifosine further upregulated DR5 expression and reduced c-FLIP levels in the metastatic HNSCC cell lines. Interestingly, the metastatic HNSCC cell lines were much more sensitive than their parental cell line to perifosine-induced apoptosis. Moreover, the combination of perifosine and TRAIL augmented induction of apoptosis in the metastatic HNSCC cells. Collectively, these data suggest that targeting activation of TRAIL/death receptor-mediated apoptosis by the combination of perifosine and TRAIL has therapeutic potential against metastatic HNSCC, thus warranting in vivo evaluation of the efficacy of the combination of perifosine and TRAIL for the treatment of metastatic HNSCC.

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Reference

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Keywords: death receptors; metastasis; TRAIL

224 Loss of Smac Expression Contributes to Resistance to Cisplatin-Induced Apoptosis in Head and Neck Squamous Cell Carcinomas

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The second mitochondria-derived activator of caspase (Smac) promotes apoptosis by sequestering inhibitor of apoptosis (IAP) proteins, abrogating their inhibitory effects on caspases. Two head and neck cancer cell lines, UM-SCC-5 and -10B, were selected for cisplatin resistance in vitro by exposure to increasing concentrations of cisplatin. Herein we show that whole-cell Smac expression is decreased at both the mRNA and protein levels in the cisplatin-resistant cell lines, UM-SCC-5PT and -10BPT, relative to the cisplatin-sensitive parental cell lines, UM-SCC-5 and -10B. To test the role of Smac in mediating cisplatin-induced apoptosis, we used a lentiviral system to stably transduce UM-SCC-10B with one of two different Smac shRNAs or a scrambled control shRNA. Western blot analysis was used to confirm the effectiveness of manipulations. Smac expression was reduced by over 80% relative to nontransduced controls in both Smac shRNA cell lines, but not the scrambled control cell line. Chemosensitivity (assessed by MTT assay) was performed 5 days after a 2-hour cisplatin exposure on UM-SCC-10B (parental, scrambled control, and Smac shRNA transduced). Knockdown of Smac expression significantly enhanced cisplatin resistance relative to nontransduced and scrambled controls (IC_{50} ≥25 μM, 8.1 μM, and 8.9 μM, respectively). Cell viability assays were performed following a 48-hour exposure to 10 μM cisplatin in UM-SCC-10B untreated, scrambled control, and Smac shRNA transduced. Knockdown of Smac in UM-SCC-10B protected cells from cisplatin-induced cell death. Untreated and scrambled control showed 46% and 51% nonviable cells while only 26% and 21% of Smac knockdown cells were nonviable. Similarly, TUNEL assays performed after a 48-hour exposure to 10 μM or 25 μM cisplatin demonstrated that Smac knockdown inhibited cisplatin-induced apoptosis. Therefore, our results suggest that loss of Smac expression contributes to escape from cisplatin-induced apoptosis in head and neck squamous cell carcinoma.

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Keywords: apoptosis; cisplatin; RNAi

225 Therapeutic Reversion of the Metastatic Phenotype in Prostate Cancer

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Prior studies by our group have identified molecular pathways that regulate prostate cancer (PCa) cell motility. We have shown that they are dysregulated during PCa progression and that genistein inhibits pro-motility pathways and metastasis of human PCa in preclinical models. Furthermore, we have characterized the pharmacokinetics of genistein administered to men with PCa. To determine whether genistein could reverse the metastatic phenotype in man, we implemented a Phase II study to treat men with localized PCa. We report here findings from our initial analysis of the effects of genistein treatment on prostate tissue. Our studies focused on morphologically normal prostate epithelial cells, an at-risk cell population. Prior in vitro studies demonstrated that genistein-mediated increases in cell adhesion were accompanied by flattening of the cell and its nucleus. Using quantitative image analysis to measure nuclear morphometric parameters in human prostate tissue, genistein was found to significantly increase nuclear length and width. In related studies, gene expression profiling was performed on cells recovered by laser capture microdissection from 14 treatment and 14 control subjects, using a 12K gene chip. Of six genes significantly altered by genistein, three have prior links to motility in other cell types. Two of these later three genes were shown by us to alter cell invasion of human PCa cells, under conditions of engineered overexpression. These findings demonstrate that therapeutically targeting pro-motility pathways in man induces cellular changes indicative of inhibition of cell detachment and selectively modulates genes that regulate cell motility. They support the notion that the metastatic phenotypic can be therapeutically reversed in man.

Keywords: cell motility; chemoprevention; TGFbeta

226 5α-Reductase Inhibition in Intermittent Androgen Ablation Therapy of Prostate Cancer

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Our recent studies showed that androgen-responsive gene U19 (upregulated 19) is a tumor suppressor. Interestingly, these genes are supra-induced by the 5α-reductase inhibitor finasteride during the regrowth of a regressed prostate but not in the full-grown prostate naive to androgen ablation. These observations led to our research hypothesis: that testosterone (T) is more potent than DHT in the induction of tumor-suppressive androgen-response genes during the regrowth of regressed prostate tumors. Blocking T to DHT conversion by 5α-reductase inhibitor during the off-cycle (when T is recovering) supra-induces tumor suppressive androgen-response genes and enhances the efficacy of intermittent androgen ablation therapy (IAAT). If the above hypothesis is correct, IAAT plus 5α-reductase inhibitor should be used to enhance the efficacy of intermittent androgen ablation therapy for prostate cancer patients.

Using the LNCaP prostate cancer xenograft model, we reported that IAAT with finasteride indeed provides the most favorable tumor growth kinetics and survival rate compared to both continuous androgen ablation and parallel intermittent androgen ablation. We have tested the effect of finasteride on several androgen-response genes and ETS genes and found that finasteride enhanced the expression of tumor-suppressive androgen-response gene U19 in LNCaP xenograft tumors during the off-cycle in IAAT. This supports our hypothesis that T is more potent than DHT in the induction of tumor-suppressive androgen-response genes during the regrowth of regressed prostate tumors. Using tumor size as the trigger point for the switch from off-cycle to on-cycle, we also showed that the first off-cycle in IAAT could be prolonged twice by finasteride or dutasteride in the LNCaP xenograft tumor model. Our current effort is trying to optimize the interval of the off-cycle and to determine whether supra-induction of U19 expression can be used to guide the switch from the off-cycle to on-cycle in IAAT.

Keywords: androgen; cancer; finasteride

227 Participation of Advocates in the Development of a Clinical Trial at the Northwestern SPORE

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The ultimate goal of a SPORE is to produce translational research, that is, laboratory discoveries that will result in better patient care. This presentation will relate to a clinical trial that is based on experimental observations at the Northwestern SPORE and may result in advancing our knowledge of how best to utilize androgen deprivation for patients with advanced prostate cancer, which may delay progression to androgen independence and prolong survival. The title of this SPORE research project is 5-Alpha Reductase Inhibition in Intermittent Androgen Ablation Therapy in Prostate Cancer.

In this Northwestern SPORE Clinical Trial, advocates have been involved in the following:

- Advocates were invited by the scientific staff to participate in the design and performance of this trial.
- Advocates developed a thorough understanding of the scientific rationale.
- Many meetings were held with the scientific staff to discuss trial details.
- Advocates recommended the use of Power Color Doppler to better localize tumors and vascularity for prostate biopsy.
- When concern was raised by clinicians about patient participation for sequential biopsies, advocates took a survey of three support groups on this issue and received a greater than 75% positive response.
- Advocates recommended the use of a local anesthetic for multiple biopsies that was accepted by clinicians.
- Advocates will be most useful in recruiting patients for this trial.
- We have started orienting patients about this trial at support group meetings explaining the purpose and participation requirements of this trial in a language they may easily understand.
- In May, Dr. Shevrin discussed this trial with a large support group, while Mr. Blair talked with his metastasis group about the details of this trial.
- We will also utilize written communication with the UsTOO Hotsheet (monthly publication) and two local Web pages related to prostate cancer.
- We have written letters of support for this clinical trial.

Our response from the prostate cancer community has been very positive and we anticipate excellent participation in this clinical trial that will focus on the potential ability of the 5-alpha reductase inhibitor dutesteride in the "off period" of treatment to enhance quality of life, time to androgen independence, and overall survival.

Keywords: advocacy; clinical; trial

228 Colorectal Liver Metastasis Has Increased HER2, Amphiregulin, and Epiregulin Expression, Which May Offer Therapeutic Targets

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Background: The EGFR receptor family has been increasingly recognized as an important therapeutic target in cancer. Herceptin for HER2-overexpressing breast cancer patients and gefitinib in lung cancer patients carrying a kinase mutation are examples. Monoclonal antibodies to EGFR, such as cetuximab and panitumumab, have up to 20% response rates when used in metastatic colorectal cancer, although there is no evidence of receptor overexpression or mutation to explain this response. This has led our laboratory to study this family of receptors and their ligands more closely in colorectal cancer liver metastasis.

Methods: We used FQ-PCR (TaqMan) to measure amphiregulin, epiregulin, EGFR, HER2, and HER3 in snap-frozen colorectal liver metastasis (n = 70), colorectal primary tumors, and normal colonic mucosa (n = 30-ligands; 70-receptors). Synthesized full-length mRNA was used to construct standard curves permitting specific and accurate ligand measurement. 10 ng/well of total-RNA extracted from snap-frozen samples was assayed in triplicate.

Results: Median EGFR copy number was actually decreased in colorectal tumors (39,918) and liver metastasis (22,477) versus normal colon mucosa (44,442) ($p = .004$). HER2 showed a slight increase in colon tumors as compared to normal mucosa (69,213 vs. 50,953), although this rose dramatically to 428,618 in liver metastasis ($p<.001$), representing a sixfold increase between

primary tumors and metastasis. HER3 had no statistically significant changes with mRNA copy numbers of 54,480 in normal colonic mucosa, 76,994 in colon tumors, and 82,999 in liver metastasis. The EGFR ligands, amphiregulin and epiregulin, were also increased in liver metastasis, with amphiregulin rising from 5,277 in normal colon and 9,089 in colonic primary tumors to 137,428 in liver metastasis ($p<.001$). Epiregulin similarly rose from 6,044 (colonic mucosa) to 9,858 (colorectal tumors) to 120,120 (liver metastasis) ($p<.001$).

Conclusions: There is no evidence of EGFR mRNA overexpression in colorectal liver metastasis when compared to normal colonic mucosa or primary colon tumors. EGFR ligands, amphiregulin and epiregulin, however, are increased in colon tumors and even more strikingly in liver metastasis, which may explain the clinical response to EGFR blockade with cetuximab and panitumumab in metastatic colorectal cancer patients. Interestingly, while there was a small increase in HER2 expression in colorectal tumors versus normal colon, this rose markedly in the liver metastasis. This increased expression may represent a therapeutic target for agents such as lapatinib, which blocks the HER2 kinase domain.

Keywords: colorectal; EGFR; metastasis

229 A Phase I Trial of CpG 7909, Rituximab Immunotherapy, and Y90 Zevalin Radioimmunotherapy for Patients With Previously Treated CD20+ Non-Hodgkin's Lymphoma

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Background: CpG 7909 is a synthetic 24 mer oligodeoxynucleotide designed to provide optimal activation of immune mechanisms through the TLR9 receptors on B cells and plasmacytoid dendritic cells. In vitro CpG can upregulate CD20 expression as well as stimulate the immune system. Radioimmunotherapy (RIT) with anti-CD20 radioimmunoconjugates such as Zevalin produces responses in approximately 80% of patients with relapsed indolent lymphoma; however, only 30% obtain a complete response and 20% have long-term disease-free survival.

Objectives: To combine CpG 7909 with rituximab and Zevalin to enhance the response to RIT.

Patients and Methods: Patients were eligible if they had biopsy-proven relapsed, refractory, or residual CD20+ non-Hodgkin's lymphoma (NHL). Histologic types included relapsed indolent and aggressive B cell NHL. Patients were required to have measurable disease, a platelet count $\geq$150,000, and an absolute neutrophil count $\geq$1,500. This was a Phase I study, in which all patients received a standard dose of Zevalin (0.4 mCi/kg with a maximum of 32 mCi for patients over 80 kg) along with a Phase I dose of CpG 7909. Four dose levels were studied: 0.08 mg/kg, 0.16 mg/kg, 0.32 mg/kg, and 0.48 mg/kg. CpG was dosed on days 6, 13, 20, and 27; rituximab was provided on days 1, 8, and 15; indium-111 Zevalin on days 1 and 8 (followed by indium-111 imaging); and yttrium-90 Zevalin on day 15. Response was assessed at weeks 4 and 12.

Results: A total of 24 patients have now been treated—6 at each of the four dose levels. One patient at dose level 4 (0.48 mg/kg) had a dose-limiting toxicity (DLT) (hematologic). The other five patients at dose level 4 did not have DLT; thus, 0.48 mg/kg was determined to be the dose for further studies. The protocol now specifies six additional patients at dose level 0.48 mg/kg, and those patients are being accrued now. The overall response rate, to date, is 92% (22/24). After followup, 6 of the 24 patients have progressed; therefore, 75% (18/24) remain in response. As part of the study, we performed indium-111 Zevalin imaging before CpG and after two doses of CpG (prior to the yttrium-90 Zevalin) to detect changes in biodistribution of the radioimmunoconjugate that could be attributed to CpG. In all 24 patients treated to date, review of the paired images does not show any deleterious effects of CpG on tumor imaging. We also have been unable to demonstrate enhancement of tumor uptake induced by CpG, at least at the level of detection of the indium-111 imaging technique. There has been no effect on normal organ uptake of indium-111 Zevalin by the CpG. Patients are also being assayed pre- and post-CpG as well as at multiple timepoints up to 1 year following treatment for changes in T cell, NK cell, and cytokine before and after treatment. To date, there has been no apparent difference in these measurements by CpG dose level.

Summary: CpG 7909 can be safely combined with Zevalin RIT. The response rate appears very promising; however, longer followup is necessary before decisions can be made regarding a Phase III trial.

Keywords: CpG; immunotherapy; lymphoma

230 Direct and Indirect Modulation of EGFR-Mediated Growth Signaling by Hsp90 Inhibition in Non-Small Cell Lung Cancers Resistant to EGFR Tyrosine Kinase Inhibitors In Vitro and In Vivo

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Activating EGFR point mutations and deletions confers sensitivity to the reversible EGFR TKIs gefitinib and erlotinib. However, reversible EGFR TKIs are not effective against non-small cell lung cancers (NSCLCs) expressing EGFRs harboring exon 20 mutations, EGFRs carrying the T790M secondary mutation, or wild-type EGFR, in a large proportion of cases. Here, we propose Hsp90 inhibition as a novel strategy for NSCLCs lacking sensitivity to reversible EGFR TKIs. Hsp90 is a crucial chaperone for both conformational maturation and stability of many cellular proteins (clients), particularly oncogenic kinases. We previously reported that Hsp90 inhibition results in degradation of EGFRs with kinase domain point mutations and deletions. To assess exon 20 insertions, we used Ba/F3 cells transformed with exon 20 insertions identified in patient samples. These cells were resistant to erlotinib and the irreversible EGFR-ErbB2 inhibitor CL-387,785 (IC_{50}s > 1 μM) but were sensitive to the Hsp90 inhibitor 17-DMAG (IC_{50}s < 50 nM). Similarly, Ba/F3 cells expressing EGFR exon 19 deletions + T790M identified in patient samples were also sensitive to 17-DMAG. In addition, an NSCLC cell line with EGFR E746_T751del_insI + T790M (H820) was resistant to both erlotinib and CL-387,785 (IC_{50} values >10 and 0.9 μM, respectively) and sensitive to 17-DMAG (IC_{50} = 68 nM). Treatment of H820 cells with 17-DMAG caused marked depletion of several growth factor receptors, including EGFR, ErbB2, and c-Met, with a diminution of downstream signals and induction of apoptosis. In contrast, treatment of these cells with CL-387,785 resulted in incomplete depletion of downstream signals and persistent ErbB3 phosphorylation. 17-DMAG was also effective against H1975 cells and in a murine inducible bitransgenic model in which EGFR harboring L858R + T790M causes lung adenocarcinoma. Finally, 17-DMAG also was effective against a variety of NSCLC cell lines harboring wild-type EGFR. For example, H1781 cells carrying wild-type EGFR and the ErbB2 G776insV_G/C mutation underwent apoptosis after depletion of mutant ErbB2 by either shRNA or 17-DMAG. 17-DMAG also compromised the proliferation and anchorage-independent growth of H1299, H460, and A549 NSCLC cell lines harboring wild-type EGFR, with acute depletion of mutant N-Ras, IGF-1R, and other Hsp90 client proteins, including CDK4. In A549 cells, erlotinib and 17-DMAG synergistically compromised cell viability. Synergism was not readily demonstrated in EGFR-mutant cell lines already exquisitely sensitive to erlotinib. The result was recapitulated by co-treatment with erlotinib and the IGF-1R inhibitor AG1024, or siRNA targeting IGF-1R. In summary, Hsp90 inhibition is effective against NSCLCs not responsive to EGFR inhibitors. The mechanism underlying the synergism of EGFR and Hsp90 inhibition in EGFR wild-type cells is under investigation.

Keywords: EGFR; Hsp90; murine adenocarcinoma

231 Chemical Genetics Approach for Dissecting Wnt Signal Transduction Using a Novel Biochemical Approach Based on Theoretical Considerations

Please see Poster Discussion Session New Technologies and Methodologies II.

232 Interleukin-13 Displaying Oncolytic Measles Virus Strains Have Tumor-Specific Activity Against Gliomas With an Improved Therapeutic Index

Please see Poster Discussion Session Therapy I.

233 Evaluation of Interleukin-2 and TGFβ Blockade in cd4TGFβ-RII DNR Mice

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Background: Our laboratory has previously demonstrated that the pleiotropic cytokine, TGFβ, is important in regulating the effects of T cells in rejecting an engrafted melanoma cell line, B16-F10. Blocking TGFβ signaling in both CD4+ and CD8+ T cells in a transgenic mouse model developed in our laboratory (cd4TFGβ-RII DNR [DNR]), results in enhanced tumor rejection by cytotoxic T cells (CTLs), making this cytokine a potential target for future therapies to treat metastatic melanoma patients. However, the effects were limited and could be overcome by increased tumor burden, suggesting that blocking TGFβ signaling alone may be insufficient as an optimal therapy in metastatic melanoma patients. Given that high-dose interleukin-2 has been demonstrated to induce a response in up to 15% of metastatic melanoma patients, with 5% of these patients achieving long-term durable responses, we evaluated the combined effects of this cytokine with TGFβ signaling blockade in the DNR mice.

Methods: Using the IVIS imaging system (Xenogen and Caliper Life Sciences, Hopkinton, Massachusetts), we evaluated the in vivo tumor kinetics of an engrafted melanoma cell line expressing luciferase (B16-Luc) in wild-type C57BL/6 (B6) mice and DNR mice with and without treatment of rhIL-2. Both B6 and DNR mice received subcutaneous doses of 1×10^5 B16-Luc cells, and the IL-2 treatment groups received intraperitoneal injections of rhIL-2 (150,000IU BID × 4 days) once engrafted tumors reached greater than 10^6 light units and 2 mm × 2 mm in size. Both luminescence and tumor measurements were obtained and mice were euthanized once the tumors reached up to 15 mm in greatest dimension.

Results: Our preliminary results suggested that the DNR mice were more resistant to engraftment of B16-Luc cells when compared to B6 mice and IL-2 administration augmented this effect. Time to development of 4 mm^2 tumors (when IL-2 was administered) was 12 days and 21 days for the B6 and DNR mice, respectively. When compared to untreated mice, adding IL-2 delayed tumor growth to 225 mm^2 by 3 days and 6 days in the B6 mice (day 21 versus day 18 for untreated mice), and the DNR mice (day 41 versus day 35 for untreated mice), respectively. IL-2 therapy was well-tolerated in the DNR mice.

Conclusions: Combined IL-2 and TGFβ inhibition enhances antitumor responses in DNR mice. Future studies will combine an antibody to TGFβ with IL-2 to determine whether antibody blockade and genetic inhibition of TGFβ demonstrate similar effects. Effects of IL-2 on lymphocyte subsets including regulatory T cells in the DNR mice will be presented.

Keywords: interleukin-2; melanoma; TGF-beta

234 Induction of Antitumor Immunity Ex Vivo Using Dendritic Cells Transduced With Fowl Pox Vector Expressing MUC-1 and a Triad of Costimulatory Molecules (PANVAC-F)

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Ovarian carcinoma cells express unique antigens, including MUC-1 and CEA, that may be targeted by host immunity. Dendritic cells (DCs) are potent antigen-presenting cells that are uniquely capable of inducing antitumor immune responses. DCs loaded with tumor antigens are being explored as cancer vaccines for patients with ovarian carcinoma. In the present study, we examined whether DCs transduced with an attenuated fowl pox virus expressing MUC-1, CEA, and costimulatory molecules (rF- PANVAC) effectively induce antitumor immunity in vitro. The impact of viral transduction on DC phenotype, tumor antigen expression, and the capacity to stimulate activated T cell responses was assessed. Partially mature DCs were generated from adherent peripheral blood mononuclear cells that were cultured for 5 days with GM-CSF and IL-4. DCs were transduced with rF-PANVAC and matured with either TNFα or the combination of PG-E2, TNFα, IL-6, and IL-1β. Transduced DCs demonstrated strong expression of MUC-1 (mean, 64.6%; MFI, 249.8). Similarly, CEA was expressed by means of 34.9% of transduced DCs with an MFI of 416.5. Transduced DCs demonstrated high levels of expression of Class II molecules (99%; MFI, 250), CD54 (98.4%; MFI, 516), CD58 (98.8%; MFI, 155), and CD80 (78.2%; MFI, 115). Transduced DCs demonstrated higher levels of the maturation marker CD83 (40.3%) as compared to untransduced DCs (19.7%) ($p = .02$). Transduced DCs matured with PGE2, TNFα, IL-6, and IL-1β as compared to TNFα alone demonstrated higher levels of CD83 and CCR7 and a more stable phenotype following withdrawal of cytokine support. To assess the ability of rF-PANVAC to stimulate tumor antigen-specific responses, the presence of T cells binding the MUC-1 specific tetramer was quantified following stimulation of autologous T cells derived from HLA*0201 healthy donors. An increase in CD8+MUC-1+ T cells was observed (8.3%) with stimulation with rF-PANVAC transduced DCs as compared to

untransduced DCs (2.5%). We further investigated the capacity of DCs transduced with rF-PANVAC to generate activated effector cells. Following stimulation with transduced DCs, an increased percentage of CD4+/CD25+ cells expressing IFNγ (mean, 58%) and CD69 (23%) was noted, consistent with activated effector cells. Consistent with their cytolytic capacity, Granzyme B and Perforin were expressed in 22.4% and 15.9% of CD8+ T cells following stimulation with PANVAC transduced DCs. Of note, stimulation with PANVAC transduced DCs resulted in 38% of cells expressing a regulatory T cell phenotype as defined by co-expression of CD4, CD25, and Foxp3. In summary, PANVAC-F transduced DCs stimulate expansion of antigen-specific activated T cell populations, suggesting their potential role as tumor vaccines for MUC-1/CEA expressing tumors. We are initiating a trial for patients with ovarian carcinoma in early (marker only) relapse in which patients will be randomized to undergo serial vaccination with rV-PANVAC/rF-PANVAC or DCs transduced ex vivo with rF-PANVAC. The capacity to generate antitumor immunity in vivo will be assessed as a primary endpoint.

Keywords: dendritic cells; immune therapy; vaccine

235 A Pilot Trial To Test the Efficacy of Broccoli Sprout Extract in Activating Nrf2 Pathway in Healthy Smoker Volunteers

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Susceptibility of the lung to oxidative injury and inflammation, such as that originating with inhalation of cigarette smoke, depends largely on its upregulation of antioxidant and cytoprotective systems. Nrf2 is a redox-sensitive bZIP transcription factor that is involved in the regulation of many carcinogen detoxification and antioxidant genes, and its role in pulmonary inflammation and carcinogenesis is emerging. Disruption of the *nrf2* gene in mice causes earlier onset and more extensive cigarette smoke (CS)-induced emphysema than is seen in wild-type littermates. Increased pulmonary inflammation in *nrf2*-deficient mice in different models (allergic asthma, endotoxin-mediated acute lung injury, as well as systemic inflammation during polymicrobial sepsis) underscores the importance of Nrf2 as a global regulator of inflammation. Responsiveness of the Nrf2 pathway may act as an important risk modifier of carcinogenesis due to chronic CS. A trial was designed to determine whether broccoli sprout extract containing an isothiocyanate Nrf2 activator compound called sulforaphane can increase carcinogen detoxification. The primary endpoint is to evaluate the effect of broccoli sprout extract ingestion on levels of DNA adducts in buccal cells and blood lymphocytes in current smokers. Subjects must be current smokers of at least one pack per day, have smoked at least 5 pack-years, and have normal kidney, hematopoietic, and liver function. They also must comply with a sulforaphane-free diet for the entirety of the study. The first 7 subjects of a total 70 planned (2 males, 5 females, median age, 54 years [range, 21-72]) have been enrolled in the trial thus far. Subjects are followed once per week for 3 weeks during a run-in phase to assess baseline DNA adduct levels. Then they are given the broccoli sprout extract once per day by mouth for 3 weeks. After the treatment phase, the subjects were followed once per week for 3 weeks during the wash-out phase. Buccal cells and blood lymphocytes are collected on a once per week basis throughout the study to assess DNA adduct levels. Subjects are required to keep diaries of their diet and smoking habits. The first five subjects were followed for an initial 9 weeks run-in period to assess the variability of DNA adducts in smokers. Of the first seven subjects, one subject withdrew consent after 1 week. The other six subjects have completed all study phases. No toxicities have been seen. DNA adducts on the first five subjects are currently being analyzed and will be reported at the time of the meeting. This study is the first step in the development of broccoli sprout extract as a potential chemoprevention strategy for lung cancer by targeting the transcription factor Nrf2.

Keywords: biomarker; Nrf2; sulforaphane

236 Models of Acquired Resistance to Gefitinib in EGFR Mutant Non-Small Cell Lung Cancer

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The epidermal growth factor receptor *(EGFR)* tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib are particularly effective therapeutic agents for patients with non-small cell lung cancer (NSCLC) whose tumors harbor activating mutations in *EGFR*. However, most if not all patients who initially develop a partial or complete response to gefitinib or erlotinib will develop progression of their cancer. We previously identified ErbB3, a kinase dead member of the *EGFR* super-family, as the key activator of PI3K/Akt signaling in *EGFR* mutant NSCLC. In addition, we previously determined that maintenance of the PI3K/Akt signaling is sufficient to confer resistance to gefitinib/erlotinib.

In order to model acquired resistance in vitro, we generated resistant clones of gefitinib-sensitive *EGFR* mutant NSCLC cell lines H3255 (L858R) and HCC827 (Del E746_A750) by exposing them to increasing concentrations of gefitinib in vitro. Both gefitinib-resistant (GR) cell lines, H3255 GR and HCC827 GR, had an IC_{50} >10 μM for gefitinib and demonstrated persistent ErbB3 and Akt phosphorylation in the presence of gefitinib. The H3255 GR, but not the HCC827 GR, cell line was also growth inhibited by the irreversible *EGFR* inhibitor CL-387,785. Although not detected by direct sequencing, the H3255 GR cells acquired an *EGFR* T790M mutation in a small fraction of the amplified alleles that confers resistance and is identified only by a highly sensitive HPLC-based technique. Similarly, this method identified *EGFR* T790M in three patients that was not detected by conventional Sanger sequencing. In the HCC827 GR cells, we identified an amplification in another growth factor receptor, MET, using single nucleotide polymorphism (SNP) arrays. Inhibition of MET, either with a specific MET kinase inhibitor (PHA-665,752) or by a *MET*-specific shRNA, was sufficient to restore gefitinib sensitivity. In the HCC827 GR cells, MET activates ErbB3/PI3K/Akt signaling in an *EGFR*-independent fashion, thus leading to gefitinib resistance. In a series of 18 patients with *EGFR* mutations that developed acquired resistance to gefitinib/erlotinib, we found *EGFR* T790M in 9/18 (50%) and *MET* amplification in 4/18 (22%) gefitinib/erlotinib-resistant tumor specimens.

Our studies demonstrate that *EGFR* mutant NSCLC cell lines can be used as effective in vitro models to identify clinically meaningful gefitinib/erlotinib resistance mechanisms. In addition, such resistant cell lines provide a means of testing novel therapeutic combinations, allowing rapid translation into clinical treatments for patients who have developed acquired resistance to gefitinib and erlotinib.

Keywords: *EGFR* mutations; *EGFR* targeted therapies; resistance

237 Targeting Transforming Growth Factor β Receptor as a Therapeutic Approach to Suppressing Pancreatic Cancer Metastasis

Please see Poster Discussion Session Therapy I.

238 Dysfunctional KEAP1–NRF2 Interaction in Non-Small Cell Lung Cancer as a Critical Factor for Chemoresistance

Please see Poster Discussion Session Therapy II.

239 Strategies for Overcoming Trastuzumab Resistance Caused by PTEN Deficiency

Please see Poster Discussion Session Therapy I.

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

240 T Cells and Dendritic Cells in High-Risk Breast Cancer Patients Given Dose-Dense Treatment With Adjuvant Chemotherapy

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Immune therapy is most effective against minimal tumor burden; however, activity during adjuvant chemotherapy is poorly defined. We undertook a longitudinal study into the effect of dose-dense chemotherapy on immune function and cellular phenotype. Patients with locally advanced breast cancers 4 cm or larger or 4+ positive nodes and p53 expression by immunohistochemistry were eligible for this study. Four cycles of dose-dense doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 (AC) therapy induced significantly more lymphopenia (T and natural killer [NK] cells) than did four cycles of dose-dense paclitaxel (P) 175 mg/m^2. In this AC $\rightarrow$ P protocol, chemotherapy was given every 2 weeks with granulocyte colony-stimulating factor (G-CSF) support. After the last P cycle, patients received 6.5 weeks of daily radiotherapy (RT).

We report the results from 18 stages II-III breast cancer patients as compared to 18 age- and menopause-matched normal female donors. Samples were obtained prior to therapy, following AC therapy, following P therapy, and 6 and 12 weeks after completion of RT. The frequency and number of naive and memory CD4$^+$ and CD8$^+$ T cells, NK cells, monocytes, and CD11c$^+$ and CD123$^+$ dendritic cells (DCs) were studied. At diagnosis, cell numbers were marginally lower than normal controls, and only monocyte numbers were significantly reduced as compared to normal female donors. Following four cycles of AC therapy, both naive and memory CD4$^+$ and CD8$^+$ T cells and NK cells were significantly decreased ($p \leq .01$, Mann Whitney U test) by at least 50%. Lymphocyte numbers increased during dose-dense P therapy to become statistically similar to pretherapy levels. Average T cell numbers (CD3) declined during AC (from $6.0 \pm 2.6 \times 10^6$/mL to $2.3 \pm 1 \times 10^6$/mL), but recovered 1 week after P ($5.0 \pm 2.0 \times 10^6$). Similarly, DC (CD11c$^+$ and CD123$^+$) levels were significantly depressed relative to normal donors after AC therapy, increasing to the levels observed in normal donors following four cycles of P therapy. In contrast, the lymphocyte populations remained significantly lower than normal donors at all timepoints. At 6 and 12 weeks following RT, CD123$^+$ DC and monocyte levels were increased as compared to post P levels, whereas naive CD4$^+$ and CD8$^+$ T cells and CD11c$^+$ DCs were decreased. T cell function, measured as phytohemagglutinin (PHA) mitogenesis, did not differ from pretherapy levels at any timepoint examined.

In summary, four cycles of dose-dense AC chemotherapy are significantly more immunosuppressive than four cycles of dose-dense P chemotherapy, suggesting that immune therapy might best be administered during P therapy.

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Keywords: breast; chemotherapy; immune

241 Determinants of Response to Type 1 Insulin-Like Growth Factor Receptor Inhibition by Human Monoclonal Antibodies in Prostate Cancer

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We have shown in preclinical and human studies that prostate cancer growth, or PSA, in early human studies indicates that there is a response to inhibition of IGF-IR signaling with the fully human IGF-IR mAb, A12, and a slowing or reversal of tumor progression. However, not all prostate cancer models or patients responded. In order to optimally target IGF-IR inhibition therapy, it is important to determine markers of tumor response. LNCaP and its castration-independent subline, C4-2, express very low levels of the IGF-IR scaffolding protein IRS-1, unlike most other prostate cancer cell lines and xenografts. Loss of IRS-1 expression occurs in approximately one-third of metastatic prostate cancers (see reference). The C4-2 cells are also resistant to A12 in the nude mouse in vivo tumor model. In vitro the C4-2 cells did not undergo apoptosis when treated with A12. However, invasion through a matrix and motility were markedly inhibited by A12 (data not shown).

In order to determine the effect of IRS-1 in these cells, we expressed IRS-1 in a temperature-sensitive conditional vector. Expression of IRS-1 resulted in increased Akt expression, phosphorylation of the androgen receptor with increased AR transcriptional activity. AR activation increased expression of IGF-I and IGF-IR. The effects of increased AR on IGF-I and IGF-IR were confirmed by decreasing AR with shRNA1, where decreased AR resulted in a decrease in IGF-I and IGF-IR mRNA and protein with decreased IGF phosphorylation. The increase in IGF-I and IGF-IR by IRS-1 activation of the AR subsequently resulted in an increased dependence on IGF signaling for survival, as seen by increased apoptosis in response to IGF-IR blockade by A12 (see figure). These data suggest that IRS-1 loss may be a marker of resistance to IGF-IR inhibition.

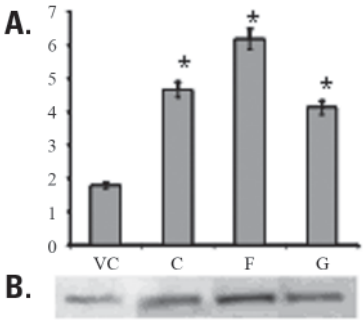

Figure A. Apoptosis as determined by cleaved PARP/intact PARP in VC-vector control and clones of IRS-1 expressing C4-2 cells C, F and G following 24 hours of exposure to the IGF-IR hmab, A12. Note the significant increase in apoptosis in response to A12 in IRS-1 expressing clones (p<.01) compared to VC cells.

Figure B. Representative Western blot of cleaved PARP.

Reference

Hellawell et al. *Cancer Res* 62:2942-50, 2002.

Keywords: androgen receptor; IGF-IR hmab; IRS-1

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

242 A Phase II Trial of Neoadjuvant Therapy With Letrozole for Stage 2 and 3 ER+ Breast Cancer in Postmenopausal Women

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The Partners in Progress Avon NCI program provided initial funding for a Phase II neoadjuvant endocrine therapy study that subsequently received continued support from NCI. This investigation examined the efficacy of 4 to 6 months of preoperative letrozole for postmenopausal women with ER+ (Allred score, 6 to 8) stages 2 and 3 breast cancer (see table). The study recently completed accrual with a total of 115 patients. The response rates in this study are consistent with our previous published experience with letrozole in the P024 trial. Eight patients were not evaluable owing to death before starting study medication, withdrawn by the principal investigator before study initiation (1), withdrawal of consent (6), and ineligible (1). No drug-related SAE has been recorded, underscoring the safety of the therapeutic approach. The surgical outcomes are also outlined in the table. Ten patients who did not have surgery immediately after preoperative letrozole included one who refused surgery, seven who went on to preoperative chemotherapy, and two who were "too ill" for surgery. The breast conservation rate of 48% was similar to our previous experience and confirms a clear benefit for the study subjects, since mastectomy was the only option for most of these patients at baseline.

Frozen sample analysis yielded 79 cases of 115 with a baseline core biopsy containing >50% tumor that could be successfully analyzed by gene expression microarray and linked to clinical outcome data (an array success rate of ~70%). This sample set also yielded enough DNA for 35 array CGH experiments. Since amplification of the ErbB2 gene at 17q12 represents a specific example of a genomic anomaly that directly causes endocrine therapy resistance, we reasoned that gene amplification in other areas of the genome could be responsible for additional cases of endocrine therapy failure. To identify amplicons, we systematically compared gene expression data and aCGH data by applying a Wilcoxon test to compare continuous gene expression levels against the matched aCGH probe data that had been presorted into amplified and not-amplified probe groups by process control.

In this analysis 1,430 genes with p<.05 were identified as differentially overexpressed in association with a significant gain in gene copy number. Repeating the analytical process on randomly permuted gene-expression levels and array CGH data showed a false positive rate of ~17% at the .05 level. We then developed a series of rules to infer the presence of an amplicon from the

gene-expression pattern by subjecting the gene-expression data to hierarchical clustering in "xcluster" (average-linkage based on a centered correlation metric). The heatmap, accompanied by gene and sample dendrograms, was graphically displayed in treeview. Expression from ~600 of the 1,430 genes formed intimate hierarchical clusters characterized by physical proximity in the human genome, corresponding to the location of the amplicons identified by array CGH. A total of 37 amplicon "signatures" consisting of at least two genes were identified. An amplicon was called "present" if a sufficient number of genes within the amplicon signature were overexpressed, i.e., with values above a gene-wise threshold in the same amplicon. Concordance analysis of ErbB2 FISH and IHC and the 17q12 binary amplicon signature derived from hierarchical clustering (80% quantile cutoff) resulted in a simple kappa coefficient of .8184, a p-value of .1573 from two-sided McNemar test, and a value of 1.613e-06 from Fisher's exact test, all at a .05 significance level. These data supported the experimental use of an expression-based amplicon binary call as a substitute for FISH test. We are currently correlating the presence of each amplicon signature with Ki67 data and clinical outcomes. Preliminary findings will be presented at the meeting.

RECIST Response	Patient Number	Percent*
CR	13	13
PR	48	48
CR+PR	61	61
SD	28	28
PD	12	12
Not evaluable	9	*excluded
Not measured	5	*excluded
Surgery 2 Bilateral	**Patient Number**	**Percent***
Mastectomy	47	45%
Lumpectomy	50	48%
Chemotherapy	7	7%
No surgery	3	*excluded
Surgery pending	1	*excluded
Not evaluable	9	*excluded

The final accrual totals were Washington University (42), Cleveland Clinic (10), University of Chicago (3), UCSF (10), UNC (15), Ireland Cancer Center (1), Duke (25), and DFCI (8). The total number accrued was 115 by 11/06/2006. The accompanying figure records clinical response data. The overall clinical response rate was 63% by WHO criteria and 61% by RECIST. 12% had PD, but 6 of these 12 cases were clinical decisions inconsistent with RECIST definitions of progression.

Keywords: letrozole; neoadjuvant; prediction

243 Effects of PEGylated Curcumin Conjugate on Pancreatic Cancer Cell Proliferation and Apoptosis

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Pancreatic carcinoma is a devastating illness with a death/incidence ratio of approximately 0.99. We have previously shown that Smad4 protein instability leads to a loss of responsiveness to TGFβ-induced growth inhibition in pancreatic cancer cells. We have also identified that Jab1 (Jun activation domain binding protein 1), integrated COP9 signalosome complex (CSN), regulates Smad4 protein instability in cells. The Jab1/CSN kinase also phosphorylates p53 and p27 for their protein degradation.

Here we show that Jab1/CSN associated kinase inhibitors, curcumin and emodin, induce apoptosis and inhibit cell proliferation in pancreatic cancer cells. Specifically, treatment with curcumin or emodin resulted in a significant elevation of Smad4 and p27 protein levels in PANC-1 and AsPC-1 cells in both Western blot and immunohistochemical analysis. As examined by MTT assays, curcumin or emodin inhibited cell growth in a dose- and time-dependent manner. BrdU analysis of proliferation yielded similar results, with the numbers of BrdU-positive cells being reduced dramatically with curcumin and emodin treatment. Furthermore, curcumin and emodin in combination with gemcitabine, resulted in a significantly enhanced inhibition of proliferation than did gemcitabine, curcumin, or emodin alone, as well as enhanced apoptosis in Annexin V staining in PANC-1 and AsPC-1 cells.

Curcumin and emodin are hydrophobic and not easy to administer systemically. We have synthesized a polyethylene glycol (PEG)-conjugated curcumin, in which curcumin is covalently linked to nonimmunogenic PEG_{35kD}. Therefore, the curcumin conjugate is a water-soluble transport form with prolonged half-life in vivo. MTT assays demonstrated that the inhibitory effect of PEG-curcumin on cell proliferation is 20 times more effective than free curcumin in PANC-1 cells. Similar effects of this conjugate were also observed in cell apoptosis assays. Moreover, immunostaining indicates that PEG-curcumin significantly elevated p27 levels, and BrdU labeling showed that this conjugate blocked cell proliferation. Most interestingly, immunostaining of PEG-curcumin-treated

PANC-1 and MiaPaCa-2 cells revealed that cells were arrested in mitotic phase and exhibited abnormal mitosis with many multinuclei cells seen. These unique features of PEG-curcumin-treated cells imply that this conjugate inhibits cell proliferation and induces cell apoptosis through interfering cell cycle progression. Thus, our results demonstrated that PEG-curcumin could represent a novel potential therapeutic treatment for pancreatic carcinoma.

Keywords: curcumin; pancreas; therapy

244 Infected Cell Carriers for Systemic Delivery of Oncolytic Measles Virus Strains in Breast Cancer Treatment

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We have previously demonstrated that engineered measles virus vaccine strains (MV) are promising candidates for oncolytic virotherapy of breast cancer. If systemic administration of oncolytic measles virus derivatives in breast cancer patients is contemplated, however, a potential problem pertains to the preexisting measles virus antibody response in cancer patients. We hypothesized that cell carriers infected with MV could allow the virus to escape neutralization in the presence of anti-measles antibodies. In in vitro experiments we demonstrated that infected carrier cell lines, including dendritic cells, endothelial cells, and the monocytoid line U-937, could effectively mediate measles virus delivery and transfer infection to breast cancer cells 20-fold to 160-fold more effectively than could free virons in the presence of neutralizing antibodies. We have established a highly reproducible breast cancer metastatic lung model in nude mice using luciferase-expressing MDA-MB-231 breast cancer cells. Chemiluminescence imaging following luciferin administration can be employed to monitor tumor growth. Systemically injected luciferase-labeled cell carriers effectively colonized mouse lungs and survived in vivo for up to 48 hours. Repeat intravenous administration of measles-infected carriers (Jurkat T cells) caused massive infection of MD-MB-231 lung metastases. Experiments to test the therapeutic efficacy of this approach in the metastatic MDA-MB-231 breast cancer model are ongoing.

Keywords: breast cancer; cell carriers; measles virus

245 Triptolide Inhibits Pancreatic Cancer Growth and Locoregional Spread by Inhibiting Hsp70 Expression

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Pancreatic cancer is the fourth leading cause of cancer death in the United States. Pancreatic cancer cells are highly resistant to apoptosis. We have shown that an increased level of Hsp70 in pancreatic cancer cells confers resistance to apoptosis and that the inhibition of Hsp70 induces apoptosis in these cells. We have evaluated the effect of triptolide (a diterpenoid triepoxide component of a Chinese herb with antitumor properties) on pancreatic cancer growth and metastasis using an orthotopic model of pancreatic cancer. Further, we have evaluated the effect of triptolide on the expression of Hsp70 in pancreatic cancer cells.

Purpose: The aims of this study are to examine the effect of triptolide on (1) pancreatic cancer cells by assessing viability, apoptosis, and Hsp70 levels and (2) pancreatic cancer growth and metastases in vivo.

Treatment (24 h)	Caspase 3 Activity (% of C)		Annexin V (% of C)	
	MiaPaCa-2	*PANC-1*	*MiaPaCa-2*	*PANC-1*
Control (C)	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Triptolide 100 nM	1078 ± 89*	507 ± 87*	194 ± 14*	198 ± 15*
Triptolide 200 nM	1282 ± 144*	801 ± 39*	247 ± 26*	306 ± 70*

*p<.05 compared to controls, n = 3, data expressed as mean ± SE.

Results: Incubating PANC-1 and MiaPaCa-2 cells with triptolide significantly reduced cell viability, but had no effect on normal duct cell viability. Triptolide induced apoptosis in the cancer cells, as evidenced by significant increases in annexin V, caspase 3 (see table), and TUNEL stain. Triptolide also decreased Hsp70 levels in both cell lines. Triptolide administered in vivo (0.2 mg/kg/day) decreased pancreatic cancer growth (tumor volume cm^3; control 1.03 ± 0.43 vs. triptolide 0.14 ± 0.05, *p<.05, n = 7, mean ± SE). In

addition, the mice treated with triptolide had minimal locoregional spread (as assessed by H&E examination by a blinded pathologist), while the control group of mice had extensive metastases in multiple organs including the spleen, liver, kidney, and small intestine.

Conclusion: Triptolide causes pancreatic cancer cell death in vitro and in vivo by inducing apoptosis, and its mechanism of action is mediated by Hsp70 inhibition. The implication of this result is that triptolide is a potential therapeutic agent that may prevent the progression and metastases of pancreatic cancer.

Keywords: apoptosis; Hsp70; triptolide

246 Hsp70 Inhibits Apoptosis in Pancreatic Cancer Cells by Two Independent Mechanisms: Stabilization of Lysosomes and Attenuation of Calcium Signaling

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Pancreatic cancer cells are resistant to apoptosis, which leads to a poor response to therapy. Studies in our laboratory have suggested that the overexpression of heat shock protein 70 (Hsp70) in pancreatic cancer cells induces resistance to apoptosis in pancreatic cancer cells by attenuating the release of cytochrome c (cyt c) from the mitochondria. However, the mechanism by which Hsp70 inhibits the release of cytochrome c is not known. Recently, we have evaluated the roles of Ca^{2+} and of the lysosomal enzymes that are the two main regulators of mitochondrial cyt c release in the apoptosis induced by downregulating Hsp70. Our initial studies have shown that downregulating Hsp70 leads to increased cytosolic Ca^{2+} and to the release of lysosomal enzymes.

Purpose: We hypothesize that Hsp70 overexpression in pancreatic cancer cells prevents apoptosis by two separate and independent mechanisms: stabilization of the lysosomes and attenuation of cytosolic Ca^{2+}. The aim of this study is to investigate the roles of lysosomes and Ca^{2+} in Hsp70 inhibition-induced apoptosis in pancreatic cancer cells.

Methods: MiaPaCa-2 or PANC-1 (pancreatic cancer cells) were pretreated with either BAPTA-AM 10 μM (an intracellular calcium chelator), with CA074me 10 μM (a cell permeable cathepsin B inhibitor), or with both at once, followed by Hsp70 inhibition via triptolide (0.2 μM) or quercetin (100 μM) for 24 hours. Cell viability was ascertained using the MTT assay. Annexin V and caspase 3 were measured as apoptotic markers.

Results: The downregulation of Hsp70 significantly decreased MiaPaCa-2 viability and induced apoptosis, which is reflected by increased annexin V (data not shown) and caspase 3 levels (see table). Treatment with BAPTA-AM or CA074me prior to Hsp70 downregulation significantly improved MiaPaCa-2 viability and reduced apoptosis, as decreased annexin V staining (data not shown) and caspase 3 activation evince (see table). Pretreatment with BAPTA AM or CA074me prior to Hsp70 downregulation increased viability and decreased apoptosis significantly, over and above that induced by BAPTA AM or CA074me alone (see table). Similar results were observed in the other pancreatic cancer cell line, PANC-1 (data not shown).

Conclusion: Lysosomal enzymes and Ca^{2+} both play independent roles in the apoptosis induced by the inhibition of Hsp70 expression.

Viability and Caspase 3 in MiaPaCa-2 (% of control, mean ± SE, n = 3, *p≤.05)

	Treatment	*Viability*	*Caspase 3*
Triptolide	alone	42.3 ± 3.5	1600.7 ± 67.7
	+ BAPTA AM	57.5 ± 2.3*	1191.2 ± 14.5*
	+ CA074me	59.0 ± 4.6*	630.0 ± 76.6*
	+ BAPTA AM + CA074me	81.1 ± 1.8*	347.7 ± 36.2*
Quercetin	alone	46.1 ± 3.3	672.0 ± 31.0
	+ BAPTA AM	63.1 ± 4.5*	464.0 ± 51.7*
	+ CA074me	59.4 ± 3.4*	412.1 ± 45.7*
	+ BAPTA AM + CA074me	81.7 ± 4.1*	256.5 ± 25.1*

Keywords: apoptosis; Hsp70; pancreas

247 Lipidic Nanoparticle Drugs Against Aggressive Breast Cancer Subtypes

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We have previously developed new approaches for breast cancer treatment using lipidic nanoparticle drugs (nanoliposomes/nLs) and immunoliposomes (ILs). Research advances included new and robust technologies for drug loading and stabilization, resulting in novel "nanoliposome" drugs, and immunoliposomes against HER2 and EGFR. These discoveries led to the translation of four novel therapeutic agents: (1) anti-HER2 immunoliposome doxorubicin (NSC701315, AP49) was GMP manufactured and licensed to industry, (2) anti-EGFR immunoliposome doxorubicin was GMP manufactured and has entered a Phase I trial at the University of Basel (Switzerland), (3) nanoliposomal CPT-11 (PEP02) completed an initial Phase I trial and continues in Phases I/II studies in Asia and Europe, and (4) nanoliposomal vinorelbine is in Phase I trials in Taiwan. We also discovered new phage library-derived monoclonal antibodies (mAbs) against subtype-specific breast cancer phenotypes, including promising scFv, that specifically bind and internalize in basal-like/basaloid cancers.

We hypothesize that lipidic nanoparticle agents can be developed against aggressive breast cancer phenotypes for which standard treatment is inadequate, especially basal-like tumors with their associated cancer stem cell characteristics. These poor-prognosis tumors, typically hormone receptor(-) and HER2(-), lack effective targeted therapies and appear to have distinct chemotherapy response profiles. To generate antibodies capable of targeting nanoparticle drugs to subtype-specific breast cancer cells, we selected cell lines representative of luminal and basal breast cancers from a panel of 55 well-characterized breast cancer cell lines. Basal and luminal subtype-specific mAbs were identified via direct selection of human phage antibody libraries against these cell lines. Phage antibodies selected upon basal-like cell lines were confirmed to be largely basal subtype-specific, with minimal binding to luminal breast cancers. When conjugated to the surface of liposomes, a number of mAb candidates mediated rapid binding and internalization of immunoliposomes in basal-like tumor cells. For antigen discovery, we used yeast display of tumor-associated antigens to identify two basal subtype-specific phage antibodies as anti-EphA2 and anti-CD44. Anti-EphA2 antibody 2D6 competed with Ephrin A1 for cell binding and inhibited the invasion of MDAMB231 cells. Dual coupling of anti-EphA2 antibody 2D6 and anti-EGFR antibody P2/4 to the surface of liposomes resulted in enhanced liposomal uptake into basal type breast cancer, suggesting a synergistic effect between EGFR and EphA2 targeting. Further studies of multiple combinations of basal type-specific phage antibodies may offer a novel approach to therapy of breast cancers associated with poor outcomes while minimizing targeting of normal tissues.

Keywords: antibodies; basal-like breast cancer; nanoparticles

248 Inhibitors of Fatty Acid Synthase in Combination With Taxanes and EGFR Inhibitors

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Inhibitors of fatty acid synthase (FAS) show significant activity as single agents for treatment of human lung cancer xenografts. However, we expect that future use of FAS inhibitors for lung cancer treatment will require combining an agent of this class with other lung cancer drugs for clinical effectiveness. Therefore, we are exploring possible implications of such combinations in preclinical investigations. For preliminary studies, we have tested combinations of C93 (a third-generation specific inhibitor of FAS) with docetaxel and with erlotinib for cytotoxicity of A549 cells in vitro. These represent two commonly used agents in lung cancer therapy—one a conventional cytotoxic drug (docetaxel), and the other (erlotinib) a targeted therapy.

Experiments to examine effectiveness of combinations administered to cultured A549 cells were designed to allow analysis using variations of the median effect method. We examined the response of A549 cultures using three different sequences of drug administration: (1) docetaxel or erlotinib treatment preceding C93, (2) simultaneous treatment with two agents, and (3) C93 treatment preceding docetaxel or erlotinib. We found that administration of docetaxel prior to, or simultaneously with, administration of C93 results in approximately additive effects. Remarkably, however, administration of docetaxel after C93 results in less than additive effects. This "inhibitory" effect of C93 on docetaxel activity was not a result of cell cycle arrest. We similarly examined combinations of C93 with erlotinib, a drug that specifically targets the EGF receptor (EGFR). Our selection of A549 cells for these studies is probably relevant to the situation of many lung cancers that have high expression of EGFR, but no

mutation of the gene, and an intermediate level of response to EGFR inhibitors. Our experiments found that administration of erlotinib following C93 resulted in additive toxicity on these cells, and more interestingly, erlotinib administered during or prior to treatment with C93 resulted in significant synergy.

These results could provide important clues regarding how these agents might be used effectively in combination therapy. Importantly, administration of C93 prior to dosing with a taxane is likely to be ineffective. However, administration of docetaxel prior to administration of C93, or simultaneous administration of these two agents, is likely to result in additive effects. Preclinical studies would support the use of taxane followed by C93, or simultaneous administration, if in vivo studies also find that the antineoplastic effects are additive and the toxicity of these two agents administered in this manner is less than additive. Even more encouraging is the finding that the effects of erlotinib treatment can be significantly enhanced by subsequent or simultaneous treatment with C93, a FAS inhibitor. This would appear to be particularly relevant to the clinical situation for a considerable portion of NSCLCs that have increased expression of EGFR and modest response to EGFR inhibitors as single agents.

Keywords: combination therapy; fatty acid synthase; lung cancer

249 Phase I Evaluation of Therapeutic, E7-Targeted DNA Vaccination in Women With HPV16+ High-Grade Cervical Dysplasia (CIN2/3)

Please see Poster Discussion Session Therapy I.

250 Preclinical Efficacy of a Campthothecin-Nano Particle Conjugate in Human Lymphoma-Bearing Mice

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First-line combination chemotherapy can result in long-term disease-free survival (DFS) in many patients with lymphoma depending on immunophenotype, genotype, and clinical prognostic factors at presentation. Patients who relapse are treated with salvage chemotherapy with either single-agent or combination therapy, and the response rates vary. Irinotecan (CPT-11) and topotecan, topoisomerase 1 analogs of campthothecin (CPT), have been used successfully in clinical trials, either alone or in combination with other cytotoxic agents as salvage treatments for patients with relapsed/refractory non-Hodgkin's lymphoma (NHL). Initial studies with CPT demonstrated significant toxicity, which led to the development of CPT-11 and topotecan. Although they are active, a disadvantage of CPT-11 and topotecan observed in early clinical trials was a high incidence of treatment-related toxicity (TRT). IT-101, a nanoparticulate conjugate of 20(S)-campthothecin and a cyclodextrin-based polymer, has recently been developed to minimize TRT and increase antitumor activity. To evaluate preclinical efficacy and toxicity of IT-101, we conducted an in vivo study in nude mice bearing subcutaneous xenografts from a human lymphoma-derived Daudi cell line, stably transfected with plasmid DNA containing firefly luciferase (ffluc) reporter gene. The tumor-derived ffluc activity was evaluated in vivo using an IVIS 100 imaging system (Xenogen); background ffluc luminescence was defined as $\leq 10^6$ p/sec/cm^2/sr. Ten days after subcutaneous tumor injection, 42 nude mice with tumor-derived ffluc activity $>10^8$ p/sec/cm^2/sr were allocated into six different treatment groups as follows: Group 1 (no therapy); Group 2 (CPT-11 100 mg/kg, IP, qwk x 3); Group 3 (IT-101 5 mg/kg, IV, qwk x 3); Group 4 (IT-101 10 mg/kg, IV, qwk x 3); Group 5 (IT-101 15 mg/kg, IV, qwk x 2); and Group 6 (IT-101 single dose of 15 mg/kg, IV). The average tumor-derived ffluc activity was equally distributed among all of the treatment groups. Tumor burden was monitored weekly by tumor-derived ffluc activity and physical tumor measurements. After completion of the treatment, there were two treatment-related deaths (TRDs; body weight loss >20%) and one non-TRD in Groups 5 and 6, respectively. At the third week after starting therapy, all treatment groups that received two or three weekly doses of IT-101 (Groups 3, 4, and 5) had 100% complete tumor regression (CTR), determined by ffluc activity ($<10^6$ p/sec/cm^2/sr), and remained tumor-free at 90 days post-therapy. In contrast, 57% (4/7) of mice in Group 2 and 83% (5/6) of mice in Group 6 had initial CTR, but all had tumor regrowth by day 69. These results demonstrate that a treatment of a single dose of IT-101 at 15 mg/kg was not sufficiently efficacious, while a treatment of two weekly doses of IT-101 at 15 mg/kg was associated with unacceptable toxicity. However, the treatment with three weekly doses of IT-101 at 5 or 10 mg/kg was well tolerated and produced superior antitumor activity against the Daudi cell line, compared to CPT-11.

These studies provide the preclinical basis for a clinical study of a cyclodextrin-based nano particle containing chemotherapy in the treatment of lymphoma, including relapsed large-cell lymphoma and mantle cell lymphoma. A Phase II trial is being developed to test the activity and toxicity of the system for delivering chemotherapy to improve the safety and efficacy of treatment.

Keywords: drug delivery; lymphoma; nanotechnology

251 Radiofrequency Ablation for the Treatment of Stage 1 Non-Small Cell Lung Cancer in High-Risk Patients

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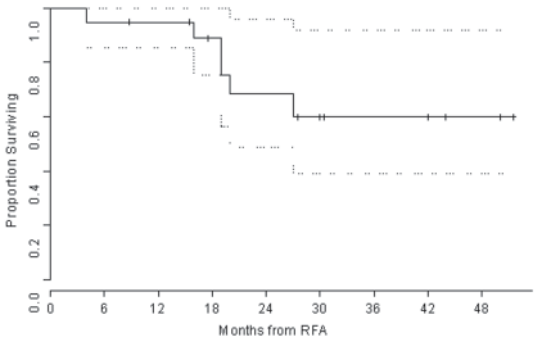
Background: Surgical resection is the standard of care for stage 1 non-small cell lung cancer (NSCLC). The objective of this study was to evaluate CT-guided radiofrequency ablation (RFA) as an alternative treatment option for high-risk patients with stage 1 NSCLC.

Methods: Medically inoperable stage 1 NSCLC patients were offered RFA. Thoracic surgeons evaluated and performed RFA under CT guidance. Response was assessed by CT and PET scan. Time to progression (TTP) and survival were monitored every 3 months.



Results: Nineteen patients with stage 1 NSCLC underwent RFA over a 3-year period. There were 8 men and 11 women with a median age of 78 years (range, 68-88). An initial complete response was observed in 2 patients (10.5%), partial response in 10 (53%), and stable disease in 5 (26%). Early progression occurred in 2 patients (10.5%). During followup, local progression occurred in eight nodules (42%) and the median TTP was 27 months. There were no procedure-related mortalities, and six deaths occurred during followup. The mean followup in the remaining patients was 29 months (9-52). The probability of survival at 1 year was estimated to be 95% (CI, .85-1.0) (see figure). The median survival was not reached.

Conclusions: Our experience indicates RFA is safe in high-risk stage 1 NSCLC patients with reasonable results in patients who are not fit for surgery.

Keywords: high-risk patients; new technology; treatment

252 Restricted, Uniform, and Constitutive Cell Surface Expression of Receptor Tyrosine Kinase-Like Orphan Receptor 1 in Chronic Lymphocytic Leukemia

Please see Poster Discussion Session Therapy I.

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

253 A Phase I Study of an Autologous GM-CSF-Secreting Cancer Vaccine for Metastatic Breast Cancer

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Background: Vaccination with tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) generates potent, specific, and long-lasting antitumor immunity in multiple tumor models. At DF/HCC, over 100 patients with melanoma, ovarian cancer, and lung cancer have been vaccinated. These studies have demonstrated that autologous vaccination can induce coordinated antitumor immune responses and have led to the identification of targets of immune-mediated tumor destruction. Here, we present interim analysis on the safety, feasibility, and biologic activity of an autologous GM-CSF-secreting breast cancer vaccine.

Methods: A total of 21 patients with metastatic breast cancer have undergone tumor procurement for vaccine from malignant pleural effusions (18), ascites (1), and tumor nodules (2), and 7 patients have received vaccine. All tumor samples were processed by the Cell Manipulation Core Facility at Dana-Farber Cancer Institute. Cells were transduced with a replication-defective adenoviral vector encoding GM-CSF and irradiated, and GM-CSF secretion was measured by ELISA. To receive vaccine, patients were required to have received at least one prior chemotherapy for metastatic disease and to have an ECOG and performance status of 0-1. Vaccine was delivered s.c. and i.d. weekly for 3 weeks and then every other week. Immune monitoring included skin biopsies of vaccine sites, measurement of leukocyte populations, and proteomic-based assessment of antibody responses.

Results: Sufficient viable tumor cell yields for vaccination were obtained from all patients, with cellular yields of 1.1-679 $\times 10^6$ cells and viabilities of 71%-100%. Dose levels were based on cellular yield, ranging from 10^5 to 10^7 cells per dose. The average yield of GM-CSF was 924 ng/10^6 cells per 24 hours (range: 24.4-5,696), higher than the average yields for lung cancer and melanoma. The seven vaccinated patients ranged in age from 49 to 66, and have received three to nine vaccines to date, with one patient having stable disease after six vaccines. Toxicities likely related to treatment include grades 1/2 local injection-site reactions (four patients), grades 1/2 inflammation at multiple tumor sites (one patient), as well as grades 1/2 fatigue, fever, nausea, and edema. Skin biopsies of vaccination sites revealed mild to moderate infiltration of lymphocytes, granulocytes, and macrophages.

Conclusion: Breast cancer cells can be harvested in sufficient number for autologous vaccine production from solid tumor as well as from malignant effusions. Autologous vaccination can induce coordinated immune responses with limited toxicity. The identification of antigen-specific immune responses following vaccination is ongoing.

Keywords: breast cancer; immunotherapy; vaccine

254 Development of Resistance to Targeted Therapy Strategies Transforms the Clinically Defined Molecular Profile Subtype of Breast Tumors

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Background: The effectiveness of therapies targeting specific pathways in breast cancer, such as estrogen receptor or HER2, are limited because many tumors manifest resistance, either de novo or acquired, during the course of treatment.

Methods: To investigate molecular mechanisms of resistance, we determined gene expression signatures in two different in vivo xenograft models of estrogen receptor-positive (ER+) breast cancer, with or without HER2 overexpression (MCF7/HER2-18 and MCF7 wt, respectively). Mice with established tumors were assigned to the following treatment groups: continued estrogen supplementation (E2), estrogen deprivation (ED), and tamoxifen (Tam), all with or without the EGFR tyrosine kinase inhibitor gefitinib (G) (to inhibit HER signaling), or the antiestrogen fulvestrant alone. E2-stimulated tumors and tumors with acquired or de novo resistance to endocrine therapies were profiled for mRNA expression using Affymetrix Genechip arrays. To assess their clinical relevance, genes arising from analysis of the xenograft tumor profile data were evaluated against public databases of clinical breast tumor gene expression.

Results: One set of genes underexpressed in endocrine-resistant tumors were estrogen inducible and associated with ER+/PR+ human breast cancers (Luminal A subtype). Another set of genes overexpressed in tumors with acquired resistance to endocrine therapy in both models represented transcriptional associated profiles of HER2 signaling and were associated with ER-/HER2+ human cancers (the ERBB2+ subtype). A third set of genes overexpressed in MCF7/HER2-18 tumors exhibiting de novo resistance to Tam were associated with ER+/PR+ human cancers but not with a group of estrogen-regulated genes identified previously from estrogen-treated cultured cells.

Discussion: In response to various endocrine therapy regimens, these xenograft breast tumors shut down estrogen signaling and activate alternative pathways such as HER2, pathways that may contribute to the resistant phenotype and may not be predictable before treatment.

Keywords: endocrine therapy resistance; expression signature; HER2

255 Convection-Enhanced Delivery of Liposome-Based Drugs for Image-Guided Brain Tumor Treatment

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We hypothesized that newly developed nanoparticle agents can be used to treat brain tumors via systemic administration or convection-enhanced delivery (CED) with MRI guidance. For systemic treatment, nanoliposomal CPT-11 is a novel liposome-based nanoparticle featuring highly stable drug encapsulation in a long circulating carrier. In the rat intracranial U87 tumor xenograft model, i.v. treatment with nanoliposomal CPT-11 produced thirteenfold higher drug exposure in tumors based on tissue AUC than did free CPT-11. Systemic treatment with nanoliposomal CPT-11 resulted in significantly improved survival, including apparent cures in some animals, as compared with free CPT-11. Based on these results, nanoliposomal CPT-11 is proceeding to Phase I clinical testing in advanced brain tumor patients.

For CED, prior studies in rodents and primates established the feasibility of MRI-based monitoring of CED of liposomal gadolinium (Gd). To evaluate this as an image-guided brain tumor treatment, we investigated the delivery, safety, and efficacy of liposome-based nanoparticles containing Gd and CPT-11 following CED into spontaneous canine gliomas. In this veterinary trial, four canine patients with biopsy-confirmed gliomas (1 astrocytoma III, 2 astrocytomas II, 1 oligodendroglioma) were infused with liposomes containing gadoteridol (1.85 mM) and CPT-11 (48.2 mg/mL). CED was monitored in real time by sequential MRI to assess localization and volume of distribution.

Successful intratumoral delivery of nanoliposomal CPT-11 was demonstrated in all animals by MRI, although significant variability was observed and appeared related to catheter placement, rate of infusion, presence of necrosis, and location in relation to ventricular and subarachnoid spaces. Infusions of up to 777 μL were feasible over 3.75 hours using one to three catheters and infusion rates up to 4 μL per minute. All infusions were limited by eventual leakage into ventricular or subarachnoid spaces within 3 hours as captured by MRI. Efficacy of this treatment was clearly observed, with decreases in tumor volume of up to 80% on MRI; also, all dogs showed improvement in clinical signs following treatment. Antitumor effects appeared to correlate with MRI-based delineation of extent and localization of infusions. Host toxicity was minimal on serial neurological evaluations. Mild and transient CSF lymphocytic pleocytosis was documented following several of the infusions. Postmortem histopathologic analysis in two dogs was correlated with in-life MRI data. Significant differences were seen between infused and noninfused tumor tissue, including presence of frank tumor necrosis and histologic evidence of treatment effect in infused areas. No tissue injury was observed in surrounding normal brain.

These studies indicate that MRI-guided CED infusion of liposome-based agents into spontaneous canine gliomas is feasible, well-tolerated, and efficacious. The findings emphasize the role of MRI monitoring of therapeutic CED infusions to optimize delivery parameters. Because CED of liposomal CPT-11 has marked antitumor activity in canine spontaneous gliomas, we conclude that this approach warrants further investigation for the treatment of human brain tumors.

Keywords: convection-enhanced delivery; image-guided; nanoparticles

256 Combination Targeted Therapy in Prostate Cancer

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Aberrant activity of signal transduction pathways plays a key role in prostate cancer (PCa), affecting both androgen receptor (AR)-dependent and AR-independent pathways. Two major pathways that have increased activity in PCa are the PI-3 kinase/Akt pathway and the p42/p44 mitogen-activated protein kinase (MAPK) pathway. Activation of these pathways in tumors can promote cell survival, proliferation, invasion, and angiogenesis. In addition, these pathways may stimulate AR expression and/or activity. PCa is an androgen-dependent disease, and the actions of androgens are mediated by AR, a ligand-activated transcription factor that plays a central role in PCa. Loss of AR activity leads to apoptosis and clinical regression of PCa in humans treated with therapies that reduce levels of circulating androgens and/or administration of AR antagonists. These therapies are a mainstay of PCa therapy, particularly in the context of locally advanced or metastatic disease. The high rate of failure of these therapies in the long term highlights the need for additional therapies targeting both AR and cell signaling pathways.

Perifosine is an alkylphospholipid that acts at the cell membrane to target signal transduction pathways, including both the Akt and p42/p44 MAPK pathways, and exhibits marked cytotoxic effects against a variety of cancer cell lines in vitro. Perifosine has mild toxicity, and a number of Phase II trials with perifosine in different tumor types are under way. We have found that perifosine has potent activity against prostate cancer cells both in vitro and in vivo. Furthermore, perifosine inhibits androgen receptor activity owing to, at least in part, its inhibition of p42/p44 MAPK. Thus, perifosine holds promise as a therapy for aggressive androgen-dependent prostate cancer.

We have carried out a neoadjuvant clinical trial of treatment with the proteasome inhibitor bortezomib (PS-341/Velcade) followed by radical prostatectomy in patients with a risk for recurrence after radical prostatectomy (i.e., clinical stage T1c or T2a with Gleason 8-10, clinical stage T2b-T2c with Gleason grade 7 and PSA >10 ng/mL, or clinical stage T3). We have found evidence of antitumor activity based on tumor histology, increased apoptosis in tumor cells, and drops in serum PSA following treatment. Based on immunohistochemical studies of the resected tissues and pretreatment biopsies and in vitro experiments, the antitumor effects of bortezomib appear to be limited by increased activation of Akt via upregulation of SRC-3, which is degraded via the proteosome. In vitro data indicate that bortezomib and perifosine may have synergistic activities against prostate cancer cells, owing to perifosine blocking Akt activation induced by bortezomib.

In summary, perifosine can target multiple key pathways in prostate cancer survival and progression, and perifosine alone or in combination with other agents, such as bortezomib or anti-androgens, is a promising therapy for aggressive, androgen-dependent prostate cancer.

Keywords: bortezomib; combination; signaling

257 Critical Role of Different Subsets of Regulatory T Cells in Suppressing Immune Responses to Prostate Cancer

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Regulatory T (Treg) cells play an important role in the maintenance of immunological self-tolerance by suppressing immune responses, thus preventing autoimmune diseases. However, such cells may also have detrimental effects on antitumor immunity. While elevated proportions of $CD4^+$ $CD25^+$ Treg cells have been demonstrated in several types of cancers, very little is known about the prevalence and subsets of Treg cells in prostate cancer. We recently generated a panel of tumor-infiltrating lymphocytes from prostate cancer. In this study, we show that high percentages of $CD4^+$ $CD25^+$ T cells are present in the majority (70%) of prostate tumor-infiltrating T lymphocytes (PTILs). Remarkably, both $CD4^+$ and $CD8^+$ T cell subpopulations possessed potent suppressive activity. T cell cloning and FACS analyses demonstrated that both $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ Treg cell clones derived from a bulk PTIL line expressed *FoxP3* and suppressed naive T cell proliferation, mainly through a cell contact-dependent mechanism. The suppressive function of Treg cells could be reversed by human Toll-like receptor 8 (TLR8) signaling, regardless of the subset of Treg cells and the suppressive mechanism involved, raising the possibility that the manipulation of Treg cell function by TLR8 ligands could improve the efficacy of immunotherapy for prostate cancer patients.

Meanwhile, we have initiated a Phase I clinical trial for prostate cancer patients using NY-ESO-1 peptides. Peptide-based vaccines can elicit antigen-specific immune responses, while treatment of TLR8-based ligands may reverse the suppressive function of Treg cells. Thus, the combination of these two strategies may improve the therapeutic potential of vaccines against cancer.

Keywords: peptide; TLR8; Treg

258 Biological Responses Following Intratumoral Injection of AdGLIPR1: Preliminary Results From a Phase I/II Neoadjuvant Gene Therapy Trial

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GLIPR1/RTVP-1 (GLIPR1) is a direct p53 target gene that encodes a member of the pathogenesis-related protein family [1,3]. The GLIPR1 gene encodes a 28-kDa protein that has a putative signal peptide sequence and a transmembrane domain suggesting extracellular functional activity. We have shown that GLIPR1 functions as a tumor suppressor through direct pro-apoptotic signaling in prostate cancer and potentially other malignancies [2,5]. Antitumor and antimetastatic effects of adenoviral vector mediated in situ GLIPR1 gene therapy and mediated locally by pro-apoptotic, antiangiogenic, and immunostimulatory activities were documented in an orthotopic mouse model of prostate cancer [6]. Mechanistic studies have shown that GLIPR1-mediated activation of $p27^{Kip1}$ and JNK activities is responsible for initiating cell growth arrest and apoptosis, respectively [5]. These preclinical studies led to an ongoing neoadjuvant Phase I/II clinical trial in which AdGLIPR1 is being tested by direct intratumoral injection prior to radical prostatectomy (IND 13033). We have enrolled and treated seven men (two completed cohorts, 1×10^{10}, 5×10^{10} vp) with clinically localized prostate cancer but high risk for recurrence into this Phase I/II clinical trial. A single intraprostatic injection was followed approximately 4 weeks later by radical prostatectomy. There was no notable toxicity associated with this experimental treatment. Pathological analysis showed tumor-specific cytopathic effects characterized by glandular and cytoplasmic dissolution and nuclear pyknosis. Some patients showed extensive changes, although the amount of affected tissues per patient was variable. Surgical specimens were embedded fully and whole mount slides were imaged and analyzed for specific immunohistochemical markers. As predicted, apoptosis was higher in the posttreatment specimens than in pretreatment biopsies. P27 is normally found in the nucleus of nonneoplastic prostatic epithelium and is lost or translocated to the cytoplasm in cancer. In the posttreatment specimens, we found that p27 levels were higher than in biopsies. More importantly, the increase was greater in nuclear p27 levels, resembling the localization of benign tissues. A systemic immune response (activated CD8+ T cells) was generated by AdGLIPR1 injection. These results suggest a tumor-specific, pro-apoptotic effect resulting from a single intraprostatic injection of low-dose AdGLIPR1.

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Keywords: AdGLIPR; gene; neoadjuvant

259 Addition of a Panel of Seven Proteomic Markers to CA125 Increases Sensitivity for Detection of Patients With Stage 1 Epithelial Ovarian Cancer

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Background: Given the prevalence of ovarian cancer, a successful strategy for early detection will require high sensitivity for early-stage disease (>90%), but even higher specificity (99.6%). The requisite specificity might be attained through a two-stage strategy that utilizes an abnormal value for serum marker(s) to prompt the performance of transvaginal sonography (TVS). To perform TVS in 2% of individuals screened, a specificity of 98% would be required. A single value of CA125 has the requisite specificity in postmenopausal subjects, but does not have adequate sensitivity for early-stage disease. Greater sensitivity might be achieved with a panel of biomarkers. The aim of this study is to validate a panel of recently reported proteomic biomarkers that can potentially distinguish individuals with ovarian cancer from healthy subjects and those with benign pelvic masses.

Methods: We analyzed preoperative serum samples from 92 patients with epithelial ovarian cancer (22 stage 1, 19 stage 2, 41 stage 3, and 10 stage 4), 40 with benign disease and 99 healthy individuals, using optimized chromatographic SELDI-TOF-MS protocols for quantitative measurements of seven proteins (transthyretin, apolipoprotein A1, transferrin, hepcidin, beta-2 microglobulin, connective-tissue activating protein 3, and inter-alpha trypsin inhibitor IV (internal fragment)). Standard curves were generated using known quantities of calibrants. Samples were processed in triplicate and randomized in 96 well bioprocessors. Using a Tecan Aquarius™ robot, samples were processed on IMAC30, H50, and Q10 ProteinChip® arrays, as appropriate for each marker. Peak intensity data were baseline subtracted and normalized using either total ion current factors or peak ratio factors.

Results: ROC curves indicated that the addition of CA125 improved the performance of the marker panel in distinguishing ovarian cancer from healthy individuals ($p = 3.6 \times 10^{-6}$), and the marker panel improved results with CA125 ($p = 4.0 \times 10^{-5}$). CA125 (>35 U/mL) alone distinguished stage 1 ovarian cancer patients from healthy women with 68% sensitivity at 98% specificity. The seven biomarker panel exhibited a sensitivity of 51%, and a combination of CA125 and the panel achieved a sensitivity of 80% at the same specificity. In addition, a combination of the marker panel with CA125 separated patients with stages 1 and 2 ovarian cancer from women with benign pelvic masses and from healthy women with 85% sensitivity at 98% specificity.

Conclusions: A combination of a seven-proteomic-marker panel and CA125 may improve detection of early-stage ovarian cancer. Larger validation studies will be required and are currently under way.

Keywords: CA125; proteomics; stage 1 epithelial ovarian cancer

260 Defining the Signals That Promote Therapeutic T Cell Responses Against Advanced Ovarian Cancers

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Background: Ovarian cancer is thought to evade immune rejection by fostering an immunosuppressive environment in ascites; however, the functional responses of ovarian cancer-specific T cells have not been directly investigated in vivo. These issues are critical to the success of our SPORE-funded Phase I clinical trial of T cell therapy for ovarian cancer (PI: Cassian Yee, Fred Hutchinson Cancer Research Center).

Methods: We developed a novel murine model to study the T cell response to ovarian cancer. An epitope-tagged version of HER2/*neu* ($Neu^{OT\text{-}I/OT\text{-}II}$) was expressed in the ovarian tumor cell line ID8. Adoptive transfer experiments were performed using naive TCR transgenic CD8+ T cells (OT-I) specific for $Neu^{OT\text{-}I/OT\text{-}II}$.

Results: After intraperitoneal injection into C57BL/6 mice, ID8 cells expressing $Neu^{OT\text{-}I/OT\text{-}II}$ gave rise to disseminated serous adenocarcinomas with extensive ascites. Upon adoptive transfer into tumor-bearing hosts, OT-I T cells underwent a striking proliferative response, reaching peak densities of ~40% and ~90% of CD8+ T cells in peripheral blood and ascites, respectively. OT-I cells infiltrated and destroyed tumor tissue, and ascites completely resolved within 10 days. By contrast, OT-I cells with a genetic deletion of the IL-2 and IL-15 receptor beta subunit (CD122) proliferated in blood but failed to accumulate in ascites or tumor tissue, or induce tumor regression. Finally, OT-I cells lacking Cbl-b, a negative regulator of signaling by the antigen receptor, demonstrated enhanced antitumor activity such that tumor regressions were achieved with a tenfold lower dose of OT-I cells.

Conclusion: Contrary to expectation, advanced ovarian cancers can support extraordinary CD8+ T cell proliferation and antitumor activity. These T cell responses are regulated positively by the IL-2/IL-15 pathway and negatively by the Cbl-b pathway. Current studies are focused on further defining the signaling mechanisms that control CD8+ T cell activity in the ovarian cancer environment. These insights will inform future clinical trials of T cell therapy for ovarian cancer.

Keywords: immunotherapy; signal transduction; T cells

261 A Phase I/II Study of Lapatinib Plus Weekly Carboplatin and Paclitaxel in Relapsed Stage 3-4 Ovarian Cancer

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Purpose: To evaluate lapatinib, an oral dual EGFR and tyrosine kinase inhibitor, in combination with weekly carboplatin and paclitaxel in relapsed stages 3-4 ovarian cancer.

Methods: The MTD in the Phase I portion of the study was found to be carboplatin AUC2 mg/M^2 and paclitaxel 60 mg/M^2 weekly × 3, then 1 week off, with 1,000 mg of lapatinib po qd continuously. Patients continue to be enrolled at this dose.

Results: Thirteen ovarian cancer patients were enrolled, three within the last month. Two were withdrawn after one cycle owing to lack of immediate response. One patient received only one cycle owing to allergic reaction to carboplatin, although she did have a dramatic decrease in ascites and CA125 level during that time. All ovarian cancer patients receiving ≥three cycles demonstrated radiographic or CA125 response. Patient 103, who is currently on cycle 17, remains NED by PET/CT.

Toxicities: Three patients developed grade 3 leukopenia. Grade 1-2 diarrhea was common but manageable. One patient had a grade 4 allergic reaction to carboplatin. Other frequent grade 2 reactions included insomnia, fatigue, stomatitis, and nausea. Continuous metronomic therapy requires C-SSF support to maintain white counts.

ID	Age	Prior Chemo Regimens	Prestudy CA125	Last CA125	Cycles Given	Radiologic Response?*
101	61	1	51	15	6	yes*
103+	53	2	1544	15	17	yes*
201	39	3	125	225	1.5	N/A
203	57	3	147	177	1.5	N/A
205	52	4	997	745	1	N/A
206	60	4	113	72	6	yes
104+	78	2	5.0	5.0	7	yes*
105+	44	5	894	4	5	yes
106	53	2	121	291	2	no
107+	69	1	9	8	3	yes
108+	81	2	117	41	3	yes
109+	52	4	21	24	2	N/A
111+	85	1	117	N/A	1	N/A

+ Remains on study.
* PR or CR confirmed by ≥2 scans per RECIST criteria.

Keywords: glioblastoma; immunotherapy; multiforme; T cell

262 Combination of Vβ Spectratyping/CD107 Degranulation Assays To Monitor Directly Ex Vivo Antitransgene T Cell Immune Responses in Lymphoma Patients Receiving Adoptive Immunotherapy

Please see Poster Discussion Session New Technologies and Methodologies II.

263 Development and In Vitro Validation of Anti-Mesothelin Biobodies That Prevent CA125/Mesothelin-Dependent Cell Attachment

Please see Poster Discussion Session New Technologies and Methodologies II.

264 A First-in-Human Clinical Trial of Adoptive Therapy Using CD19-Specific Chimeric Antigen Receptor Redirected T Cells for Recurrent/Refractory Follicular Lymphoma

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The immunotherapeutic ablation of lymphoma is a conceptually attractive treatment modality that is the subject of intense translational research activities. One approach being piloted through the City of Hope Lymphoma SPORE is the application of cellular immunotherapy employing cytolytic T lymphocyte (CTL) grafts genetically engineered for CD19-specific effector functioning. This has been achieved by the construction of a chimeric T cell antigen receptor (CAR) composed of a CD19-specific single-chain immunoglobulin variable fragment (scFv) extracellular targeting domain molecularly coupled to the CD3 complex zeta chain. Cytolytic T cells that are genetically modified to express this CAR display HLA-independent CD19-specific recognition and killing of lymphoma target cells. Using clinically applicable plasmid gene transfer/selection systems, our group has demonstrated that anti-CD19 $CAR^+/HyTK^+$ $CD8^+$ effectors can be generated from CTL precursors present in the peripheral blood and expanded to clinically relevant cell doses. We have designed an FDA-authorized (BB-IND 11411) clinical trial in which patients with recurrent/refractory follicular lymphoma undergo leukapheresis and a polyclonal CTL product derived that (1) expresses the anti-CD19 CAR, (2) kills $CD19^+$ lymphoma targets, (3) remains dependent on exogenous IL-2 for survival and proliferation, and (4) is sensitive to ganciclovir ablation by virtue of co-expression of the selection-suicide transgene HyTK. The trial is designed to assess the safety of intra-patient dose escalation beginning at a cell dose of 100×10^6 cells/m^2 and escalating to a dose of $2{,}000 \times 10^6$ cells/m^2. In addition, this trial employs rituximab to clear circulating B cells prior to the first T cell dose and fludarabine following the first T cell infusion to both induce temporary lymphopenia and limit anti-transgene rejection responses. The protocol employs low-dose subcutaneous rhuIL-2 to support the numerical and temporal persistence of transferred CTLs. Treated patients are monitored with immunologic correlative studies that include Q-PCR for tracking persistence of transferred CTLs, RT-QPCR for following the levels of $CD19^+$ B cells in the peripheral blood, bone marrow, and lymph nodes, and CT and PET imaging studies to follow disease responses. Here, we report on our initial clinical experience treating patients on this trial.

Keywords: adoptive therapy; chimeric antigen receptor; genetic engineering

265 Efficacy of Combined VEGFR/EGFR Inhibition Against Non-Small Cell Lung Cancer Xenografts With Primary and Acquired Resistance to EGFR Tyrosine Kinase Inhibitors

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EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib lead to significant tumor regressions in a subset of non-small cell lung cancer (NSCLC) patients, but the majority of patients treated do not respond (i.e., they have primary resistance) and almost all patients with an initial response eventually present with tumor progression (i.e., they have acquired resistance). Resistance to EGFR tyrosine kinase inhibitors (TKIs) has been associated with secondary mutations in the EGFR tyrosine kinase domain (i.e., T790M), mutated Kras, and VEGF overexpression, although in many cases the cause remains unknown. In this study, we investigated the impact of inhibition of the VEGF and EGFR pathways in vitro and in vivo in human NSCLC animal models that were sensitive or resistant to EGFR TKIs, using either the dual VEGFR/EGFR TKI vandetanib (ZD6474) or a combination of erlotinib and the VEGF monoclonal antibody bevacizumab. This included xenografts sensitive to EGFR TKIs (HCC827 cells), with primary resistance (A549 xenografts, bearing wild-type EGFR and mutated Kras), and three different xenograft models of acquired resistance: A549 tumors that had progressed after prolonged treatment with erlotinib; H1975 tumors, bearing EGFR exon 19 deletions in addition to a secondary T790M mutation; and HCC827 tumors that had been transfected with EGFR bearing an activating deletion and T790M mutation. In vitro vandetanib had a similar profile of activity against NSCLC cells expressing wild-type and mutated EGFR. Gefitinib did not inhibit cell lines bearing T790M EGFR mutation. In vivo erlotinib, gefitinib, and vandetanib caused sustained tumor regressions in the sensitive HCC827 animal model. In all four resistant in vivo models, however, vandetanib was more effective than erlotinib or gefitinib at inhibiting tumor growth, as was a combination of erlotinib and bevacizumab in the xenograft models tested. This enhanced activity appeared to be a result of enhanced antiangiogenic activity and not improved EGFR inhibition. These studies suggest that combined VEGF and EGFR inhibition is more effective than EGFR inhibition alone, particularly in EGFR TKI-resistant disease.

Keywords: animal model; primary and acquired resistance; VEGFR and EGFR

266 Enhancing the Replication and Antitumor Activity of Avirulent HSV-1 Oncolytic Vectors

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Oncolytic herpes simplex virus (HSV) $\gamma_1$34.5-deletion viruses ($\Delta\gamma_1$34.5) are a promising agent for treatment of malignant glioma and other tumors. The attenuating mutation not only renders the virus aneurovirulent but also limits late viral protein synthesis and efficient replication in many tumors. We sought to determine whether substitution of $\gamma_1$34.5 with human cytomegalovirus (HCMV) genes that allow late viral protein synthesis would improve HSV replication and antitumor efficacy without restoring neurovirulence. To test this hypothesis, two recombinant $\Delta\gamma_1$34.5 HSV vectors were constructed that express the HCMV TRS1 (C130) or IRS1 gene (C134). These recombinants were then tested against a $\Delta\gamma_1$34.5 recombinant in human glioma cell lines in vitro to determine whether the HSV/HCMV chimeric viruses could overcome PKR-mediated inhibition of protein translation and replicate more efficiently. These viruses were subsequently tested in vivo to determine neurovirulence and antitumor efficacy in two brain tumor models. The results show that the chimeric HSVs are safe, are more effective than $\Delta\gamma_1$34.5 virus in reducing tumor volume, and prolong survival in both xenogeneic and syngeneic tumor models. These findings suggest that selective complementation of avirulent HSV late viral protein synthesis improves the antitumor effect. Due to its high level of efficacy and low neurovirulence profile, the IRS1-expressing $\Delta\gamma_1$34.5 recombinant exhibited the most advantageous therapeutic ratio.

Keywords: brain tumor; dsRNA-activated protein kinase (PKR); oncolytic vectors

267 Separation of Graft-Versus-Leukemia From Graft-Versus-Host Disease via Modulating Dendritic Cells' Ability in Imprinting Donor T Cell Tissue Tropism

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We recently reported that donor CD8$^+$ T cells mediated graft-versus-leukemia (GVL) without causing graft-versus-host disease (GVHD) in recipients conditioned with a radiation-free anti-CD3 regimen, and the separation of GVL from GVHD was associated with confinement of donor CD8$^+$ T cells to host lympho-hematological tissues (see reference). Dendritic cells (DCs) in anatomic lymph nodes (LN) have been shown by others to imprint T cell tissue tropism, and CD103$^+$DCs in mesenteric (M) LNs regulate CD8$^+$ T cell expression of integrin $\alpha4\beta7$ and chemokine receptor CCR9 that mediate CD8$^+$ T cell entering gut tissues. Therefore, we hypothesize that anti-CD3-conditioning modulates host DCs in anatomic LNs so that their ability in induction of donor T cell expression of homing and chemokine receptors is downregulated.

To test our hypothesis, we first tested whether anti-CD3 conditioning prevents GVHD but retains GVL in recipients treated with lethal total body irradiation (TBI). Accordingly, BALB/c mice were injected with anti-CD3 mAb (20 µg/g body weight) or PBS, and 7 days after anti-CD3 conditioning, the mice were given lethal TBI. 8-12 hours after TBI, mice were IP injected with Luc$^+$/BCL1 cells (1×10^6) and were IV injected with donor spleen cells (2.5×10^6) and BM cells (2×10^6) from allogeneic C57BL/6 or syngeneic BALB/c mice. We found that although 100% of the recipients given syngeneic donor cells died of tumor growth, the anti-CD3-conditioned recipients given allogeneic donor cells eliminated tumor cells with little sign of GVHD. In contrast, the non-anti-CD3-conditioned recipients given allogeneic donor cells all died of GVHD, although they also eliminated tumor cells as judged with in vivo bioluminescent imaging (BLI). We also observed a marked reduction in donor T cell infiltration of GVHD target tissues, and a marked reduction in donor T cell expression of $\alpha4\beta7$ and CCR9 in MLN of the anti-CD3-conditioned recipients as compared to the non-anti-CD3-conditioned ones. Furthermore, we found that although donor CD8$^+$ T cells markedly upregulated $\alpha4\beta7$ and CCR9 when cocultured with host MLN DCs from recipients treated with TBI only, donor CD8$^+$ T cells did not upregulate the receptors when cocultured with host MLN DCs from recipients treated with anti-CD3 or anti-CD3 followed by TBI. In addition, we observed that anti-CD3 conditioning markedly reduced the percentage of CD103$^+$ DCs among MLN DCs. Taken together, anti-CD3 conditioning reduces the DC subsets that induce donor T cell expression of homing and chemokine receptors in the anatomic LN so that donor T cell expression of homing and chemokine receptors is downregulated and migration to GVHD target tissues is prevented.

Reference

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Keywords: dendritic cells; graft-versus-host disease; graft-versus-leukemia

268 Expanded/Activated Gamma/Delta T Cells as Immunotherapy for Glioblastoma Multiforme

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Background: Current therapies for glioblastoma multiforme (GBM) remain largely ineffective. Clearly, novel approaches are required, including cellular therapies that incorporate innate immunity. Unlike $\alpha\beta$+CD8+ cytotoxic T lymphocytes that require MHC Class I-based presentation of processed antigen for activation, $\gamma\delta$ T cells can directly recognize stress-associated antigens, many of which are expressed on GBM. Our laboratory has developed clinically approvable procedures for expansion/activation of large numbers of apoptosis-resistant $\gamma\delta$ T cells that retain cytotoxicity against GBM lines and primary tumors.

Methods: Allogeneic peripheral blood mononuclear cells were incubated for 24 hours in RPMI with 9% human serum supplemented with anti-CD2, IL-12, and IFN-γ, followed by addition of OKT-3 and IL-2. Cultures were maintained for 18 days, harvested, and depleted of CD4+ and CD8+ T cells for a final product of >80% $\gamma\delta$ T cells. In vitro cytotoxicity (CTX) was evaluated against both adherent and suspension cultures of primary GBM explants; U87, U251, and 1047 GBM cell lines; and astrocytes. In vivo activity of $\gamma\delta$ T cells was evaluated against luciferase-transduced human U251 and U87 GBM intracranial xenografts in 2 groups of 10 athymic nude mice that received intracranial injections of either 1.25×10^6 $\gamma\delta$ T cells or vehicle only.

Results: Allogeneic $\gamma\delta$ T cells readily killed U251, U87, and 1047 GBM cell lines and GBM-primary explant cultures in vitro, but spared normal astrocytes. Mice treated with $\gamma\delta$ T cells at the time of tumor placement showed significantly slower progression of U251 intracranial xenograft tumors compared to mice receiving vehicle only ($p = .008$). Survival was significantly improved in mice treated with $\gamma\delta$ T cells ($p = .007$) as determined by Kaplan-Meier analysis. Mice treated with $\gamma\delta$ T cells 1 week after establishment of U87 tumors also showed slower tumor progression as well as improved survival ($p = .002$)

Conclusion: Intracranial therapy with expanded/activated allogeneic $\gamma\delta$ T cells represents a potentially effective post-resection immunotherapeutic strategy against GBM.

This study was supported by National Cancer Institute grant P50 CA097247.

Keywords: glioblastoma multiforme; immunotherapy; T cell

269 NK Cell Activation Induced by Rituximab-Coated Target Cells Is Inhibited by the C3b Component of Complement

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Antibody-dependent cellular cytotoxicity (ADCC) and complement fixation both appear to play a role in mediating antitumor effects of monoclonal antibodies (mAbs). We evaluated the relationship between complement fixation and NK cell activation induced by mAb-coated target cells using rituximab and a human lymphoma cell line as a model. Human serum inhibited phenotypic signs of NK activation induced by mAb-coated target cells, including NK cell CD16 downmodulation and CD54 upregulation. C1q and C3 in the serum were required for these effects, while C5 was not. An antibody that stabilizes C3b on the target cell surface enhanced the inhibition of NK cell activation induced by mAb-coated target cells. Serum inhibited NK cell CD16 downmodulation induced by killed and fixed mAb-coated target cells and by mAb-coated plates in the absence of target cells. These data suggest that C3b deposition induced by mAb-coated target cells inhibits the interaction between the human IgG1 Fc and NK cell CD16, thereby limiting the ability of mAb-coated target cells to induce NK activation. Ongoing studies are exploring the impact of complement fixation on ADCC, and whether mAbs that fail to fix complement will be more effective at mediating ADCC.

This research was supported by P50 CA97274 (Iowa/Mayo Lymphoma SPORE) and a translational research grant from the Leukemia and Lymphoma Society.

Keywords: antibody; complement; mechanism

270 RNA-Loaded Dendritic Cells Targeting Cytomegalovirus and CTLA-4 Blockade in Malignant Gliomas

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Introduction: A Phase I/II clinical trial targeting the CMV integument protein pp65 in patients with newly diagnosed GBM using pp65-LAMP RNA-transfected DC has been completed. Our supportive preclinical laboratory efforts have been focused recently on reducing the impact of tumor-induced immunosuppression by systemic CTLA-4 blockade.

Methods: The clinical trial opened in February 2006 has now accrued six CMV seropositive and seven seronegative patients. Patients were treated with standard doses of temozolomide (TMZ) throughout vaccination and were randomized to receive an autologous lymphocyte transfusion (ALT) prior to vaccination. Our preclinical studies tested systemic delivery of a monoclonal antibody (9H10) to CTLA-4 in mice with well-established malignant astrocytomas. Survival and autoimmune toxicity in the form of experimental allergic encephalomyelitis (EAE) were assessed, as were $CD4^+$ T cell numbers and function in the peripheral blood, spleen, and cervical lymph nodes.

Results: No vaccine-induced toxicities were observed. Standard TMZ therapy, however, induced transient, grade 3 lymphopenia (<500 cells/μL) in 70% of patients after the first TMZ cycle with nadirs occurring 14-21 days after treatment. Regulatory T cell (T_{Reg}) ($CD4^+CD25^{++}CD45RO^+FOXP3^+$) levels increased from 5.2 ± 1.5% (3.3-7.5) to 11.8 ± 2.6% (6.9-13.8) ($p = .0004$; paired t-test) with TMZ and XRT and averaged 12.2 ± 4.0% (6.4-18.1) after the second TMZ cycle ($p = .007$). Despite these changes, we have data showing that immune responses can be successfully generated and may be enhanced in this context. Nearly complete radiographic responses were observed. Median TTP is 60.5 weeks and median survival has not been reached. There are no statistically significant differences based on serologic status ($p = .3633$) or ALT ($p = .0447$). In our preclinical studies, we found that CTLA-4 blockade conferred long-term survival on 80% of treated mice, without eliciting EAE. Tumor-induced $CD4^+$ lymphopenia was reversed and increases in T_{Reg} fraction were abrogated. $CD4^+$ T cell proliferative capacity is restored and the cervical lymph node antitumor response is enhanced. Treatment benefits are bestowed exclusively upon the $CD4^+CD25^-$ T cell population, and not T_{Regs}, as $CD4^+CD25^-$ T cells from treated mice demonstrate improved proliferative responses and resistance to T_{Reg}-mediated suppression, while T_{Regs} from the same mice remain anergic and exhibit no restriction of their suppressive capacity.

Conclusions: Chemotherapy and immunotherapy can be delivered concurrently without negating the effects of immunotherapy, and TMZ-induced lymphopenia may prove to be synergistic with tumor-specific immunotherapy. CTLA-4 blockade is a rational means of reversing glioma-induced changes to the CD4 compartment and enhancing antitumor immunity. These benefits may be synergistic with approaches that target T_{Regs}.

Keywords: antigens; brain tumors; immunotherapy

271 Therapeutically Targeting the Tumor-Specific EGFR Mutation, EGFRvIII

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Most well-characterized tumor antigens are overexpressed normal proteins that have triggered immunologic tolerance to some degree. This compromises their effectiveness as tumor rejection antigens and poses a risk of autoimmunity if they are effectively targeted. Conversely, tumor-specific antigens derived from mutations in somatic genes are less prejudiced by central tolerance and less likely to be associated with autoimmunity. Some studies also suggest that the autonomous immune response to human tumors is dominated by such neoantigens. These mutations, however, often arise randomly as a result of the genetic instability of tumors and, as such, tend to be patient-specific and incidental to the oncogenic process.

EGFRvIII, however, is a frequent and consistent tumor-specific mutation, central to the neoplastic process, that consists of an in-frame deletion of 801 base pairs from the extracellular domain of the EGFR that splits a codon and produces a novel glycine at the fusion junction. This mutation encodes a continuatively active tyrosine kinase that enhances tumorigenicity and migration and confers radiation and chemotherapeutic resistance to tumor cells. In addition, cells appear to become "addicted" to the

signaling pathways induced by EGFRvIII and die without it. The EGFRvIII mutation is most frequently seen in patients with glioblastoma multiforme (GBM), but has been found in a broad array of other common cancers. The new glycine inserted at the fusion junction of normally distant parts of the extracellular domain results in a tumor-specific epitope not found in any normal adult tissues. The exquisite tumor specificity of EGFRvIII, its clonal expression in GBMs and other common tumors, its absence in any normal tissue, and its importance in the pathobiology of tumors make EGFRvIII an ideal target for antitumor targeted therapy.

Our human in vitro studies have shown that EGFRvIII is a suitable immunologic target for inducing T cell proliferation, cytokine secretion, and specific lysis of GBM cell lines. We also have now completed two consecutive and one multi-institutional Phase II studies vaccinating with an EGFRvIII-specific peptide conjugated to KLH (PEPvIII-KLH). These studies have produced remarkably consistent and impressive extensions of median survival to >126 weeks in patients with GBM, which is significantly better than temozolomide-treated historical controls matched for treating institution, known prognostic factors, and all eligibility criteria ($p<.001$). In addition, although patients with residual disease were excluded from our Phase II studies, a number of patients with residual disease were treated in our Phase I study. All of these patients had a nearly complete radiographic response to vaccination. Furthermore, even in patients with recurrent tumors, PEPvIII-KLH vaccination appears to universally eliminate EGFRvIII-expressing tumor cells. Recurrent tumors, however, continued to express wild-type EGFR, suggesting that the PEPvIII-KLH vaccine-induced immune response is highly specific.

MR1-1 is a novel immunotoxin that also specifically targets EGFRvIII with high affinity. MR1-1 consists of a recombinant chimeric protein composed of a disulfide-stabilized variable region fragment (dsFv) of an EGFRvIII-specific monoclonal antibody and a genetically engineered form of the *Pseudomonas* exotoxin (PE) PE-38. Replacing the native binding domain of PE-38 with the EGFRvIII-specific dsFv selectively targets the toxin to tumor cells expressing EGFRvIII. MR1-1 targets EGFRvIII with high affinity ($K_d + 1.5 \times 10^{-9}$ M), as measured by surface resonance analysis, and specificity, as demonstrated by inhibition with competing unlabeled EGFRvIII-specific scFv fragments. Preclinical studies have demonstrated that MR1-1 is efficacious at a level not associated with significant toxicity. On the basis of our prior studies with similarly sized constructs, where only 0.0006% to 0.0043% of the total injected dose localized to the tumor after systemic delivery, we did not believe that significant intracerebral localization of MR1-1 (61 kD) would occur after systemic delivery. Therefore, the innovative intracerebral regional delivery technique of convection-enhanced delivery (CED) has been selected as the delivery method to evaluate this promising new compound.

Keywords: antigens; brain tumors; immunotherapy

272 Hedgehog and EGF Pathway Interaction in Pancreatic Cancer

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Interactions between oncogenic pathways are key molecular events in the initiation, progression, and maintenance of the transformed phenotype. Several studies suggest that the ability of EGF pathway, an oncogenic cascade that is activated in more than 90% of pancreatic tumors, to transform cells depends on cooperation with other oncogenic pathways. However, the potential EGF-interacting oncogenic pathways as well as the molecular mechanisms underlying this phenomenon in pancreatic cancer cells remain elusive. Therefore, further characterization of EGF signaling interactions will be crucial for the understanding of the complex and varied networks of signaling cascades that lead to pancreatic neoplastic transformation. Here we show evidence of a molecular interaction between EGF and hedgehog (HH), a novel pancreatic carcinogenic cascade. Specifically, we demonstrate the ability of EGF to activate GLI transcription factors, essential effectors of the HH oncogenic function in pancreatic cancer cells. Luciferase reporter assay in combination with genetic and pharmacological manipulations of the EGF pathway show that this cascade is able to activate GLI transcriptional activity, even in the absence of active HH signaling. Further analysis of this molecular interaction suggests that EGF-mediated activation of GLI transcription factors requires an intact Src-Ras-Raf-Mek-Erk pathway. Finally, sequence analysis identifies candidate EGF-dependent phosphorylation sites within GLI domains that are essential for their transcriptional activity, thus suggesting potential molecular mechanisms underlying the modulation of GLI function by EGF in pancreatic cancer cells. Taken together, these results provide a novel insight into the complex network involved in pancreatic carcinogenesis and present new avenues for the development of therapeutic regimens for this dismal disease.

Keywords: signaling; therapy; transformation

273 The Dual Tyrosine Kinase Inhibitor, Lapatinib, Prevents ER-Negative Breast Cancer by Suppressing the Development of Premalignant Lesions

Please see Poster Discussion Session Therapy I.

274 Activation of LMP1- and LMP2-Specific T Cells for the Immunotherapy of EBV-Positive Lymphomas With an Adenoviral Vector Encoding Full-Length LMP1 and LMP2

Please see Poster Discussion Session Therapy II.

275 Redirected T Cells to Selectively Target Mature B Cell-Derived Malignancies

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Adoptive transfer of CD19 or CD20 chimeric artificial receptors (CARs) to T lymphocytes has been proposed to treat B cell malignancies. If successful, however, the approach would likely severely impair humoral immunity. Since chronic lymphocytic leukemia (B-CLL), follicular lymphoma, and mantle cell lymphoma express surface monoclonal immunoglobulins (Igs) carrying kappa (κ) or lambda (λ) light chain, we explored whether chimeric T cells targeting these immunoglobulin chains could be used instead. This approach would selectively eliminate κ+ or λ+ clonal tumor cells, while sparing λ+ and κ+ normal B cells, respectively. We generated an anti-κ scFv from the κ-specific monoclonal antibody (hybridoma HP6053), using RT-PCR and phage display. The specific scFv was cloned in SFG-retrovirus vector in frame with the IgG1-CH2CH3 domain, CD28 endodomain, and ξ chain of the TCR complex (CAR46/CD28/ξ). T cells obtained from six normal donors were activated with OKT3/CD28 antibodies and then transduced. Seventy-one percent (range, 68%-76%) of T cells were CAR46/28/ξ^+. The cytotoxicity of transgenic T cells was tested using the ^{51}Cr release assay against several B cell malignancies (Daudi, BJAB, JEKO-1, and CLL-120 as κ^+/λ^-targets; SP53: λ^+/κ-target; RAJI, κ-/λ-target; K562). CAR46/28/ξ^+ T cells efficiently killed all κ+ tumors (44%; range, 48%-80% at 20:1 ratio) with <15% killing of SP53, RAJI, and K562 cells. Control T cells (GFP+) did not show any significant activity (<15%). In addition, T cells isolated from three patients with κ^+B-CLL were transduced with CAR46/28/ξ. They efficiently killed both autologous and allogeneic κ+B-CLL cells (55%; range, 28%-71%), but not allogeneic κ^+B-CLL cells. These transgenic T cells produced IL-2 (>2,000 pg/mL $\times$ 10^6cells) and significantly expanded (elevenfold; range, 7-16) in response to autologous tumor cells (κ^+B-CLL). No significant expansion was observed in control T cells or T cells transduced with CAR46/ξ receptor lacking the CD28 domain. To discover whether free Ig-kappa in serum competed for the chimeric receptor, we co-cultured CAR46/28/ξ^+ T cells with Daudi cells (ratio 10:1). Although the presence of plasma (1:1) reduced killing (residual activity, 25% $\pm$ 6%) in short-term cytotoxicity assays, the chimeric T cells retained the ability to eliminate all viable tumor cells by days 5-7 of co-culture, while tumor overgrowth was observed in the presence of control T cells. To explore the function of transduced cells in vivo, we used a xenograft mouse model and in vivo bioluminescence. We injected IP Daudi cells (5×10^6) expressing renilla luciferase (RL), and 5 days later, T cells (10 x 10^6) expressing both CAR46/28/ξ^+ and firefly Luciferase (FL) or T cells expressing only the FL. No exogenous cytokines were given. By day 16, FL signal was significantly enhanced in mice receiving CAR46/28/ξ^+ cells (ROI = 3.2×10^6 counts), compared to mice receiving control cells (ROI = 0.3×10^6), suggesting an in vivo expansion of CAR46/28/ξ^+ T cells. In parallel, RL signal was reduced in mice receiving CAR46/28/ξ^+ T cells (ROI = 0.6×10^6), compared to mice receiving control T cells (ROI = 1.2×10^6) suggesting control of tumor growth. Adoptive transfer of T cells targeting the appropriate Ig light chain could be a useful immunotherapy approach to treat clonal B cell malignancies.

Keywords: chimeric antigen receptor; chronic lymphocytic leukemia; lymphoma

276 Epstein-Barr Virus-Specific Cytotoxic T Lymphocytes Expressing the Anti-CD30ξ Artificial Chimeric T Cell Receptor for Immunotherapy of Hodgkin's Disease

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Adoptive transfer of Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes (EBV-CTLs) has shown that these cells expand and persist in patients with EBV+ Hodgkin's lymphoma (HD), to produce complete tumor responses. Treatment failure, however, occurs if a subpopulation of malignant cells in the tumor lacks or loses expression of EBV-antigens. Therefore we set out to determine whether we could prepare EBV-CTLs that retain the antitumor activity conferred by their native receptor while expressing a chimeric antigen receptor (CAR) specific for CD30, a molecule highly and consistently expressed on malignant Hodgkin Reed-Sternberg cells. We made a CD30CAR and were able to express it on 26% ± 11% and 22% ± 5% of EBV-CTLs generated from healthy donors and HD patients, respectively. These CD30CAR+ CTLs killed both autologous EBV+ cells through their native receptor and EBV-/CD30+ targets through their MHC-unrestricted CAR. A subpopulation of activated T cells also expressed CD30, but the CD30CAR+ CTLs did not impair cellular immune responses, which is likely because normal T cells express lower levels of the target antigen. In a xenograft model, CD30CAR+ EBV-CTLs could be costimulated by EBV-infected cells and produce antitumor effects even against EBV-/CD30+ tumors. EBV-CTLs expressing both a native and a chimeric antigen receptor therefore may have added value for treatment of HD.

Keywords: chimeric artificial receptor; Hodgkin's lymphoma; immunotherapy

277 Generation of B-CLL-Specific Cytotoxic T Lymphocytes Capable of Recognizing $CD5^+CD19^+$ Side-Population Cancer Stem Cells

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An apparent stem cell population can be identified in many normal and malignant tissues by their side-population (SP) phenotype, characterized by Hoechst dye efflux. Malignant SP cells have increased resistance to many cytotoxic drugs and may contribute to patient relapse, suggesting that therapies targeted against these cells may improve survival in cancer patients. In a previous clinical study, we observed a dramatic decrease in $CD5^+CD19^+SP^+$ B-CLL tumor following vaccination with autologous, gene-modified (CD40L/IL2) tumor cells that corresponded to an increase in B-CLL-specific $CD4^+$ and $CD8^+$ immune responses, indicating the possibility of an anti-SP immune effect. To test this hypothesis, B-CLL-specific T cells were generated from post-vaccine patient samples by coculture with CD40L activated B-CLL tumor cells in the presence of IL-7, IL-12, and IL-21. After weekly stimulations, CTL lines (n = 3), were tested against autologous and allogeneic B-CLL tumors, using IFN-γ ELISpot assays to verify specificity. Next, bulk CTL lines were tested against sorted SP and non-SP. We found a moderate increase in IFN-γ release against SP compared to non-SP. To examine this effect in greater detail, we then cloned CTL by limiting dilution, which generated 13 B-CLL-specific clones. Autologous and allogeneic SP and non-SP cells were then sorted and used as stimulators in ELISpot assays. We observed that 9 of 13 clones showed SP reactivity and that this response was 8.2-fold (±7-fold) higher than reactivity against non-SP tumor cells. None of the clones recognized allogeneic SP or non-SP. These data indicate that vaccination with gene-modified tumor cells can induce anti-SP, show T cell mediated immunity, and may be responsible for the observed decrease in SP frequency during immunization. These results also suggest that targeting cancer stem cell populations in B-CLL may be a promising approach for eliminating long-lived, chemoresistant tumor cells.

Keywords: cancer stem cell; gene modification; immunotherapy

278 RGS GAIP Interacting Protein C-Terminus as a New Target in Pancreatic Cancer Treatment

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Previously, we have shown that GIPC is highly expressed in human pancreatic adenocarcinoma and is a central protein for the stability of IGF-1R in pancreatic adenocarcinoma cell lines (see reference). The goal of this study was to prove the importance of GIPC in vivo and to evaluate possible therapeutic strategies that target this protein and its PDZ domain. In vivo effects of GIPC knockout were studied after lentiviral transduction of luciferase-expressing MiaPaCa2 pancreatic cancer cells with shRNA against GIPC. Growth characteristics were monitored with bioluminiscence. Knockdown of GIPC led to a significant inhibition of pancreatic tumor cell growth in vivo in an orthotopic and heterotopic mouse model by downregulation of IGF-1R. To test a possible therapeutic approach, the PDZ domain of GIPC was targeted by a short peptide composed of the amino acid sequence PSQSSSEA. This octapeptide was designed based on the C-terminal binding motif of GAIP. Targeting GIPC with this peptide inhibited the association between IGF-1R and GIPC. The subsequent downregulation of IGF-1R decreased proliferation in vitro and in vivo. In conclusion, our findings suggest that targeting GIPC and its PDZ domain-mediated interaction with the tyrosine kinase receptor IGF-1R could be a promising new treatment option for pancreatic cancer.

Reference

Muders MH, Dutta SK, Wang L, Lau JS, Bhattacharya R, Smyrk TC, Chari ST, Datta K, Mukhopadhyay D. Expression and regulatory role of GAIP-interacting protein GIPC in pancreatic adenocarcinoma. *Cancer Res (Priority Report)* 66(21):10264-8, 2006.

Keywords: angiogenesis; metastasis; therapeutics

279 Breast Cancer Therapeutic Agents Based on Telomerase Misfunction

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The goal is to develop and assess the translational potential of agents we have developed that force telomerase interference in breast cancer. This project focuses on exploitation for clinical use of a new strategy: to turn the action of active telomerase against the breast cancer cells. We have demonstrated that a low threshold of expression of mutant-template telomerase RNA (MT-hTer) genes in human breast cancer cells is sufficient for a potent killing and growth inhibitory effect on these cells. The telomeres that result from MT-hTer action are "toxic" to cells, inducing a robust apoptotic response. Additionally, during the previous SPORE funding period, new science arising from our research on telomerase also led to two unanticipated discoveries: first, that simply decreasing the endogenous telomerase level by ribozyme or RNA targeting methods rapidly decreased cancer potential. Specifically, we found that lowering overall telomerase diminished the metastatic potential of cancer cells in vivo, and rapidly inhibited the growth of breast and other cancer cells in vitro. Second, cell death induced by MT-hTer expression was dominant and did not require the p53 or pRb checkpoint pathways. Based on these findings, we then showed that combining the expression of MT-hTer with small interfering RNA directed against the endogenous WT-hTer of cancer cells synergistically increases the potency of the MT-hTer effects in killing cancer cells. With the overall goal of bringing this work to the clinic, our first goal is to further test and characterize the previously developed immunoliposome (ILS) constituted with HER2-targeting antibody containing the MT-hTer/anti-hTer siRNA construct ("MT-Rx" agent). In order to monitor MT-Rx efficacy we will use relevant biomarkers of response to the agent, suitable for early-stage clinical trials. Our second goal is to identify telomere/telomerase-based biomarker patterns predictive of apoptotic response to anticancer treatments and to specific MT-Rx therapy. We will identify the subsets of breast cancer that will be most responsive to existing therapies and to MT-Rx using (1) a panel of 60 breast cancer cell lines grouped by genomic and expression profiling, telomere maintenance status, and other clinically relevant characteristics and (2) patient-derived primary breast cancer cells, including stem/progenitor cell lines that target the most sensitive patient subpopulation, as identified. Our third goal is to validate and optimize the assays for biomarkers of telomerase and telomere status on tumor and biopsy specimens, with the goal of validating these assays per CLIA regulations in a CLIA-certified laboratory such that the results can be used clinically. Toward translation of MT-Rx, we will finalize the product configuration, perform initial manufacturing scaleup, and evaluate initial toxicology targeted systemic delivery of MT-Rx agent in rodent models.

Keyword: telomerase

280 Results From the Prostate Cancer Clinical Trials Consortium

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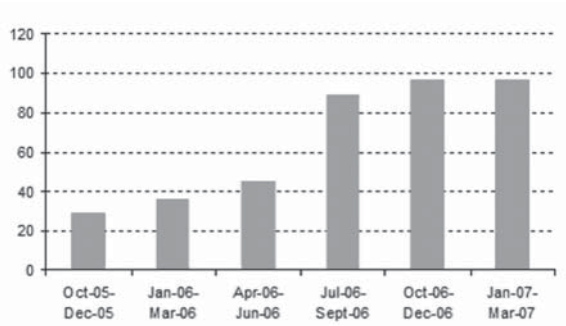
Purpose and Scope: In 2006 seven institutions with SPOREs in prostate cancer received an infrastructure award from the Department of Defense (DOD) Prostate Cancer Research Program to develop the infrastructure for the Prostate Cancer Clinical Trials Consortium (PCCTC). This 10-institution consortium emphasizes Phases II and I/II clinical trials. Memorial Sloan-Kettering Cancer Center (MSKCC) serves as the coordinating center and has focused on creating the consortium structure to expedite the design, initiation, and completion of trials through multicenter collaboration, especially trials with correlative studies to better identify patients that are most likely to benefit and with pharmacodynamic assessments to optimize dose and schedule.

Major Progress: MSKCC has developed the overall consortium structure with Web-based dissemination of new trial concepts for rapid review. We have created standard operating procedures, a PCCTC protocol template, and the PCCTC membership and master clinical trials agreements.

Results: To date 43 LOIs were submitted, and 31 were approved. Overall 366 patients have been accrued since 1/2006.

Current Status of PCCTC Clinical Trial Activity (May 2007)

Status	*N*
Protocols approved	31
Open trials	26
Open and accruing	22
Open and closed to accrual	4
Current activity	
Consortium (2 + sites)	14
Open at lead site only	8
Pending activation at lead site	5
Lead sites represented	9 (90%)
Average number of participating sites per protocol	3 (range, 2–5)
Protocols with non-consortium sites	8



Total Patient Accrual by Quarter (October 2005 to Present; Total = 395)

Significance: By creating a mechanism for the rapid design, development, and implementation of early-phase clinical trials on new agents and novel approaches, consortium researchers ensure that promising scientific initiatives are evaluated rapidly in the clinic, that results are disseminated broadly, that the drug development process is accelerated, and, ultimately, that patients with prostate cancer receive the highest level of care.

This research is supported by funds from DOD PC051382, the Prostate Cancer Research Proposal Clinical Consortium Award.

Keywords: clinical trials; infrastructure; research consortium

281 Pharmacologic Modulators of Cellular Senescence: Further Defining the Molecular Pathways Critical for Pro-Senescence Therapy in Prostate Cancer

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The irreversible cell growth arrest, cellular senescence, is emerging as an intrinsic tumor-suppressive mechanism that restricts the progression of early cancerous lesions in humans. The tumor suppressor gene PTEN is frequently mutated or deleted in primary and metastatic human prostate cancers, demonstrating its important role in both cancer initiation and progression. We have recently reported in a mouse model for prostate cancer a new type of cellular senescence, which occurs after the complete inactivation of the tumor-suppressor PTEN and significantly opposes tumorigenesis in vivo. PTEN-induced cellular senescence (PICS) occurs at early timepoints after complete PTEN inactivation, is dependent on p53 upregulation, and offers potential prognostic and therapeutic implications for prostate cancer. To better define the molecular pathways involved in PICS, potential pharmacologic modulators of senescence were evaluated in PTEN null cells translated from our previous in vivo work. We will discuss the multiple functions of PICS in tumorigenesis, define a basic molecular framework for "pro-senescence" therapy, and point to fundamental constraints for therapeutic approaches that may suppress the potential tumor cell's inherent senescence response.

Keywords: cellular senescence; prostate cancer; PTEN

Biomarkers I

065 A Tissue Biomarker Panel That Predicts Which Men With a Rising Prostate-Specific Antigen Postdefinitive Prostate Cancer Therapy Will Have Systemic Progression

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Background: The majority of men with prostate cancer are diagnosed with cancers with low mortality. Such men are treated with radical prostatectomy (RP), external beam radiotherapy, or brachytherapy and followed by serum prostate-specific antigen (PSA) evaluations. Some men with a rising PSA therapy will have local recurrence or metastasis, but many will have no other evidence of recurrent disease other than a rising PSA. The PSA doubling time has been used to determine which of these men deserve adjuvant hormonal ablation, radiation therapy, or observation. We hypothesize that additional biomarkers will predict which men with a rising PSA postdefinitive therapy would benefit from additional therapy.

Methods: We designed a custom array containing 526 RNA targets whose expression has been reported to be altered in association with prostate cancer progression. Importantly, probe targets for eight previously published expression biomarker panels associated with prostate cancer progression were included. We also included targets from Mayo Clinic prostate cancer research. Together with a second commercial array, 530 genes implicated in prostate cancer progression and 420 other cancer-related genes were evaluated. A case-control design was used to test the association of the expression results with outcome. Cases were men with a post-RP PSA recurrence followed by systemic progression within 5 years (n = 213). Controls were matched men with a post-RP PSA recurrence but no evidence of systemic progression in the next 5 years (n = 213). Median followup post-RP was 15 years. Median followup after PSA recurrence was 13 years. The case and control cohorts were split into training and validation sets to develop an RNA expression panel for the prediction of prostate cancer systemic progression.

Results: Of 426 eligible patients, paraffin blocks were available on 418 (98.1%). RNA was prepared from all 418 blocks, and both arrays were successful on 405 (96.9%) RNAs. Upon univariate analysis, 45 genes were highly significantly overexpressed or underexpressed in the cases versus controls ($1 \times 10^{-10} < p < 1 \times 10^{-5}$). Using the training set and performing random forest and logistic regression analyses, we selected 17 genes as the final RNA expression panel for the prediction of prostate cancer systemic progression. A multivariable ROC analysis using these 17 genes generated an AUC of 0.85 (95% CI, 0.81-0.90) for the training set. When relevant clinical variables were included, the training set AUC increased to 0.88 (95% CI, 0.84-0.92). When applied to the validation set, and including the clinical variables, the AUC decreased to 0.72 (95% CI, 0.63-0.81). Similar AUCs were observed when the expression data for three of the previously published panels were applied to the validation set.

Conclusions: A gene expression panel was developed using RNAs isolated from archival paraffin-embedded prostate cancer specimens with long clinical followup. This gene expression panel was significantly associated with systemic progression after PSA recurrence. Several other previously published expression panels were also able to predict prostate cancer progression. The measurement of gene expression patterns may be useful for determining which men may benefit from additional therapy after PSA recurrence.

Keywords: expression profiling; PSA recurrence; systemic progression

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

072 Multiplexed In Situ Analysis of Protein Expression (AQUA™) To Predict Breast Cancer Prognosis and Response to Trastuzumab

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HER2 belongs to a family of tyrosine kinase receptors, including EGFR, HER3, and HER4, that homo- and hetero-dimerize to activate distinct programs of proliferation, survival, migration, and angiogenesis. In breast cancer, this family also demonstrates cross-talk with the hormone receptors for estrogen (ER) and progesterone (PR). Trastuzumab is the primary and most effective antibody-based targeted therapy in advanced breast cancer, and recently evidence of its effectiveness has been extended to the

adjuvant setting. In this study, we quantitatively assessed the expression of ER, PR, and the four HER family proteins in four subcellular compartments using automated quantitative analysis (AQUA®), a novel, immunofluorescence-based quantitative method of measuring protein expression in situ with the accuracy and precision of an ELISA assay. We compared expression patterns in breast cancer specimens with patient survival in one cohort and with response to trastuzumab therapy in a second cohort.

We used unsupervised hierarchical clustering to examine the co-expression of these proteins in the archival Yale breast cohort (n = 656). Six classes were identified, including a small class enriched for HER2 expression. Disease-specific survival of this class was compared to other HER2 classification methods by Kaplan-Meier analysis. The separation of classes by survival was optimal when multiple markers were considered, as the median survival for the HER2 group dropped from 98 months in the IHC 3+ group, to 55 months by univariate AQUA HER2 and 43 months by multiplexed clustering.

Individual, pairwise, and multivariate logistic models were constructed with the data from expression of the six proteins in the BC Cancer Agency trastuzumab-treated cohort (n = 152). Prediction error estimates for dichotomized outcome (complete/partial response vs. stable/progressive disease) were assessed with leave-one-out cross-validation and the corresponding intervals with bootstrap resampling. Using continuous AQUA scores, both low estrogen receptor expression (p = .027) and high HER2/neu (p = .0007) expression were significantly associated with favorable clinical response. A multivariate model including ER, HER2, EGFR, and HER4 had a misclassification estimate of 31.8% (95% CI, 18.9-44.7), which was superior to any one protein, IHC, or FISH for prediction of trastuzumab response.

In summary, using AQUA, we found that combined analysis of ER, PR, and ErbB family members improved prognostic classification, and we developed a multivariate logistic model that optimized prediction of trastuzumab response in the metastatic setting. As trastuzumab treatment is extended to patients in the adjuvant setting, development of prediction methods such as these will be essential to accurately identifying patients who will respond favorably to treatment.

Keywords: HER2; quantitative immunofluorescence; trastuzumab

128 Genomewide and Promoter-Specific Analyses Identify Novel Targets of Aberrant DNA Methylation in Adult Acute Lymphocytic Leukemias

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This study described a genomewide analysis of DNA methylation in the promoter regions of genes using methylated CpG island amplification (MCA) coupled with representational difference analysis (RDA) and DNA promoter microarray techniques. By microarray analysis of 17,000 human promoter-associated transcripts that span all human chromosomes, several hundred genes were statistically identified as hypermethylated in acute lymphocytic leukemia (ALL) peripheral blood mononuclear cells (PBMCs). 31 novel candidate genes were further selected for pyrosequencing verification since they are also cloned by MCA/RDA strategy. 27 genes were identified as hypermethylated in 23 human leukemia cell lines but not in normal PBMCs. The methylation status of these genes was found to be differentiated leukemia cell lines from T and B lymphocytic, chronic myeloid, and other subtypes. In ALL patient samples, the frequencies of DNA methylation in the promoter regions of these genes were from 23% to 87% for MAGI1, CAST1, ADCY5, HSPA4L, OCLN, EFNA5, GFPT2, GNA14, ZNF382, and MN1, and 96% to 100% for GIPC2, RSPO1, MSX2, SALL1, and MYO5B. Expression analysis of eight selected genes (GIPC2, RSPO1, MAGI1, ADCY5, HSPA4L, OCLN, EFNA5, and GNA14) in leukemia cell lines further confirmed methylation-associated gene silencing, and treatment of methylated/silenced cell lines with 5'-aza-2'-deoxycytidine and trichostatin resulted in gene re-expression. In addition, specific chromosomal methylation hotspots and major signaling pathways were found to be associated with ALL. This overall strategy provides a powerful means to globally identify methylation-regulated, tumor-related genes in ALL and provides a panel of novel methylation biomarkers that could be useful in profiling ALL and other cancers.

Keywords: acute lymphocytic leukemia; DNA methylation; MCA/microarray

074 Validation and Streamlining of Ovarian Cancer Prognostic Profiles With a Customized Genechip

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Background/Hypothesis: A number of ovarian cancer prognostic microarray profiles have been developed using different array platforms and processing algorithms. Earlier statistical methods and limited sample sizes often resulted in large signatures, and it is unclear whether all the identified genes were necessary for prognostication. In addition, improvements in array technology also necessitate reexamining the validity of previously published signatures, before they are used for clinical tool development. We hypothesized that prospective, external validation of a customized chip, containing carefully selected prognostic gene subsets from several previously published microarray datasets, will allow (1) external, independent confirmation of these signatures using a new cohort of tumor specimens, (2) identification of an optimal subset of genes with robust performance across different studies and platforms, and (3) streamlining of this gene signature to facilitate its use as a clinical and investigational tool.

Methods/Chip Design Summary: A customized Affymetrix Chip was designed with a total of 1,200 probe sets. RNA from a new cohort of 80 advanced stage tumors from SPORE institutions is being procured for array hybridization. Three microarray datasets with clinical information from SPORE-funded programs were used totaling approximately 190 advanced-stage tumors (Boston (DF/HCC), M.D. Anderson, Duke). The following probe set groups were spotted on the custom chip: (1) previously published signatures (Ovarian Cancer Prognostic Profile [OCPP], ChemoResponse Profile-JCO 2004, 2005, Duke prognostic signature) (*Clin Canc Res*, 2005) totaling approximately 230 probe sets, (2) additional probe sets that were identified by reanalyzing the Boston dataset using more recently developed, and now recognized as optimal, signal processing algorithm (RMA) and statistical methodology more specifically directed toward survival analysis (Supervised Principal Components, Richard Simon, NCI)-400 probe sets, (3) additional probe sets identified by applying Supervised Principal Components to the M.D. Anderson dataset (approximately 300 probe sets), and (4) approximately 250 probe sets as normalization and quality controls to allow comparison of the custom array data to previously run datasets.

Preliminary Analysis: Using Supervised Principal Components, subsets of 30-70 probe sets were identified within the OCPP with virtually indistinguishable prognostic performance and very small loss of prognostic power compared to the original OCPP. Because of the risk of overfitting, it is not possible to select a final streamlined signature without the new sample cohort. In addition, analysis of the M.D. Anderson dataset identified 50-80 prognostic probe sets overlapping with the Boston dataset with comparable performance. Further analysis of these datasets is under way.

Specific Aims/Future Steps: Using the new cohort of 80 tumors from SPORE institutions in Boston, we will generate microarray expression profiles in order to (1) test the prognostic power of the previously published signatures (OCPP/CRP/Duke) without further modification, (2) test the prognostic power of the modified, reduced size, 30-70 probe-set signatures from the Boston dataset, (3) test the prognostic power of a common subset of probe sets displaying optimal performance when the three datasets are combined, and (4) compare the performance of these signatures as defined by statistical significance, accuracy, and hazard ratio. It is anticipated that these data will allow the creation of a manageable and robust gene expression signature. This signature will then be tested as a clinical tool, either by real-time RT-PCR (if fewer than 20-30 genes), or by a final tailored array (up to 100 genes).

Keywords: microarrays; review; survival

042 Clinical Classification of BRCA1 and BRCA2 Variants of Uncertain Significance

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Interpretation of results from mutation screening of the breast and ovarian cancer predisposition genes *BRCA1* and *BRCA2* is becoming an increasingly important part of clinical practice. In most cases, this is quite straightforward, but classification of rare missense variants in these genes presents a difficult problem because it is not known whether these subtle changes in the proteins alter function sufficiently to predispose cells to cancer development. As missense and intronic mutations account for approximately 43% of all known variants detected by clinical mutation screening in *BRCA1* and *BRCA2,* this has become a

significant clinical genetics issue. Clinical utility of BRCA testing would be greatly increased if such missense mutations could be reliably classified in terms of their predisposition to disease.

We report on the assessment of the clinical significance of 196 *BRCA1* and 351 *BRCA2* variants of uncertain significance identified through clinical genetic testing. The classification of these variants was achieved using a combination of genetic and functional studies. The genetic studies involved determination of the odds of cancer causality of each mutation using a combined likelihood model encompassing information on co-occurrence in *trans* phase of variants with known deleterious mutations, detailed analysis of personal and family history of cancer in variant-carrying probands, and analysis of co-segregation with disease in pedigrees. Genetic data were derived from the Myriad Genetics Laboratories database of nearly 70,000 full-sequence tests and additional families ascertained as part of an ongoing study of breast cancer families carrying missense *BRCA1* and *BRCA2* mutations at the Mayo Clinic. A total of 133 variants had odds of at least 100:1 in favor of neutrality with respect to risk, while another 43 had odds of at least 100:1 in favor of being deleterious and 12 had odds of 1,000:1 in favor of being deleterious. These data will prove useful for improved genetic counseling of patients and their families and for validation of other approaches to mutation classification.

As the genetic approach to classification is limited by availability of family data, we further characterized a series of *BRCA2* missense mutations using functional assays that measure the ability of wild-type and mutant forms of *BRCA2* to repair DNA damage by homologous recombination and to maintain the normal complement of centrioles. Analysis of eight missense mutations classified as deleterious using the genetic data showed that all of the variants disrupted homologous recombination repair. Likewise seven known neutral variants had no effect on *BRCA2* recombination activity. In addition, seven of the deleterious mutations induced centrosome amplification. Overall, both assays displayed strong correlations with the results of the genetic studies. These data suggest that it may be possible to classify missense mutations in *BRCA2* by functional assays alone in the absence of genetic data. This finding has important implications for classification of the many variants in *BRCA2* that have been found in small numbers of families.

Keywords: BRCA2; missense; testing

073 Identification of microRNAs That Modulate Sensitivity to Paclitaxel of Non-Small Cell Lung Cancer

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We are trying to determine whether microRNA expression profiles correlate with chemotherapy sensitivity/resistance in lung cancers, whether microRNAs play a functional role in drug sensitivity/resistance of lung cancer cells, and whether microRNA expression levels can be manipulated to increase drug sensitivity in cultured lung cancer cells. We have developed a panel of lung cancer and immortalized normal lung epithelial lines for which we have in vitro sensitivity profiles to drugs commonly used in the treatment of lung cancer which can show variations of >1,000-fold, as well as Affymetrix mRNA expression profiles (providing gene expression signatures for sensitivity/resistance). We profiled miRNA expression in a subset of 20 lung cancer lines and identified miRNAs that are differentially expressed between cancer and normal and between drug-sensitive and drug-resistant non-small cell lung cancers (NSCLCs). We have identified several miRNAs with expression profiles that are inversely correlated with response to drug treatment in cultured cells. We have transfected chemoresistant NSCLCs with stabilized mimics of these miRNAs and observed a 200- to 300-fold increase in sensitivity to paclitaxel. We also developed a computational method to predict interactions between miRNAs and mRNAs. On the basis of this method, we predicted that a number of genes potentially related to drug resistance are regulatory targets of miRNAs, including tubulin- and microtubule-associated protein kinases. Several of these predicted targets confer sensitivity to paclitaxel when knocked down by siRNA. These data suggest that miRNAs can be both diagnostic and modulate the response of lung cancers to chemotherapy.

Keywords: drug sensitivity; genomics; microRNA

114 A Mayo SPORE in Brain Cancer (P50 CA108961) and University of Iowa/Mayo Clinic Lymphoma SPORE (P50 CA97274) Inter-SPORE Project: Specific Chromosomal Abnormalities in Primary Central Nervous System Lymphoma Differ From Those of Systemic Diffuse Large B Cell Lymphoma, Suggesting a Distinct Pathogenesis

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The molecular pathogenesis of primary central nervous system (CNS) lymphoma (PCNSL) is largely unknown. Small preliminary studies have suggested immunoglobulin heavy chain (IGH) gene translocations play a role, particularly those involving *BCL6*. The aim of this study was to determine the incidence of IGH, BCL6, and MYC gene rearrangements in PCNSL affecting immunocompetent patients.

The cohort comprised 44 cases of PCNSL affecting patients without clinical evidence of immunosuppression (including HIV infection) whose lymphomas were diagnosed and treated at Mayo Clinic between 1992 and 2006. All cases were classified as diffuse large B cell lymphoma (DLBCL) according to the World Health Organization classification and were confirmed as confined to the CNS. Interphase fluorescence in situ hybridization (FISH) was performed using a two-color IGH-BCL6 dual-fusion probe and a two-color MYC breakapart (BAP) probe on thin sections of paraffin-embedded tumor samples. Two-color BCL6 or IGH BAP FISH probes were also used in cases showing extra FISH signals without fusion using the IGH-BCL6 probe.

Sufficient tumor cells for FISH analysis using the IGH-BCL6 and MYC probes were available in 43 and 39 cases, respectively. *IGH-BCL6* fusion was present in six (14%) cases, one of which also had a separated *MYC* signal. Three (7%) cases showed three intact BCL6 signals using both the IGH-BCL6 and BCL6 BAP probes, indicating trisomy 3. One (2%) showed three intact IGH signals using both the IGH-BCL6 and IGH BAP probes, indicating trisomy 14. The remaining 33 (77%) cases lacked abnormalities involving *IGH*, *BCL6, or MYC*.

In this study, *IGH-BCL6* fusion was present in 14% of cases, one of which also had a translocation involving *MYC* and an unknown partner gene. All other cases lacked translocations involving *IGH*, *BCL6,* and *MYC*. The frequency of *IGH-BCL6* translocations in PCNSL is less than that seen in systemic DLBCL. However, other translocations involving *IGH* that are seen in systemic DLBCL, such as *IGH-BCL2* and *IGH-MYC*, are rare to absent in PCNSL. These data suggest that PCNSL has a distinct pathogenesis from systemic DLBCL.

Keywords: cytogenetics; IGH-BCL6; PCNSL

159 Heterogeneity of Breast Cancer Metastases: A Rapid Autopsy Study Using Matched Primary-Metastases Tissue Microarrays

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Background: A comprehensive comparison of biomarker expression between a patient's primary breast carcinoma (PBC) and metastases (metastatic breast carcinomas [MBCs]), as well as between different MBCs from different sites of the same patient, has not been performed.

Design: We performed 10 rapid autopsies (post mortem intervals, 1-4 hours) on consenting patients who died of MBC. Paraffin tissue blocks from the patients archived PBC and multiple different MBCs were used to construct single patient TMAs. Ten TMA slides containing PBCs and in total 655 spots derived from 120 MBC sites were immunohistochemically labeled for the following: estrogen receptor (ER), progesterone receptor (PR), HER2/neu, E-cadherin, fascin, EGFR, C-Met, Cox-2, CK 5/6, and mesothelin. Methylation of the *RASSF1a*, *HIN1*, *Cyclin D2*, *Twist*, *ER*, *E-cadherin*, and *RAR*β gene promoters was assessed quantitatively on dissected PBC and MBC samples.

Results: Extensive heterogeneity was observed between PBCs and their paired MBCs, as well as among multiple MBCs from the same patient. The patterns observed are summarized into three categories: (1) Markers Downregulated Uniformly in All Metastases of a Case: ER, PR. Four cases were ER-PR- and three cases were ER+PR+ in the PBC and all MBC. However, one ER+PR+ PBC was ER-PR- in all its MBC, one ER+PR- PBC was ER-PR- in all its MBC, and one ER+PR+ PBC was ER+PR- in all its

MBC. (2) Markers Consistently Expressed Between Primary and Metastasis. Fascin, implicated in the lung metastasis gene expression signature of PBC (see reference), was overexpressed in the PBC and all MBC in one of eight cases. Interestingly, this was the only case with bulky lung metastasis. Promoter hypermethylation of *RASSF1a*, *HIN1*, *Cyclin D2*, *Twist*, *ER*, *E-cadherin*, and *RAR*β was overall very similar in the PBC and all MBC in all seven evaluable cases. (3) Markers Variably Expressed among Metastases. E-cadherin was variably downregulated in the MBC of one case; the E-cadherin positive invasive ductal PBC gave rise to both E-cadherin positive ductal MBC and E-cadherin negative MBC with lobular morphology. Variable overexpression in MBC compared to the PBC was observed for Cox-2 (five cases), EGFR (four cases), C-met (two cases), and mesothelin (one case). No case strongly overexpressed HER2/neu by IHC, but two cases showed variable expression ranging from negative to weakly positive (2+) in different MBCs. In one case, variable low-level HER2/neu amplification was found. Interestingly, EGFR and Met overexpression were restricted to ER-/PR- cases that were CK5/6+ and HER2/neu negative, and hence qualify as basal-type cancers.

Conclusions: Therapeutic targets identified in the PBCs or even some MBCs may not reflect targets present in all metastatic sites. Overexpression of receptor tyrosine kinases in metastases of basal-type triple-negative MBC offers a potential therapeutic target.

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Reference

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Keywords: metastasis; methylation; tyrosine kinases

Biomarkers II

053 New-Onset Diabetes Precedes Onset of Symptoms and Diagnosis of Pancreatic Cancer: Implications for Early Diagnosis of Pancreatic Cancer

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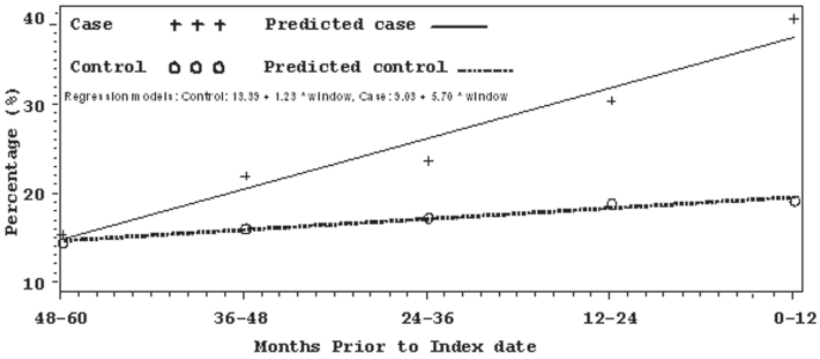
Background and Aims: The value of pancreatic cancer (PaC)-associated new-onset diabetes mellitus (DM) (PaCDM) as a marker of PaC needs further study. Our aims were to compare the overall prevalence and duration of DM in subjects with and without PaC.

Methods: PaC (n = 1,172) subjects living within 120 miles of Rochester and seen at our institution between 1/15/1981 and 7/9/2004 were identified from the Mayo Diagnostic Index. Subjects without PaC (n = 2,141) were identified from among Olmsted County residents seen at the Clinic and matched with PaC subjects for gender, age at index year, and year of registration at Mayo. We excluded from analyses 436 subjects with PaC and 266 without PaC who had no fasting blood glucose (FBG) values in the medical records. From the medical records, the following data were abstracted: All FBG values grouped into 12 monthly



Time	48-60 months	36-48 months	24-36 months	12-24 months	0-12 months
Window	1	2	3	4	5
Cases (n)	157	160	165	168	685
Controls (n)	891	1027	1071	1172	1162
P-value	0.76	0.06	0.04	0.001	<0.0001

Observed and Predicted Prevalence of Diabetes at 12-Month Time Intervals Prior to Index Date in Cases vs. Controls

time intervals up to 60 months prior to index date, height and weight at each FBG, parental history of DM, and smoking history (ever/never). Diabetes was defined as any FBG ≥126 mg/dL or treatment with antidiabetic medications. The duration of cancer-related symptoms (abdominal pain, back pain, anorexia, weight loss, and jaundice) and duration of first symptom were noted.

Results: The proportion of subjects meeting criteria for DM at any time in the 60 months prior to index date was higher in PaC than in subjects without PaC (40.2% vs. 19.2%, p<.0001). Compared to subjects without PaC, a greater proportion of subjects with PaC met criteria for DM in the 0 to -12 (p<.001), -12 to -24 (p<.001), and -24 to -36 (p = .04) month time intervals, while it was similar in the -36 to -48 and -60 to -48 month time intervals. Duration of DM was <24 months in a greater proportion of PaC than non-cancer subjects (52.3% vs. 32.6%, p<.0001). The first cancer-related symptom occurred a median of 2 months (range, 0-36 months) before diagnosis.

Conclusions: PaC is associated with a very high (40%) prevalence of DM, which is frequently new onset. PaCDM precedes onset of symptoms and diagnosis of PaC by up to 24 months. Identification of a specific mediator of PaCDM may allow screening for asymptomatic PaC in new-onset DM.

Keywords: diabetes; early diagnosis; pancreatic cancer

112 Protein Expression Profiling To Determine Potential Markers of Therapeutic Response in Breast Cancer

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The current management of high-risk, operable breast cancer includes the use of taxanes. The decision to use taxane-based treatment is made using clinical parameters such as age, size, grade, and lymph node status. Because the treatment benefits only a small minority of patients, it is important to identify predictive factors of taxane response. We sought to identify protein markers that predict response to paclitaxel treatment in breast cancer patients by analyzing pretreatment biopsies in the neoadjuvant setting and correlating the data to status of response at the time of surgery. Patients with high-risk, operable stages 2/3 breast cancer were treated with three cycles of paclitaxel 175 mg/m^2, followed by twice weekly paclitaxel 30 mg/m^2 and concurrent radiation. Patients underwent definitive surgery 4 weeks later. Pretreatment core biopsies were analyzed for proteomic profiles using histology-directed MALDI-TOF mass spectrometry to determine proteins that are differentially expressed between nonresponding and pathological complete response (pCR) patients. The high resolution of this technique allows for the enrichment of tumor and stroma protein expression profiling. Mass spectra from each matrix droplet were baseline-corrected, normalized, and aligned using ProTS-Marker. Multiple spectra from an individual patient were averaged, and candidate features were extracted from the average spectrum. Significance analysis of microarrays (SAM) and linear mixed effect model were used to identify potential features that were significantly correlated with the clinical outcomes of nonresponding and pCR patients. The false discovery rate (FDR) was controlled. In the profiling comparison of invasive tumor tissue (n = 21, 15 nonresponding patients and 6 pCR patients), five features (m/z = 5677, 6955, 7007, 7769, and 14015) were identified as underexpressed by at least a twofold change in patients with pCR. Particularly, one feature (m/z = 7769) was identified as overexpressed by a sevenfold change in patients (nonresponders) with progressive disease. Interestingly, none of the six patients with pCR exhibited this feature in their MS spectra. In comparison, three features (m/z = 3371, 3442, and 3485) were identified as overexpressed in both stroma and tumor by at least thirtyfold change in patients with pCR. These features have previously been identified by mass spectrometry as being alpha-defensins 1-3. Parallel microarray and immunohistochemical analyses confirmed defensin expression in the tumor specimens. Defensins play a role in the innate immune defense against a number of infectious inflammatory diseases, including epithelial cancers. Interestingly, all pCR patients had no evidence of disease recurrence at their last clinical followup, suggesting the importance of identifying predictive markers of response.

Keywords: defensin; histology-directed MALDI-TOF; taxane

121 Functional Proteomic Profiling of AML Predicts Response, Relapse, and Clinical Outcome

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Proteins and, more importantly, protein function regulate the phenotypic characteristics of the hallmarks of cancer. Thus, a functional proteomic classification of tumors has the potential to provide important information for prognosis and prediction of response to therapy. In order to develop a useful proteomic-based classification of acute myeloid leukemia (AML), we performed reverse-phase protein arrays (RPPA) on 266 blood and marrow-derived samples from newly diagnosed AML patient samples assayed for 52 total and phospho-proteins from apoptosis, cell cycle, and signal transduction pathways. Protein expression in matched samples from blood and marrow were similar for 44 proteins, and, after scaling, the fold changes for the other 8 proteins were only minimal, suggesting that the functional proteomics of cells in the marrow and periphery were similar. For the proteins assessed, protein expression patterns were independent of clinical characteristics. Twenty-three proteins were significantly different between FAB subtypes, and those proteins formed distinct profiles for each FAB group. Protein expression patterns in tumors with unfavorable cytogenetic changes involving -5, -7, alone or in combination, were similar, suggesting mechanistic commonalities despite unique cytogenetic abnormalities. Supervised clustering according to cytogenetics and Flt3 status yielded distinct expression patterns harboring clues to disease biology. Principal component analysis defined seven protein signature groups (signatures) that correlated with likelihood of remission, time to relapse, or overall survival that were distinct from prognostic information from cytogenetics. In conclusion, the protein signatures correlate with known morphological features (FAB), cytogenetics, and outcome and, following confirmation in independent studies, may also provide pathophysiological insights facilitating triage of patients to emerging targeted therapies.

Keywords: clinical; proteomic; relapse

016 Distinct Classes of Chromosomal Rearrangements Create Oncogenic ETS Gene Fusions in Prostate Cancer

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Recently we identified recurrent gene fusions involving the 5′ untranslated region of the androgen-regulated gene TMPRSS2 and the ETS family genes, ERG, ETV1, or ETV4, in a majority of prostate cancers. Here we explore the mechanism of ETS family overexpression in prostate tumors. Remarkably, we identified novel 5′ fusion partners in prostate tumors with outlier expression of ETS family members, including untranslated regions from a prostate-specific androgen-induced gene and endogenous retroviral element, a prostate-specific androgen-repressed gene, and a strongly expressed housekeeping gene. As the commonality of these rearrangements is the aberrant overexpression of ETS genes, we recapitulated this event in vitro. We demonstrate that ETS overexpression in multiple benign prostate cells induces a marked increase in invasion, confirming the role of ETS gene rearrangements in prostate cancer development. Identification of distinct classes of ETS gene rearrangements demonstrates that dormant oncogenes can be activated in prostate cancer by juxtaposition to tissue-specific or ubiquitously active genomic loci. Subversion of active genomic regulatory elements may serve as a more generalized mechanism for carcinoma development. Furthermore, the identification of androgen-repressed and insensitive 5′ fusion partners has important implications for the antiandrogen treatment of advanced prostate cancer.

Keywords: ETS family; gene fusion; translocations

087 PSA Dynamics Do Not Improve the Predictive Accuracy of a Single Prostate-Specific Antigen Measurement in Either Long-Term Prediction of Prostate Cancer Diagnosis or Outcome From Radical Prostatectomy

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Background: It has been proposed that prostate-specific antigen (PSA) dynamics can predict both risk of being diagnosed with prostate cancer and disease outcome in patients diagnosed and treated for prostate cancer. However, published definitions for PSA velocity (PSAV) and doubling time (PSADT) vary.

Objective: We initially tested whether adding total PSA velocity (tPSAV) to a predictive model using a single tPSA (tPSA) value improves prediction of risk of prostate cancer diagnosed about two decades later in men with no prior history of PSA testing. We then assessed whether various pretreatment PSA dynamic definitions could predict outcome after radical prostatectomy (RP) and compared which definition best predicted outcome.

Methods: Part 1: A large, representative population-based cohort of men ages 33-50 years participated (74% rate) during 1974-86 in a study of risk factors for cardiovascular, hypertensive, and metabolic disease in Malmö, Sweden. A total of 5,722 men agreed to give two blood samples about 6 years apart. We measured free (fPSA) and total PSA (tPSA) in archived plasma samples from 4,907 participants. Prostate cancer was subsequently diagnosed in 443 men (9%).

Part 2: The cohort included 3,155 patients with two or more PSA values prior to treatment by RP at MSKCC, 468 of whom developed BCR. We examined 13 definitions for PSADT and 9 for PSAV. Univariate Cox proportional hazards regression was used in part 1 to evaluate tPSA and tPSAV as predictors of prostate cancer diagnosis, and in part 2 to evaluate the association between each definition and biochemical recurrence (BCR; PSA ≥0.1ng/mL and rising) following RP. Predictive accuracy was assessed by the concordance index (c-index).

Findings: Part 1: median time from second blood draw to cancer diagnosis was 16 years (range, 1.7-22.0). Median followup for men without cancer was 21 years. In univariate models, both tPSA level at the second assessment and tPSAV were associated with prostate cancer ($p<.001$), and tPSAV was highly correlated with tPSA level ($r = 0.93$). Twenty-year probabilities of cancer diagnosis for men at the 50th, 90th, and 95th percentiles of tPSA and tPSAV were 10.6%, 17.1%, and 21.2%, vs. 9.1%, 11.8%, and 14.1%. The c-index for tPSA level was 0.771. Adding tPSAV or fPSA velocity did not importantly increase predictive accuracy (c-index 0.771 for tPSA+tPSAV). Adding tPSAV also failed to increase accuracy of predicting advanced prostate cancer at diagnosis.

Part 2: On individual analysis, most PSAV but only two PSADT definitions were associated with BCR ($p>.02$). For 12 of 22 definitions, comparison was not possible owing to limited numbers of patients for whom the definition could be calculated, because PSA value inclusion criteria varied between definitions. For the remaining 10 definitions, there were 2,016 patients, with 196 developing BCR. Median followup for patients without recurrence was 2.2 years. On univariate analysis, one of five definitions of PSAV, and no PSADT definitions, was significantly associated with BCR ($p = .02$). The c-index for PSA level was 0.735. After adjustment for pretreatment PSA, no PSA dynamics definition enhanced predictive accuracy when compared to pretreatment PSA alone.

Conclusion: Our data, based on a large, representative population, show that although tPSAV is significantly increased in men diagnosed with prostate cancer up to two decades before diagnosis, it does not aid long-term prediction of cancer diagnosis. Furthermore, pretreatment PSA dynamics does not appear to improve the predictive accuracy of a single pretreatment PSA value. There is no evidence to suggest that PSA dynamics should replace a single PSA value alone in clinical practice.

Keywords: dynamics; prediction; PSA

110 Disseminated Tumor Cells in Prostate Cancer: Implications for Systemic Progression and Tumor Dormancy

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Introduction and Objectives: We have previously reported our refined technique for isolating disseminated tumor cells (DTCs) from the bone marrow (BM) of patients with prostate cancer (PCa). By obtaining BM aspirates in patients with PCa at varying stages in their disease course, we sought to investigate the significance of DTCs in individuals with this malignancy.

Materials and Methods: Since 2002, we have obtained 931 BM aspirates from 826 patients with PCa at the time of radical prostatectomy (RP) and/or during followup. BM was also obtained from healthy men with PSA <4.0 ng/mL as controls. We used density gradient centrifugation with Ficoll-Hypaque to obtain enriched mononuclear and DTC cell populations. This was followed by magnetic bead fractionation initially with antibodies to CD45 and CD61 (negative selection) and then with antibodies to human epithelial antigen (HEA; positive selection). The DTCs were stained with FITC-labeled anti-HEA antibodies (BerEp4), viewed under ultraviolet light, and isolated with a micropipette. Genomic DNA from these cells was amplified by ligation-mediated PCR and used for array comparative genomic hybridization (aCGH).

Results: DTCs were detected in 74% (395/537) of patients prior to RP. There was no correlation with stage, Gleason grade, PSA, or tumor volume. Of 34 controls, 3 (8.8%) had DTCs present. In patients post-RP and without evidence of disease, DTCs were present in 56/98 (57%). Of these patients, 14 have recurred to date and 12/14 (86%) had DTCs present while still NED. Presence of DTCs in patients NED post-RP was an independent predictor of disease recurrence (HR 6.9; CI, 1.03-45.9). DTC samples from 54 patients pre-RP were subjected to aCGH analysis: All had low-level (1-3) genomic aberrations. Additionally, DTC samples from six patients with metastases were similarly analyzed. All had numerous, more pronounced genomic aberrations, which included those commonly deviant in PCa, i.e., 8p loss and 8q gain.

Conclusions: Nearly three-quarters of men undergoing RP had DTCs detected in their BM prior to surgery, suggesting that these cells escape early in the disease course. These early DTCs had low-level copy-number changes, suggesting that they are tumor cells. Though pre-operative DTC status does not correlate with pathologic risk factors, persistence of DTCs after RP in NED patients was an independent predictor of recurrence. Additionally, the high rate of detection of DTCs in NED patients (some >10 years out from surgery) suggests that these may be dormant tumor cells. In advanced-stage patients, DTCs had pronounced genetic changes, implying an evolution in genomic aberrations. Further work with aCGH as well as cDNA microarrays is ongoing to characterize these cells on a molecular level.

Keywords: dissemination; dormancy; metastasis

084 Evidence From Transgenic Mouse Models, Preclinical Studies, and In Silico Data Analysis Point to a Role for HOXB7 in Tamoxifen Resistance and Poor Disease Outcome

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Microarray analysis of invasive breast carcinomas and distant metastasis showed that HOXB7 is expressed at high levels in about 50% of invasive breast carcinomas. Our previous work had also shown that overexpresssion of HOXB7 in breast cancer cells promotes cell proliferation, induces epithelial-mesenchymal transition, and promotes tumor invasion [1]. To study the function of HOXB7 in vivo, we generated a MMTV-HOXB7 transgenic mouse model. By crossing MMTV-HOXB7- with MMTV-HER2/neu-transgenic mice, we found that HOXB7 plays a dual role in HER2-induced tumorigenesis: a delay in tumor onset and lower multiplicity, but promotion of tumor growth rate and metastasis. Proteomic and genomic analysis showed that HOXB7 may negatively regulate HER2-signaling pathways but activate ER-signaling pathways. Overexpression of HOXB7 converted the HER2-induced tumor from ER-negative phenotype to ER-positive phenotype. In keeping with their ER-negative status, the HER2/neu mammary tumor cell lines were tamoxifen- and fulvestrant-resistant. HOXB7xHER2/neu mammary tumor cells remained resistant to tamoxifen but were susceptible to fulvestrant. Very much reflecting these findings, our preclinical data derived from cell culture and xenograft studies showed that HOXB7 expression in the ER-positive human breast cancer cell line MCF7 is associated with tamoxifen resistance. But, like the double transgenic mouse mammary tumor cells, MCF-7/HOXB7 cells retained

their responsiveness to fulvestrant. Molecular analysis revealed that HOXB7 could play a critical role in the regulation of, and interaction among, various growth-factor pathways associated with endocrine resistance. Further, analysis of published microarray data [2] showed that high levels of HOXB7 predict a poor outcome in the HER2-positive (p = .046), but not in the HER2-negative subset of breast cancers. In particular, high HOXB7 expression levels strongly correlated with poor survival outcome (p = .002) in patients with HER2+/ER+ breast cancer. This observation has potential clinical implications: It is known that the subset of patients with HER2+/ER+ disease, on average, are less likely to benefit from endocrine therapy. Our data suggest that HOXB7 may serve as a prognostic marker within the ER+/HER2+ subset, which could potentially predict for endocrine responsiveness.

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Keywords: HOXB7; progression; transgenic

082 A Systems Approach to Marker-Guided Therapy

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Therapeutics agents designed to counter specific genomic or transcriptional abnormalities have improved standard of care for cancer patients. This has stimulated development of over 400 new therapeutic agents that are now being evaluated in Phases II and III clinical trials. Unfortunately most of these drugs will fail to gain FDA approval even after extensive clinical evaluation. This is partly due to the fact that only a fraction of patients selected to have the target aberration will respond to treatment. This is, in part, due to the wide spectrum of accumulated genomic, biological, and epigenomic abnormalities found in individual breast cancers. We present here a model system and computational approach for the discovery of predictive biomarkers that can be used to identify and select patients most likely to respond to specific therapeutics. Our approach evaluates drug responses in a panel of 50 breast cancer cell lines that mirror the recurrent genomic and transcriptional abnormalities found in primary tumors as well as the biological variability defining tumor subtypes. Prior detailed molecular analysis (DNA, RNA, and protein profiles) of these cell lines enables direct association to be established between drug sensitivity and biomarkers of response. The strength of this approach lies in a focused assessment of drug sensitivities in a single indication of cancer, and a robust computational method for defining biomarkers from small datasets.

Responses for both targeted therapeutics and chemotherapeutics are presented. We show that for rationally designed drugs targeting HER2, MEK, and Akt, the resultant biomarkers are frequently associated with the target biology. In addition we identified remarkable subtype specificity for a number of the compounds. For example, basal tumors are resistant to therapeutics targeting the PI3K pathway, but sensitive to a PLK inhibitor. In contrast luminal tumors are resistant to MEK inhibition, but luminal tumors harboring PIK3CA or PTEN mutations are particularly sensitive to inhibition of Akt (in contrast to p110δ-inhibition by LY294002). Subsequent studies will validate these candidate biomarkers in clinical samples. These observations set the stage to systematically identify drugs that target tumor subtypes with few therapeutic options as well as improve outcomes across all patients. By employing such predictors to prescreen patients prior to receiving therapy in the clinic, it may become possible to reach the goal of individualized therapeutics.

Keywords: automation; predictive markers; systems biology

068 Comparison of Sample Preparation and Solubilization Techniques for Shotgun Proteomic Analysis of Laser Capture Microdissected Cells From Human Breast Tumors

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Background: Proteomic evaluation of clinical samples has enormous potential for deciphering the complex molecular events underlying neoplastic transformation. The development of assays for the analysis of tissue is often confounded by the presence of multiple cell types, small size of most biopsies, and focal presence of tumor. The use of laser capture microdissection (LCM) allows for isolation of nearly pure cell populations, enhancing specificity of subsequent analyses. Unfortunately, the number of cells typically dissected by LCM limits proteome coverage to primarily abundant proteins, severely compromising biomarker discovery achievable by standard proteomic platforms. We present an improved technique for facilitating sample cleanup and analysis of LCM-acquired cells utilizing a short-stack SDS-PAGE and tandem mass spectrometry.

Methods: Human invasive mammary carcinoma cells were LCM-acquired (Arcturus Pix Cell II) from a frozen tissue section. Proteins were isolated by boiling the thermoplastic membrane in SDS loading buffer and run 2 cm into a 10%-20% Tricine gel followed by in-gel trypsin digestion and peptide extraction. The tryptic peptides were analyzed by reversed-phase (RP) liquid chromatography-tandem mass spectrometry (LC-MS-MS) on a Thermo LTQ ion trap instrument. Tryptic peptides resulting from 500 to 800 cells were analyzed for all single RP separations. A strong cation exchange (SCX) chromatography fractionation step was performed on lysates consisting of 5,000 to 8,000 cells prior to the RP separation in attempts to decrease the complexity of the peptide mixture and increase the number of protein and peptide identifications. Data were searched against the International Protein Index (IPI) human database using SEQUEST and proteins were required to be identified by two or more unique peptides. False discovery rates were estimated using reverse database searching. The SDS-PAGE/in-gel digestion method with a single RP separation was then compared to in-solution digests with 0.2% PPS and 50% trifluoroethanol (TFE). In order to evaluate the relationship between the number of LCM-acquired cells and the number of identified proteins, the SDS-PAGE/in-gel digestion method with a single RP separation was used to analyze cells from 1, 2, and 4 caps.

Results: One-dimensional RP analysis of the LCM digests identified 1,333 peptides and 267 proteins after the SDS-PAGE/in-gel digestion. SDS-PAGE/in-gel digestion combined with SCX fractionation identified 4,906 peptides and 828 proteins. When the three different preparation methods were compared using a one-dimensional LC-MS-MS analysis, SDS-PAGE/in-gel digestion identified an average of 1,372 peptides and 272 proteins, PPS digestion identified an average of 825 peptides and 190 proteins, and the TFE method identified an average of 903 peptides and 185 proteins. A total of 169 proteins were common to the 3 methods and the SDS method identified the most unique proteins (more than twofold) compared to the other methods. The number of identified peptides and proteins using the SDS-PAGE/in-gel digestion method and a single RP separation was not significantly increased by combining cell equivalents from either 2 or 4 caps. False discovery rates for these data at both the protein and peptide levels were below 1%.

Discussion: These results demonstrate that a surprisingly large number of proteins can be identified in shotgun analyses of lysates prepared from as few as 500 LCM-acquired cells and highlight the potential feasibility of this approach for in-depth proteomic analysis of human tumor samples. Based on function, the identified proteins cover a wide range of biological functions as well as distribution within cells. The SDS-PAGE/in-gel digestion method was superior to PPS and TFE; however, identification of unique proteins by each method indicates that several sample preparation methods may still be required for the most extensive protein coverage. Addition of an SCX separation to LC-MS-MS analysis increases identifiable proteins and peptides threefold to fivefold. Although an increase in the number of identifications was not seen with increasing numbers of cells in the single RP run, this is likely due to a saturation of the number of detectable peptides in the system. We expect to see increased identifications with increasing cell numbers with the addition of an SCX separation to LC-MS-MS analysis.

Keywords: LC-MS-MS; prognosis; proteomics

092 Automated, Whole-Slide-Based Multiplexed Molecular Marker Assessment in Formalin-Fixed, Paraffin-Embedded Tissues

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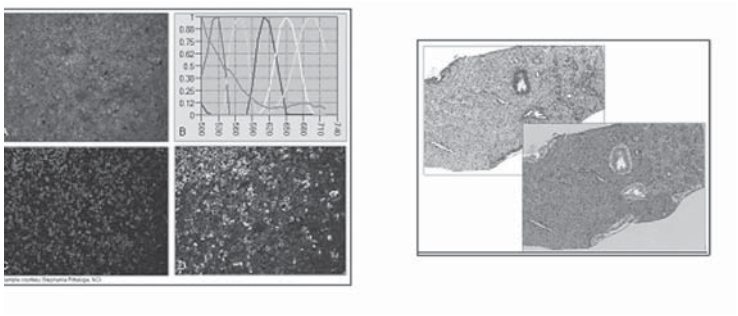
The purpose of this work is to provide reliable, cellularly resolved molecular pathway assessment in cancer biopsies to assist pharmaceutical drug development and provision of patient-specific prognosis and therapy guidance ("personalized medicine"). The organizing theme is that the appropriate unit of analysis should be the individual cell, as opposed to averaged tumor extracts that are typically used for genomic, gene expression, and proteomics analysis. To this end, we have combined multispectral imaging techniques with automated slide scanning and powerful tissue segmentation-based software. Spectral imaging and advanced image analysis tools permit multitarget immunohistochemical (IHC) and/or immunofluorescence (IF) detection, at the cellular and subcellular levels in intact, formalin-fixed, paraffin-embedded (FFPE) tissue sections. CRI-developed image processing and machine-learning tools provide automation and hands-off quantitation options and can be combined with multiplexed staining protocols that yield independent, potentially stoichiometric labeling.

Multispectral imaging, using either modulation of the light source to provide wavelength-specific illuminants, or detection of emitted or transmitted light using a liquid-crystal tunable filter-based system was used to resolve multiple molecular markers in FFPE tissues. Co-expression of nuclear markers, such as estrogen receptor (ER) and progesterone receptor (PR), using chromogenic stains, is easily quantified and can be combined with membrane markers and counterstain in the same sample. In fluorescence, we have been able to image five quantum-dot labeled antigens and a DAPI nuclear stain using a single spectrally resolved acquisition (requiring no switching of filter wheels or filter cubes). Autofluorescence, which degrades sensitivity and clarity, can be spectrally removed, providing dramatic improvements in signal-to-background for low-abundance targets.



Whole-slide scanning with multispectral capabilities has been developed at CRI, permitting automated slide handling and imaging. The final piece of the puzzle is the ability to detect appropriate regions for extraction and quantitation of molecular data. For example, ER and PR scores are usually of interest only from cancer deposits, and data of normal breast epithelium and stroma should be excluded. We have developed a machine-learning, neural net-based capability that can be readily trained to segment tissues into appropriate classes. We have combined this with nuclear segmentation tools and can therefore extract molecular data from appropriate nuclei. Preliminary comparisons with human scoring (so far of single molecular markers) indicate high concordance. Future work will include investigation of coordinated subcellular location of "pathway" molecules, with simultaneous assessment of cell-surface receptors (e.g., EGFR, VEGF, HER2/neu), downstream signaling proteins and phosphoproteins (e.g., pAKT, pERK), nuclear proteins (e.g., ER, Ki67), and novel players such as protein-folding mediators (e.g., BIP1).

The sum of these parts is a system that can scan whole slides, isolate regions containing only cancer cells, spectrally unmix multiple molecular markers, and provide quantitative metrics, including cell-by-cell colocalization indices. This combination of capabilities should prove to be of value for tissue-based molecular investigations for basic research, preclinical studies, and, ultimately, patient-based molecular medicine in the clinic.

Keywords: imaging; markers; pathology

058 Genomewide Approach to Identifying Genetic Variants That Contribute to Chemotherapy-Induced Cytotoxicity

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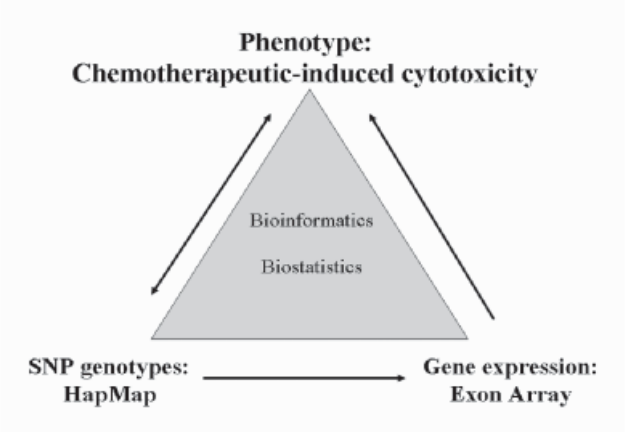

The multigenic nature of sensitivity to drugs has limited the success of candidate gene approaches. We present a genomewide model utilizing human lymphoblastoid cell lines from the International HapMap Consortium, of which extensive genotypic information is available, to identify genetic variants contributing to chemotherapeutic agent-induced cytotoxicity. Our model integrates genotype, gene expression, and sensitivity of HapMap cell lines to chemotherapeutic drugs. Cell lines derived from 30 trios of European descent (CEU) and 30 trios of African descent (YRI) were utilized. Cell growth inhibition at increasing concentrations of chemotherapeutic agents (etoposide, daunorubicin, cisplatin, or carboplatin) for 48 or 72 hours was determined using the alamarBlue® assay. Gene expression on 176 HapMap cell lines (87 CEU and 89 YRI) was determined using the Affymetrix GeneChip® Human Exon 1.0 ST Array. SNP genotype and chemotherapeutic IC_{50} were linked through whole genome association in cell lines from combined and independent CEU and YRI populations. A second association test was performed between SNP genotype and gene expression. Additionally, linear regression was utilized to evaluate the correlation between gene expression and drug IC_{50} (concentration required to inhibit 50% cell growth). We identified 2-54 genetic variants that significantly were associated with cytotoxicity induced by these four chemotherapeutic agents through differences in gene expression in the combined and separate CEU and YRI populations. A multivariate model indicated that these limited numbers of genetic variants derived from >2 million filtered SNPs from the human genome explained approximately 20%-65% of the observed human variation in cellular sensitivity to chemotherapy. In addition, we have identified genetic variants that may facilitate our understanding of underlying genetic cause for observed population differences in response to chemotherapeutic agents. This novel, unbiased method can be used to elucidate genetic variants contributing to a wide range of cellular phenotypes induced by chemotherapeutic agents.

Keywords: gene expression; genomewide association; pharmacogenomics

171 Establishment and Molecular Characterization of Mouse Xenografts of Organ-Confined Human Prostate Tumors

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As a result of prostate-specific antigen (PSA) screening, most adenocarcinomas now appear confined to the prostate at presentation. At the same time, traditional histopathologic parameters used to guide clinical decision-making are beginning to lose their discriminatory power. The amount of tissue available from primary human tumors is insufficient to carry out the appropriate molecular analyses essential to subclassifying prostate cancer according to biologic behavior. In recent years considerable efforts have therefore been devoted to generating clinically relevant models of prostate tumors. While a number of cell lines and in vivo models reflecting advanced disease have been generated, to date, no models of primary human prostate cancer (PCa) are available. We proposed to generate orthotopic as well as subrenal capsule murine xenograft of primary localized human prostate tumors and to genetically characterize them through their lifespan.

Twenty-eight fresh human prostate samples from patients undergoing radical prostatectomy for clinically localized PCa (Gleason score, 6-8) were used for injection/implantation in mice. Two mm^3 of tumor (as well as normal) tissue was either shortly digested with collagenase (n = 5) to obtain a cell suspension to be injected mixed with matrigel in the mouse prostate, or implanted

directly both under the renal capsule (n = 21) and orthotopically in the anterior lobe of the prostate (n = 2) of nude mice supplemented with testosterone pellets.

Mice were sacrificed 3 months after implantation. While no tumors grew either after collagenase treatment or orthotopic implantation, 13 of 21 (62%) cases grafted under the renal capsule developed malignant xenografts, showing a phenotype consistent with the original human tumors by histopathology and immunohistochemistry (PSA, androgen receptor, alpha-methylacyl-CoA racemase, p63). Only normal prostate tissue was found in three cases while three additional xenografts underwent regression showing calcifications surrounded by fibrotic stroma. Both high (Gleason, 6-7; n = 8) or low (Gleason, 8-9; n = 5) grade human tumors grew as xenografts.

PSA serum levels were above baseline only in mice bearing viable human prostate tissues. Tumor-bearing mice in general expressed higher PSA levels (5.7 ± 2.08) than those in which normal human prostatic tissue grew (1.47 ± 0.9) while PSA dropped to undetectable levels during regression. Finally, PSA levels increased proportionally to the growth of the xenograft.

Genetic characterization included array comparative genomic hybridization (aCGH) analysis in laser microdissected tumor xenografts and in the original tumors. In addition, both parental tumors and xenografts have been characterized by FISH analysis for the presence of TMPRSS-ETS gene fusions.

In conclusion, this mouse xenograft model, capable of mimicking the morphology and genetic- and micro-environment of primary, organ-confined human tumors could provide an important tool in preclinical trials—drug validation as well as biomarkers discovery.

Keywords: genomics; mouse xenografts; organ-confined prostate cancer

147 High Cancer-Specific Expression of an Overexpressed Biomarker Is Attributable to an Upstream Enhancer

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Constitutively upregulated activities have been central to the biology of several tumor-suppressor genes and oncogenes, but finding them has often been serendipitous. Identification of genes with cancer-specific overexpression offers the potential for efficiently discovering such events in an unbiased manner after which a pathway can be interrogated biochemically. We apply this paradigm to studying an overexpressed cancer-specific biomarker. Such markers are a nearly ubiquitous and diagnostically and therapeutically useful characteristic of many cancer types. We identified an upstream enhancer, termed CanScript, that strongly activated transcription from an otherwise weak tissue-nonspecific promoter and functioned selectively in cells with aberrant cancer-specific marker overexpression. Subsequent functional analysis demonstrated the enhancer consisted of two functionally distinct DNA elements. This enhancer and others that might be identified by this technology represent optimal candidates for cancer-specific targeting. They are thus potentially specific and suitable for the vast majority of human cancers, unlike prior enhancer elements identified through studies of noncancer models.

Keyword: gene therapy

006 Urine Cytology for Prostate Cancer Detection: Immunocytostains of Urine Sediment in the Era of New Prostate-Specific Biomarkers

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Background: Patients with negative initial biopsies represent a serious clinical dilemma because we currently lack effective means for differentiating men who are at high risk for harboring cancer from those unlikely to have cancer. A definitive diagnosis of prostate cancer occurs when a pathologist finds cancer cells in a prostate needle biopsy. The decision to perform a biopsy (of which >1,000,000 are performed annually in the United States alone) is driven by abnormal findings on a digital rectal exam (DRE) or a prostate-specific antigen (PSA) blood test, leading to a suspicion of prostate cancer. Unfortunately, these indicators lack specificity for prostate cancer; thus numerous unnecessary biopsies are performed. The biopsy procedure itself has suboptimal

sensitivity for detecting cancer, leading to many missed cancers. A test that would improve upon our current methods for cancer detection is therefore highly desirable and would be of significant clinical benefit. Prostate cancer markers are shed into urine after prostatic manipulation, suggesting that whole prostate cells might also be detectable and useful for diagnosis. However, identification of prostate cancer cells by traditional cytology has been hampered by relatively low proportions of prostate cancer cells present, and by high false-negative rates. Considering recent progress in identifying new molecular markers of prostate cancer, plus significant advances in antibody and in situ nucleic acid detection methods, it may be productive to revisit the cell-based approach to prostate cancer detection in urine samples.

Design: We sought to optimize methods for sample collection and cell preservation and to find an optimal combination of antibodies plus telomere FISH for identification of prostate cancer cells in urine specimens. We used nuclear Nkx3.1 antibody staining as a marker of prostatic epithelial cells and a combination of punctate cytoplasmic alpha-methylacyl CoA racemase (AMACR) positivity, enlarged and/or multiple nucleoli (delineated by antinucleolin antibody), and short telomeres as markers of prostate cancer cells. For these optimization studies, LNCaP (prostate cancer) and TR4 (bladder cancer) cells were spiked into human urine, isolated, cytospun onto glass slides, and stained. In addition, a small number of post-biopsy urine samples were evaluated.

Results: LNCaP cells were successfully visualized by the staining of NKx3.1, AMACR, nucleolin, and telomeres. The nuclei of LNCaP cells were stained by anti-NKX3.1 antibodies, and their cytoplasm was stained by anti-AMACR antibodies. Large or multiple nucleoli of LNCaP cells were stained by anti-nucleolin antibodies. The LNCAP cells had shorter telomeres than did normal cells by FISH. In normal cells from urine, telomeres were longer than those of the malignant cells, the nucleolin staining was small and scant, and the AMACR and Nkx3.1 stains were negative. Importantly, similar results were obtained from post-biopsy urine specimens collected in the clinic.

Conclusion: Multiply staining for AMACR, NKx3.1, nucleolin, DAPI, and telomeres has the potential to diagnose prostate cancer cells from voided urine. Our data demonstrate that prostate cancer cells, when spiked into urine samples, can be isolated intact and detected by a reproducible, multistaining, cytology protocol and suggest that we may be able to use a similar protocol for studies of voided urine cytology from men being screened for prostate cancer.

Keywords: diagnosis; multiplex fluorescence imaging; urine cytology

020 Multislice High Spectral and Spatial Resolution Magnetic Resonance Imaging of Entire Human Breast

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Introduction: Early and accurate diagnosis of breast cancer greatly improves survival and reduces morbidity. Conventional magnetic resonance imaging (MRI) has little spectral resolution and represents each spatial location or voxel using a single complex number. To improve breast imaging, we have introduced the use of high spectral and spatial resolution (HiSS) MRI. Echo-planar spectroscopic imaging and high resolution are used to acquire detailed water and fat spectra in each voxel at the spatial resolution of conventional imaging or greater. The central Fourier component of the water resonance—the peak of the water spectrum—can be used to produce images with excellent anatomic detail and contrast and complete fat suppression. Previous work demonstrated quantitatively that HiSS water peak height images are significantly improved relative to conventional fat-saturated images and that HiSS reliably detects small tumors in the absence of contrast injection. However, HiSS datasets are extremely large and acquisition times are long. As a result, we previously acquired images from only two to three slices through suspicious lesions. Here we demonstrate the application of a sagittal HiSS sequence through the entire breast in 9-15 minutes, using conventional echo-planar spectroscopic imaging (EPSI).

Methods: 30 women were scanned after informed consent was obtained, using high-resolution EPSI on Philips 1.5T Achieva scanner. (1.00-1.25 mm spatial resolution in 3 mm thick axial slices, 11 Hz spectral resolution). To generate pure water peak height images, the fat peak in each small (3-4.5 mm^3) voxel was fitted using a Lorentzian function, and the fitted peak was then subtracted to leave a spectrum of water only. Maximum intensity projections (MIP) images were generated from individual water peak height images.

Results: Acquisition of HiSS images with modest spectral resolution allowed coverage of most of the breast tissue—in some cases, the entire breast—in 9-15 minutes of scan time. This increase in scan time was well tolerated by the patients. Excellent fat suppression resulted in MIP images showing parenchyma against a dark background. Lesions were visible before contrast

injection. Background suppression and high dynamic range resulted in improved detail and higher contrast, as compared to the standard clinical images. The moderately reduced spectral resolution needed to cover a large volume of tissue sometimes obscures the fine structure of the water resonance, but earlier work shows that most of the image contrast is preserved.

Conclusions: HiSS images can be acquired at modest spectral resolution to allow coverage of the entire breast. Further optimization of data acquisition by improved k-space sampling, reducing the spectral bandwidth, and/or using parallel imaging will lead to faster imaging. In the clinical setting, a full-breast HiSS multislice scan could be obtained prior to contrast agent injection to evaluate lesion morphology in the absence of contrast media. In addition, the location of suspicious lesions on HiSS images could be used to design the subsequent DCEMRI scan. Additional postcontrast HiSS scans could provide more information concerning contrast media distribution. HiSS could substitute for conventional spin echo and/or fat-saturated images, and, as a result, the increase in scan time required for HiSS would be modest.

Keywords: detection; diagnosis; magnetic resonance imaging

094 Arterial Spin Labeling MR Perfusion Provides Radiologic-Pathologic Correlation for Monitoring Responses to Antiangiogenic Therapy in Renal Cell Cancer Animal Models

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Purpose: To determine whether arterial spin labeling (ASL) MR imaging can detect early changes in therapeutic response to antiangiogenic therapy in an animal tumor model.

Methods and Materials: Three human renal cell carcinoma (RCC) xenographs were implanted in 28 nude mice, including Caki-1 (sorafenib resistant, n = 6), A498 (sorafenib sensitive, n = 6), and 786-0 (early response followed by rapid development of resistance by day 5, n = 15). Animals received 80 mg/kg of sorafenib qd beginning at 12 mm diameter. ASL imaging (2 mm slice thickness at greatest axial diameter) was performed at baseline and day 14, with additional timepoints performed for 786-0 at days 3-9. ASL values were analyzed quantitatively (i.e., using mean blood flow values) and qualitatively (i.e., comparing spatial serial blood flow changes in the generated perfusion maps) and were compared to the histopathologic reference standard for viability.

Results: Baseline flow was 67.4 ± 15.2 (mL/100 mg/min) forA498, 78.1 ± 29.6 for 786-0, and 10.3 ± 9.5 for Caki-1. Treated Caki-1 showed no significant change (15.6 ± 7.5) in blood flow on day 14, whereas blood flow decreased variably in all treated A498 (39.3 ± 22.6, 41.7% mean decrease). 786-0 showed decreased mean blood flow on day 3 (22.5 ± 10.2, 71.2% decrease), with no significant further alterations in mean blood flow observed thereafter (days 5-14, 10.9 ± 15.5 to 31.8 ± 28.8, $p>.10$). Qualitatively, no significant change in ASL signal or pathologic appearance was seen after treatment for Caki-1. A498 on day 14 and 786-0 on days 5-9 had central loss of ASL signal, with patchy return of signal beyond day 9 in 786-0. Although blood flow change was variable, its extent corresponded tightly to histopathologic appearance ($r^2>0.7$). High signal regions correlated with viable tumor, with zones of absent or diminished signal (i.e., less than 2 mL/100 mg/min) representing necrosis.

Conclusion: ASL imaging provides important information regarding tumor viability in RCC lines that respond to sorafenib, as manifested by tight radiologic pathologic correlation to induced intratumoral necrosis. Low perfusion might predict lack of responsiveness to antiangiogenic therapy. If further characterized and validated, ASL may provide additional useful information upon which to base therapeutic decisions for patients with RCC.

Keywords: angiogenic therapy; perfusion imaging; resistance

New Technologies and Methodologies II

009 Noninvasive Imaging Metrics To Profile Treatment Response in Models of Colorectal Cancer

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Noninvasive imaging has considerable potential to visualize and quantify relevant molecular events associated with disease initiation, progression, and response to treatment. Toward translational imaging of treatment response in patients with colorectal cancer (CRC), we are developing and implementing synergistic imaging probes and methodologies that facilitate comprehensive characterization of tissue physiology. Our overall approach capitalizes upon the advantages of multimodality imaging, combining complementary optical, nuclear, and magnetic resonance imaging (MRI) techniques to yield a collection of research tools spanning multiple imaging scales. With such capability, we aim to employ multidimensional imaging to profile the microenvironment of tumors at the cellular, subcellular, and molecular levels. For example, we have developed a number of targeted optical molecular imaging reagents aimed at profiling molecular events relevant to CRC, including expression of EGFR, angiogenesis, and apoptosis. To our collection of optical probes, we are now adding complementary MRI methods that characterize tumor vascularity and perfusion at the near cellular level, including dynamic contrast enhanced (DCE) MRI and MR angiography. Furthermore, to these imaging metrics, we are implementing assessments of glucose metabolism and tumor cell proliferation using the positron emission tomography (PET) tracers [^{18}F]-FDG and [^{18}F]-FLT. We envision that fusion of these unique multidimensional imaging techniques and reporters will enable a rigorously thorough, yet noninvasive assessment of disease physiology. Within the preclinical setting, concerted use of these imaging metrics may significantly improve our ability to discover and optimize experimental therapeutic regimes that can be subsequently advanced to clinical practice. Additionally, we also envision advancing the most promising of imaging metrics to the clinic for patient stratification, as well as individualized efficacy monitoring and treatment optimization in patients with CRC.

Keywords: imaging; noninvasive; response

077 Adenovirus-Mediated Gene Expression Imaging To Directly Detect Sentinel Lymph Node Metastasis of Prostate Cancer

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Background: Prostate cancer is the second leading cause of death in men in the United States, and pelvic lymph node metastasis is one of the most reliable indicators of poor patient prognosis. Despite the importance of lymph node metastasis, there are currently no reliable noninvasive techniques available to detect small nodal lesions. In the present report we utilized a prostate-restricted, PSA-promoter-based adenoviral vector to express firefly luciferase (AdTSTA-FL) or herpes virus thymidine kinase (enhanced variant sr39tk) gene (AdTSTA-sr39tk) [1,2]. We and others have demonstrated the preferential distribution of adenovirus in the lymphatic circulation [3]. This study exploits the prostate-restricted imaging reporter adenoviral vectors and their preferential lymphotropic properties and applies them toward the specific detection of nodal metastasis of prostate cancer by bioluminescence and positron emission tomography (PET) imaging.

Materials and Methods: The PSA- and androgen receptor-positive LAPC-9 human prostate xenograft was induced to metastasize to regional lymph nodes by overexpression of lymphatic endothelial growth factor, VEGF-C. Tumors were implanted subcutaneously (on the right shoulder) or intraprostatically in SCID/beige mice. Lymph node metastasis was targeted by subcutaneous injection of AdTSTA-FL in the lymphatic rich forepaw (for subcutaneous upper back implants) or hindpaw (for intraprostatic implants). Bioluminescent signals were monitored over time beginning at 2 days postinjection. In addition, PET imaging of herpes simplex virus thymidine kinase (sr39tk) expression was also investigated.

Results: In the subcutaneous LAPC-9/VEGF-C tumor model, AdTSTA-FL-mediated bioluminescent signal was detected 3 days after virus injection only in the brachial and axillary lymph nodes, ipsilateral to the implanted tumor, where metastatic lesions were present. Conversely, no signal could be detected in lymph nodes contralateral to the tumor implantation site or in non-tumor-bearing animals. Similarly, bioluminescence was detected in inguinal and peri-aortic lymph nodes of mice bearing tumors in the prostate. Metastatic lesions in regional lymph nodes were readily detected by AdTSTA-sr39tk-mediated PET imaging by using ^{18}F-FHBG as tracer.

Conclusion: Prostate-specific adenovirus vectors can enter lymphatic circulation and transport to the sentinel lymph nodes (SLNs). Transduction of metastatic tumor cells present in regional lymph nodes and the subsequent expression of imaging reporter gene lead to specific diagnostic imaging signals. These findings suggest that the prostate-targeted adenoviral vector can be an effective diagnostic imaging agent to detect SLN metastasis of prostate cancer and improved patient care.

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Keywords: imaging; lymphangiogenesis; metastasis

113 Dual-Labeled Trastuzumab-Based Imaging Agent for the Detection of Human Epidermal Growth Factor Receptor-2 (HER2) Overexpression in Breast Cancer

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Introduction: Recently, we demonstrated the ability to use microdose traces of near-infrared (NIR) fluorophore to map lymphatics in breast cancer patients noninvasively with optical imaging. In this work, we seek to develop an imaging agent for detecting cancer-positive lymph nodes in a translational study involving HER2-positive cancer patients. We exploited the extracellular binding property of Herceptin® (trastuzumab), a clinically therapeutic anti-HER2 monoclonal antibody, to design a diagnostic imaging agent that is dual-labeled with Indium-111, a gamma emitter, and a near-infrared fluorescent dye, IRDye® 800CW, to detect HER2 overexpression in breast cancer cells in a preclinical model prior to clinical translation.

Methods: Fluorescence and confocal microscopy were used to determine the molecular specificity of $(DTPA)_n$-trastuzumab-$(IRDye800)_m$ in vitro in SKBr3 (HER2+) and MDA-MB-231 (HER2-) breast cancer cells. SKBr3 cells were incubated with $(DTPA)_n$-trastuzumab-$(IRDye800)_m$ or IRDye® 800CW, or were pretreated with trastuzumab or human IgG followed by $(DTPA)_n$-trastuzumab-$(IRDye800)_m$, and were examined under a fluorescence microscope. For in vivo characterization, athymic nude mice bearing HER2-overexpressing SKBr3-luc subcutaneous (SC) xenografts were injected with $(^{111}In\text{-}DTPA)_n$-trastuzumab-$(IRDye800)_m$ intravenously (IV), and imaged using SPECT and near infrared (NIR) fluorescence imaging at 48 hours. Tumor-bearing mice were also injected IV with trastuzumab 24 hours before following up with $(^{111}In\text{-}DTPA)_n$-trastuzumab-$(IRDye800)_m$. Nonspecific uptake in the SKBr3-luc tumors was analyzed by injecting the mice with IRDye® 800CW and $(^{111}In\text{-}DTPA)_p$-IgG-$(IRDye800)_q$.

Results: $(DTPA)_n$-trastuzumab-$(IRDye800)_m$ binds significantly higher to SKBr3 cells compared to MDA-MB-231 cells. Confocal imaging revealed that this binding occurs predominantly around the cell membrane. Competitive binding studies using excess trastuzumab prior to incubation with $(DTPA)_n$-trastuzumab-$(IRDye800)_m$ abolished this binding affinity, while pretreatment with nonspecific IgG did not alter binding. In vivo nuclear and optical imaging of SKBr3-luc xenografts injected with $(DTPA)_n$-trastuzumab-$(IRDye800)_m$ showed significant uptake in the tumor region compared to the contralateral muscle region. The tumor-to-muscle ratio (TMR) decreased in mice pretreated with trastuzumab, and was further reduced in mice injected with IRDye® 800CW and $(^{111}In\text{-}DTPA)_p$-IgG-$(IRDye800)_q$. Ex vivo imaging of dissected organs confirmed this result. Co-registering histological H&E stains with autoradiography signals from tumor and muscle tissue slices indicates that $(^{111}In\text{-}DTPA)_n$-trastuzumab-$(IRDye800)_m$ binds only to tumor tissue and not nonspecifically to muscle. Finally, we show that $(^{111}In\text{-}DTPA)_n$-trastuzumab-$(IRDye800)_m$ clears through normal lymph nodes.

Conclusion: Dual-labeled $(^{111}In\text{-}DTPA)_n$-trastuzumab-$(IRDye800)_m$ may be an effective diagnostic biomarker capable of tracking HER2 overexpression in breast cancer patients using both optical and nuclear imaging techniques.

Keywords: antibody; molecular imaging; nodal staging

132 Restoration of E-Cadherin in Colon Cancer Cells: Identification by High-Throughput Screening

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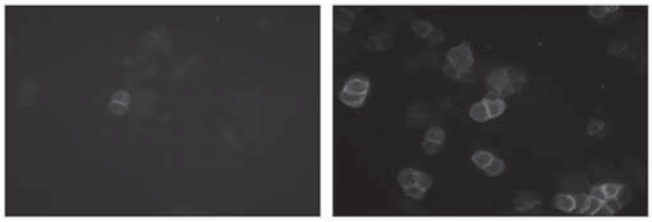
Introduction: E-cadherin is a calcium-dependent transmembrane product of the CDH1 gene and critical intercellular protein at the adherens junction of epithelial cells. Loss of E-cadherin results in epithelial to mesenchymal transition (EMT) and is a hallmark of malignant transformation in GI tumors including colorectal cancer. Restoration of E-cadherin to the cell membrane reduces the metastatic potential of colon cancer cells.

Methods: We are using high-throughput screening (HTS) to identify compounds that restore E-cadherin to the cell membrane in SW620 cells, a human metastatic colorectal cancer cell line deficient in E-cadherin. We have optimized a slide-based E-cadherin immunofluorescent protocol to be used in a 384 well plate assay. The HTS system is facilitated by an articulated arm controlled by robotic scheduling to assay multiple plates simultaneously. Our goal is to screen a chemical library containing 160,000 compounds and identify "hit" compounds that re-express E-cadherin. SW620 cells treated with trichostatin-A (TSA), an HDAC inhibitor known to cause E-cadherin re-expression, are used as control. To determine pharmacologic effects in another common malignancy, selected compounds from the screen are tested in H520 lung cancer cells.

Results: To date, 83,200 compounds have been screened. Analysis is complete on 72,000 compounds from which 1,611 "hits" were identified for an overall hit rate of 2%. Validation on 153 of these hit compounds has been completed. The figure shows restoration of E-cadherin in SW620 cells by a hit compound labeled #7. At present, 200 analogs of compound 7 have been obtained for concentration response curve (CRC) analysis. Seven of these analog compounds were found to restore E-cadherin to equal or greater levels than the native compound 7 in both SW620 and H520 cancer cells. These compounds are under further analysis, including structure activity relationship, while the remaining chemical library is screened.



Control Compound 7

Conclusion: This robotic HTS system allows screening of large chemical compound libraries. We have successfully adapted and optimized an immunofluorescent protocol for low-volume, micro-plate readouts using HTS. Hit compounds from the initial screen have been identified and individual compound analysis is under way. HTS of chemical compounds that restore E-cadherin in colorectal cancer is a powerful resource for identifying potential novel therapeutics.

Keywords: E-cadherin; metastasis; therapeutics

263 Development and In Vitro Validation of Anti-Mesothelin Biobodies That Prevent CA125/Mesothelin-Dependent Cell Attachment

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Preventing peritoneal implantation of carcinoma cells could prolong ovarian cancer patient remission and survival. Peritoneal cells constitutively express mesothelin, a ligand for CA125 that is expressed by tumor cells. Thus, blocking CA125/mesothelin-dependent cell attachment may prevent or delay peritoneal metastatic recurrence. We developed a high-throughput screening system for reagents able to block CA125/mesothelin-dependent cell attachment with a sensitive quantitative readout. Using a novel yeast expression system to produce secreted, in vivo biotinylated proteins and biobodies (BBS), we generated anti-mesothelin Bbs. Anti-mesothelin Bbs derived from high-affinity yeast-display scFv detected both membrane-bound and soluble mesothelins and inhibited CA125/mesothelin-dependent cell attachment.

Keywords: biobody; peritoneal metastasis; tumor cell attachment

231 Chemical Genetics Approach for Dissecting Wnt Signal Transduction Using a Novel Biochemical Approach Based on Theoretical Considerations

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We have performed a novel, high-throughput screen to identify small-molecule regulators of the canonical Wnt pathway that combines mathematical modeling, biochemistry, and embryology approaches. Inappropriate activation of the canonical Wnt pathway is a critical event in the genesis of many common human cancers. Although it is well accepted that perturbation of the Wnt pathway plays a critical role in neoplastic transformation, the mechanisms by which Wnt signal transduction is regulated under normal and pathological conditions are poorly understood. A critical step in the control of Wnt signal transduction is regulation of the stability of the transcriptional coactivator, β-catenin. The Wnt pathway protein, Axin, has been proposed to play a critical role in inhibition of the Wnt pathway via a variety of mechanisms, including (1) promoting degradation of β-catenin, (2) sequestering β-catenin, and (3) translocating β-catenin from the nucleus. We have previously shown that Xenopus egg extract accurately recapitulates the degradation of β-catenin by components of the Wnt pathway and developed a mathematical model of Wnt signal transduction. Based on these theoretical considerations, we have developed a biochemical assay that recapitulates activation of the canonical Wnt pathway by the Wnt coreceptor LRP6 in Xenopus egg extracts. Using a chemical genetics-based approach to identifying compounds that perturb LRP6-mediated stabilization of β-catenin and degradation of Axin in Xenopus extracts, we have identified 38 lead compounds, several of which are FDA approved for non-neoplastic conditions.

One of these FDA-approved drugs, WS30, blocks induction of secondary axis formation in Xenopus embryos in a dose-dependent manner (an indication of Wnt pathway inhibition in vivo). In cultured mammalian 293 HEK cells, WS30 inhibits Wnt3a-induced expression from a TOPFLASH reporter, as well as the endogenous Wnt gene targets, Dkk1 and Axin2. Furthermore, inhibition of Wnt-mediated gene transcription by WS30 correlates with decreases in cytoplasmic β-catenin levels in these cells. WS30 inhibits gene transcription from the TOPFLASH reporter in 293 HEK cells and inhibits viability of the cancer cell lines MDA-MB231, SW620, and SW480 at similar concentrations (EC_{50} ~55 nM). We further show that WS30 inhibits growth of these cancer lines in a dose-dependent manner. FACS analysis was performed on MDA-MB231 and HCT-116 cells treated with WS30, and an enhancement of the Sub-Go fraction was detected over time, suggesting the decrease in cell viability is likely due to activation of the apoptotic pathway. MDA-MB231, SW480, and HCT-116 cancer lines show decreased Ki-67 staining following treatment with WS30, indicating that they have exited the cell cycle. In addition, cytoplasmic β-catenin levels appear dramatically reduced, and there is a pronounced redistribution of β-catenin within the nucleus. Actin staining reveals an alteration in cellular morphology suggestive of a reversal of an epithelial-mesenchymal transition. Finally, we show that WS30 is able to inhibit Axin degradation in Xenopus extracts, suggesting that WS30 downregulates the canonical Wnt pathway by potentiating the function of Axin. We predict WS30 will be a valuable pharmacological agent to biochemically dissect the Wnt pathway, to explore the role of Wnt signaling during development, and to serve as a potential agent for the treatment of Wnt-driven cancers.

Keywords: Axin; chemical genetics; Wnt

030 BioMark™ System: Integrated Fluidic Circuits Enable the Use of Real-Time qPCR for Cancer Gene Expression Panels, Drug Resistance Monitoring, and Circulating Tumor Cell Detection

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Precise quantitative measurement of gene expression panels in precious clinical samples and detection of rare mutations are challenging for microarrays and conventional qPCR systems. We have developed the BioMark™ System, which miniaturizes and highly parallelizes qPCR in nanofluidic circuits called dynamic arrays and digital arrays.

We compare the cost and logistics of a 48 sample by 48 assay *dynamic array* in prostrate and lung cancer panels and show transcript profiling for *single* CTC/stem cells. Dynamic arrays enable parallel multiplexing of up to 48 genes for each of 48 samples yielding 2,304 ten-nanoliter qPCR reactions per run. The dynamic array configuration is completely flexible, allowing the researcher to use *any* set of 48 samples and *any* set of 48 genes. The efficiencies realized in terms of time, effort, and costs are revolutionary compared to traditional microplate-based systems.

Digital arrays provide single-copy sensitivity and dramatically enhance detection of mutants in a high background of wild-type, enabling new applications such as true absolute quantification and somatic cell mutation detection. We demonstrate detection of single T315I imatinib resistance mutations in backgrounds of greater than 20,000 copies of wild-type.

Keywords: cancer detection; gene expression; nanofluidic circuits

262 Combination of Vβ Spectratyping/CD107 Degranulation Assays To Monitor Directly Ex Vivo Antitransgene T Cell Immune Responses in Lymphoma Patients Receiving Adoptive Immunotherapy

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Adoptive transfer of gene-modified immune cells to enhance immune function holds significant promise for therapy of malignancy. A significant limitation associated with this approach is the development of host immune responses against transgenes expressed by transferred immune cells. It is important to be able to detect and follow the development of anti-infused cell immune responses, from a correlative perspective both to understand why infused cells may not persist and to allow for the potential to modulate treatment if such responses are observed.

Detection of an immune response against transgene-containing cells has typically involved repeated in vitro stimulation of patient PBMCs with infused cells, followed by the measurement of anti-transgene cell-specific cytolysis and/or proliferation. This approach suffers from limitations, since it is time consuming, requires a considerable lag from sample procurement to evaluation, and involves repetitive in vitro stimulation to expand cells for evaluation.

Using a combination of two assays, TcR Vβ spectratyping and CD107a/b degranulation, we have developed a methodology to detect the presence of cellular anti-transgene responses in patient PBMCs directly ex vivo. This approach is rapid and avoids the pitfalls associated with in vitro expansion protocols. We have applied this methodology to evaluate directly ex vivo the expansion of anti-transgene cytotoxic T cells in patients enrolled in a pilot/feasibility study to evaluate the safety of cellular immunotherapy for CD19+ follicular lymphoma using autologous T cells genetically modified to be CD19-specific and co-express HyTK.

Consistent with the potential development of an immune response against the infused cells, we demonstrate the lack of persistence of infused T cells in a patient who received gene-modified autologous T cells using Q-PCR. Using Vβ spectratyping, we demonstrate the specific expansion of distinct T cell populations in patient PBMCs within 2 weeks of the first T cell infusion. By combining antibodies specific for the defined Vβ T cells with a flow-based CD107 degranulation assay, we demonstrate the development of a robust anti-transgene-specific T cell response detectable within 2 weeks of the first T cell infusion and maintained throughout the infusion course. Finally, using Vβ spectratyping, we show that the T cell repertoire of T cell cultures generated using in vitro expansion methodologies is dramatically different from the repertoire shown to be specific for infused cells directly ex vivo.

Keywords: clinical trials; correlative studies; immunotherapy

Therapy I

237 Targeting Transforming Growth Factor β Receptor as a Therapeutic Approach to Suppressing Pancreatic Cancer Metastasis

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Most pancreatic cancer patients present with inoperable disease or develop metastases after surgery. Conventional therapies are usually ineffective in treating metastatic disease. It is evident that novel therapies still need to be developed. Transforming growth factor β (TGF-β) plays a key role in cancer metastasis, signaling through the TGF-β types I/II receptors (TβRI/II). We hypothesized that targeting TβRI/II kinase activity with the novel inhibitor LY2109761 would suppress pancreatic cancer metastatic processes. We generated pancreatic cancer cell lines Colo357FG (nonmetastatic) and Colo357L3.6pl (metastatic), expressing both luciferase and green fluorescence proteins (FG/GLT and L3.6pl/GLT). The effect of LY2109761 has been evaluated on soft-agar growth, migration on a wound-closure assay, invasion using a fibroblast co-culture model, and detachment-induced apoptosis (anoikis) by annexin V flow cytometric analysis. The efficacy of LY2109761 on tumor growth, survival, and reduction of spontaneous metastasis was evaluated in an L3.6pl/GLT orthotopic nude mouse model. To determine whether L3.6pl/GLT cells or the cells in the liver microenvironment were involved in LY2109761-mediated reduction of liver metastasis, we used a model of experimental liver metastasis. LY2109761 significantly inhibited the L3.6pl/GLT soft-agar growth ($p<.0001$); suppressed both basal and TGF-β1-induced cell migration ($p = .0107$; $p<.0001$) and invasion ($p = .0493$; $p = .0176$); and induced anoikis ($p = .0004$, $p<.0001$, and $p = .0005$ at 2, 4, 8 hours). In vivo LY2109761 in combination with gemcitabine significantly reduced the tumor burden ($p<.05$), prolonged the survival ($p = .0018$), and reduced spontaneous abdominal metastases ($p<.0225$). Results from the experimental liver metastasis model suggested an important role for targeting TβRI/II kinase activity on tumor and liver microenvironment cells in suppressing liver metastasis. Targeting TβRI/II kinase activity on pancreatic cancer cells or the cells of the liver microenvironment represents a novel therapeutic approach to preventing pancreatic cancer metastasis.

Keywords: metastasis; pancreatic cancer; TGF-beta receptor

249 Phase I Evaluation of Therapeutic, E7-Targeted DNA Vaccination in Women With HPV16+ High-Grade Cervical Dysplasia (CIN2/3)

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HPV-associated neoplasia of the cervix presents a compelling opportunity to test antigen-specific immunotherapies because expression of two non-self viral antigens, E6 and E7, are together functionally required to initiate and maintain high-grade dysplastic lesions (CIN2/3), the lesion which is the immediate precursor to invasive cervical cancer. Globally, one genotype, HPV16, accounts for over one-half of all cervical malignancies. We developed a therapeutic DNA vaccine construct, pNGVL4a-Sig/E7(detox)/HSP70. Preclinical work showed that this vaccine elicited CD8+ T cell responses that were capable of recognizing and eliminating TC-1, an epithelial tumor expressing E7. We have completed a Phase I dose-escalation clinical trial in women with HPV16+ CIN2/3 lesions. Eligibility criteria included age >18, colposcopically directed biopsy-confirmed CIN2/3, with visible residual lesion after diagnostic biopsy, and HPV16+ lesions. A total of 70 patients enrolled for screening after referral for HGSIL pap smears, of whom 47 (67%) were Caucasian, 6 (9%) were Hispanic, 15 (21%) were African American, and 2 (3%) were Asian. Reasons for ineligibility included not HPV16, no visible lesion, and no biopsy-proven CIN2/3. A total of 15 underwent vaccination. Three dose levels were evaluated: 500 mcg, 1 mg, and 3 mg, given intramuscularly in the deltoid. Study participants underwent a total of three vaccinations at 4-week intervals. Patients then underwent standard therapeutic resection of the cervical squamocolumnar junction 7 weeks after the last vaccination. Vaccination has been well tolerated, with no dose-limiting or severe adverse events reported. Patients in the highest dose cohort are currently completing the study protocol. Immune response data and lesion regression data will be mature by the end of May 2007 and will be presented.

Keywords: cervical dysplasia; immunotherapy; Phase I clinical trial

239 Strategies for Overcoming Trastuzumab Resistance Caused by PTEN Deficiency

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ErbB2 overexpression is found in ~30% of human breast cancers and leads to a very aggressive, highly metastatic breast cancer phenotype and poor patient survival. The ErbB2-targeting antibody trastuzumab (Herceptin) has shown remarkable therapeutic efficiency in some patients with ErbB2-overexpressing breast cancer. However, the overall trastuzumab response rate is limited and the causes of trastuzumab resistance are poorly understood. We found that PTEN activation contributes to the antitumor activity of trastuzumab and that reduced PTEN expression in ErbB2-overexpressing human breast cancer cells confers trastuzumab resistance in vitro and in xenograft models. More importantly, in a small cohort of patients with ErbB2-overexpressing breast cancers treated by trastuzumab plus taxanes, those with PTEN-deficient tumors had significantly worse response than those with normal levels of PTEN (see reference). Thus we hypothesized that PTEN deficiency in an ErbB2-overexpressing breast tumor is a sensitive and specific predictor of trastuzumab resistance and that combination treatment with trastuzumab and agents targeting PI3K pathways will overcome trastuzumab resistance caused by PTEN loss.

Cell culture and xenograft models were used to test a panel of clinically applicable, small-molecule inhibitors of the Akt/mTOR signal transduction pathway, a critical pathway downstream of ErbB2, and to identify compounds with the ability to restore trastuzumab sensitivity to PTEN-deficient cells. When trastuzumab was combined with the Akt inhibitor triciribine, breast cancer cell growth was inhibited and apoptosis was induced. In a xenograft model, combination therapy with trastuzumab and triciribine dramatically decreased the average tumor size. The combination of trastuzumab and the mTOR inhibitor RAD001 also slowed breast cancer cell growth in vitro and in vivo.

A Phase I/II clinical trial was initiated to test the efficacy of combining the mTOR inhibitor RAD001 with trastuzumab in patients with resistance to trastuzumab-based therapy for metastatic breast cancer. Of six patients treated to date, four exhibited stable disease for >5 months, as defined by RECIST criteria.

Our findings demonstrate that combining trastuzumab with inhibitors of the PI3K/Akt/mTOR pathway is a clinically applicable strategy, and that combinations of trastuzumab with triciribine or RAD001 are promising regimens for rescue of trastuzumab resistance caused by PTEN loss.

Reference

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Keyword: breast cancer

273 The Dual Tyrosine Kinase Inhibitor, Lapatinib, Prevents ER-Negative Breast Cancer by Suppressing the Development of Premalignant Lesions

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Background: Lapatinib, a selective oral dual tyrosine kinase inhibitor (TKI) of ErbB1 (EGFR) and ErbB2 (HER2) receptor tyrosine kinases, is a promising agent for the treatment of breast cancer. This signal transduction inhibitor is approved for the treatment of women with metastatic breast cancer and is being evaluated for the treatment of women with early-stage breast cancer. We are investigating whether lapatinib will be useful for the prevention of breast cancer. We hypothesized that blockade of peptide growth factor signaling pathways by lapatinib would effectively prevent the development of ER-negative breast cancer.

Methods: We first assessed the ability of lapatinib to block signal transduction pathways in breast epithelial cells using Western blotting to detect phosphorylation changes of signaling molecules. We next determined the effect of lapatinib on the growth of normal, immortal, and malignant breast cells using the MTS assay. We then determined the effect of lapatinib on ER-negative mammary tumorigenesis by treating MMTV-ErbB2 transgenic mice with two doses of lapatinib (75 mg/kg or 30 mg/kg twice daily by oral gavage). For these animal studies, we investigated the effect of this drug on the development of invasive mammary tumors and on premalignant lesions.

Results: Our results demonstrate that lapatinib effectively blocks EGF-induced signaling through the ErbB1 and ErbB2 receptors, inhibits cell cycle progression, suppresses cyclin D1 and epiregulin expression, and stimulates P27 expression. Results from our studies of MMTV-erbB2 transgenic mice demonstrated that chronic lapatinib treatment suppressed ER-negative invasive mammary tumor development, while short-term treatment decreased the incidence of mammary epithelial cell hyperplasia, and microscopic invasive breast cancer.

Conclusions: Lapatinib effectively blocks EGF-induced signaling through the ErbB1 and ErbB2 receptors in normal and malignant human breast cancer cells, inhibits the growth of ER-negative premalignant and malignant cells, and suppresses the development of ER-negative breast cancer in mice. In addition, our results show that the ErbB family of receptors, intracellular kinases, and cyclin D1 represent useful biomarkers of lapatinib response and demonstrate that lapatinib is a promising agent for the prevention of ER-negative breast cancer.

Keywords: prevention; signal transduction; therapeutics

210 Stimulation of Antitumor Immunity Using Dendritic Cell/Tumor Fusions and Anti-CD3/CD28

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We have developed a promising cancer vaccine in which autologous tumor cells are fused with dendritic cells (DCs). In animal models, vaccination with DC/tumor fusions potently stimulates antitumor immunity resulting in the eradication of metastatic disease. In clinical trials, a majority of patients demonstrate evidence of immunologic responses, but clinical efficacy is likely limited by the immunosuppressive environment and increased presence of regulatory T cells that characterizes the patient with malignancy. Ligation of the T cell receptor/costimulatory complex (CD3/CD28) delivers a potent antigen-independent signal promoting T cell expansion. The nature of the resulting T cell phenotype is dependent on the immunologic milieu in which the signal is delivered. We postulated that stimulation with DC/tumor fusions followed by anti-CD3/CD28 would result in the significant expansion of activated T cells targeting tumor antigens. DCs were generated from adherent mononuclear cells cultured with rhIL-4, GM-CSF, and TNFα and fused with RCC by coculture in 50% solution of polyethylene glycol. T cells were stimulated by DC/tumor fusions prior to or following exposure to anti-CD3/CD28 antibody coated plates for 48 hours. A dramatic, statistically significant increase in T cell proliferation was observed following the sequential exposure to DC/RCC fusions and anti-CD3/CD28 (SI 13.2). In contrast, stimulation by anti-CD3/CD28, DC/tumor fusions, or anti-CD3/CD28 followed by fusion cells did not result in significant T cell proliferation. Similarly, sequential stimulation by DC/RCC fusion cells followed by anti-CD3/CD28 resulted in an increase in the mean percentage of T cells co-expressing CD4+ and CD25+ from 2.8% to 25.3% ($n = 11$, $p = .001$). A 42-fold increase in the percentage of cells co-expressing CD4, CD25, and CD69+ was observed, consistent with the expansion of activated T cells. In contrast, exposure to anti-CD3/CD28 alone or anti-CD3/CD28 followed by stimulation with fusion cells resulted in a modest expansion of CD4/CD25/CD69+ cells. In concert with these findings, IFNγ production by CD4+ T cells was most pronounced following stimulation with DC/tumor fusions and expansion with anti-CD3/CD28 ($p<.01$). In nine experiments, stimulation with DC/RCC fusions followed by expansion with anti-CD3/CD28 also resulted in a fifteenfold expansion of regulatory T cells expressing CD4/CD25/Foxp3+. We subsequently examined whether T cells stimulated by DC/RCC fusions and anti-CD3/CD28 demonstrate cytolytic capacity as evidenced by expression of granzyme B. Sequential stimulation with DC/RCC fusions and anti-CD3/CD28 induced a 21-fold expansion of granzyme B + cells, while only modest increases were seen with stimulation with fusions alone, antiCD3/CD28 alone, or anti-CD3/CD28 followed by fusion stimulation. To determine whether sequential stimulation with DC/RCC fusion and anti-CD3/CD28 resulted in the selective expansion of tumor reactive lymphocytes, we evaluated the effect on T cells that recognized the tumor-associated antigen, MUC1. Sequential stimulation with DC/RCC fusions followed by anti-CD3/CD28 resulted in a dramatic increase in MUC1 tetramer+ cells (17.3%; $p = .02$ and .004 as compared to stimulation with fusions or antiCD3/CD28 alone, respectively). In contrast, nonspecific stimulation with anti-CD3/CD28 followed by co-culture with fusions did not induce the expansion of MUC1 tetramer + cells (0.19%). In conclusion, we have demonstrated that stimulation of T cells by DC/RCC fusions followed by exposure to anti-CD3/CD28 antibodies results in the expansion of tumor reactive T cells that predominantly express markers of activation. We are developing a clinical trial in which patients will receive fusion/CD3/CD28 expanded T cells following in vivo depletion of regulatory T cells.

Keywords: adoptive immunotherapy; anti-CD3/CD28-activated T cells; dendritic cell vaccines

232 Interleukin-13 Displaying Oncolytic Measles Virus Strains Have Tumor-Specific Activity Against Gliomas With an Improved Therapeutic Index

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Glioblastoma multiforme (GBM) is the most common brain tumor in adults and has a dismal prognosis despite use of multimodality treatment. The majority of GBM (80%) overexpress interleukin-13 receptor alpha 2 (IL-13Rα2), which is not expressed in normal brain. We have previously demonstrated that vaccine strains of measles virus have significant antitumor activity against gliomas (Phuong, 2003). We tested the hypothesis that retargeting measles virus entry via the IL-13Rα2 by displaying IL-13 on the measles virus H protein, while eliminating natural receptor binding through CD46 and SLAM, could result in improved therapeutic index, as compared to unmodified vaccine strains.

Methods and Results: The recombinant measles virus MV-GFP-HAA-IL13 was generated from the Edmonston-NSe vaccine strain by displaying IL-13 at the C-terminus of the H protein and introducing a CD46 ablating mutation (Y481A) and a SLAM ablating mutation (R533A) in H. The virus also contains the GFP gene at position 1. Unmodified MV-GFP was used as a positive control.

Of 14 primary or established GBM lines tested, 10 demonstrated IL-13Rα2 receptor overexpression by FACS. The IL-13 retargeted virus showed significant cytopathic effect against IL-13Rα2 overexpressing glioma lines, which was comparable to MV-GFP. One-step viral growth curves demonstrated comparable growth of the IL-13R retargeted strain to MV-GFP. In contrast to MV-GFP, however, treatment with the IL-13 expressing retargeted strain resulted in complete lack of cytopathic effect or viral replication in untransformed cells such as normal human astrocytes (NHA) and normal human dermal fibroblasts (NHDF), which do not express IL-13Rα2. Pretreatment with IL-13, but not IL-4 or IL-2, inhibited infection and replication of the retargeted virus in IL-13Rα2 expressing U87 cells, indicating IL-13 specific entry. In vivo, treatment of orthotopically implanted, IL-13Rα2 expressing, GBM12 xenografts demonstrated statistically significant prolongation of survival in mice treated with the retargeted strain (total dose, 9e5TCID50) as compared to control treated animals ($p<.0001$), and comparable activity between the IL-13R retargeted strain and the unmodified strain MV-GFP ($p = .6377$). Administration of the retargeted strain in the CNS of measles replication permissive Ifnar^{ko} CD46 Ge mice resulted in lack of neurotoxicity. In contrast, four of five mice administered MV-GFP had to be euthanized by 7 days post-administration of a single dose of 1e5 TCID50 because of development of neurologic toxicity.

Conclusions: Measles virus strains retargeted by a combination of IL-13 display and ablation of entry via the natural CD46 and SLAM receptors have comparable therapeutic efficacy in vitro and in vivo as compared to the unmodified measles virus strains in IL-13Rα2 expressing glioma lines and improved therapeutic index in nontransformed cells, thus representing excellent candidates for clinical translation.

Keywords: glioma; IL-13 receptor; measles virus

165 Arsenic Trioxide Enhances the Antitumor Effects of Radiation Treatment While Protecting the Bone

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Therapeutic radiation is commonly used in the treatment of squamous carcinoma of the head and neck. Despite the proven efficacy of this form of anticancer therapy, high-dose radiation treatment is invariably associated with numerous side effects that adversely affect the growth and function of normal cell populations at the treatment site. This is particularly true for bone cells, where radiation often leads to bone destruction and impaired healing. We and others have shown that p38 MAPK is a key cell death pathway in endothelial cells and a number of tumor cell types. In contrast, p38 MAPK mediates osteoblast proliferation and survival. This dual property of p38 MAPK provides a unique opportunity in the treatment of head and neck cancers. This leads us to hypothesize that activation of p38 in combination with radiation therapy can accelerate tumor and tumor vessel destruction while sparing bone from radiation-induced osteonecrosis. The aim of this study was to investigate whether targeting of p38 MAPK with arsenic trioxide (As_2O_3) could enhance the antitumor effect of radiation treatment while protecting the bone. We selected As_2O_3 for this study because it has been used in clinic for acute promyelocytic leukemia and has low systemic toxicity. As_2O_3 treatment induced a dose-dependent (1-20 μM) inhibition of endothelial (HDMEC) and tumor cell (OSCC-3) survival. In

contrast, As_2O_3 treatment of osteoblast (NHOst) induced proliferation up to 7.5 μM dose. At 5 μM dose, As_2O_3 treatment significantly enhanced radiation-induced HDMEC and OSCC-3 cell death, whereas As_2O_3 treatment at the same dose (5 μM) protected NHOst against radiation-induced cell death. To test the effectiveness of As_2O_3 treatment in vivo, we developed a SCID mouse model that has a bone ossicle and tumor growing side by side subcutaneously. In this model, we implanted Gelfoam sponges seeded with bone marrow stromal cells (BMSCs) in the flank of the animals to form bone ossicles. After 2 weeks, OSCC-3 cells (1×10^6) and HDMEC (1×10^6) were mixed with 100 μL of Matrigel and implanted adjacent to the bone. Tumors were allowed to grow and vascularize for 10 days and then three doses of As_2O_3 (10 mg/kg) and ionizing radiation (fractionated dose of 7.5 Gy × 3) were administered. One week after the last treatment, tumors and lungs were removed and analyzed for tumor growth, tumor angiogenesis, and tumor metastasis to lungs, and bone ossicles were analyzed for bone mineral density. Animals treated with As_2O_3 and radiation treatment showed significant inhibition of tumor growth, tumor angiogenesis, and tumor metastasis to lungs as compared to As_2O_3 treatment or radiation treatment alone. In contrast, As_2O_3 treatment protected bone ossicles from radiation-induced bone loss. These results suggest a novel strategy to enhance the therapeutic efficacy of radiation treatment while protecting bone from the adverse effects of therapeutic radiation.

Keywords: angiogenesis; metastasis; radiotherapy

252 Restricted, Uniform, and Constitutive Cell Surface Expression of Receptor Tyrosine Kinase-Like Orphan Receptor 1 in Chronic Lymphocytic Leukemia

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Chronic lymphocytic leukemia (CLL) is a biologically and clinically heterogeneous cancer characterized by the gradual accumulation of CD5+ CD19+ malignant B cells. Gene expression profiling on a genomic scale led to the discovery of a distinctive CLL gene signature composed of genes selectively expressed by CLL cells. However, the expression and function of the corresponding proteins and their suitability as targets for therapeutic intervention have remained largely unexplored. In the present study, we analyzed the cell surface expression of one such protein, the receptor tyrosine kinase-like orphan receptor 1 (ROR1), an embryonic protein involved in organogenesis. We show that ROR1 protein is selectively expressed on the surface of CLL cells, whereas normal B cells, other normal blood cells, and normal adult organs do not express ROR1 protein. Moreover, cell surface expression of ROR1 is uniform and constitutive, i.e., independent of anatomical niches, independent of biological and clinical heterogeneity of CLL, independent of B cell activation, and found at similar levels in all CLL samples tested (n = 28). The antibody binding capacity of CLL cell surface ROR1 was determined to be in the range of 10^3-10^4 molecules/cell. A portion of CLL cell surface ROR1 was actively internalized upon antibody binding as shown by inhibition with phenyl arsine oxide. Soluble ROR1 protein was detectable in sera of less than 25% of CLL patients at concentrations below 200 ng/mL. The restricted, uniform, and constitutive cell surface expression of ROR1 protein in CLL provides a strong incentive for the development of targeted therapeutic strategies such as monoclonal antibodies.

Keywords: antibody; antigen; leukemia

Therapy II

211 Systemic Therapy With Tumor Suppressor FUS1-Nanoparticles for Stage 4 Lung Cancer

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The tumor suppressor gene FUS1 is frequently inactivated early in the development of lung cancer. We examined Fus1 immunohistochemical (IHC) expression in 301 lung cancers, and loss and reduction of expression was detected in 100% of small cell lung cancers (SCLCs) (n = 22) and 82% of non-small cell lung cancers (NSCLCs) (n = 281), without significant differences between adenocarcinoma and squamous cell carcinoma histologies. In NSCLCs, loss of FUS1 IHC expression was associated with significantly worse overall survival. FUS1 mediates apoptosis in cancer cells but not normal cells through its interaction with Apaf1 and downregulates tyrosine kinases including EGFR and c-abl. We developed a DOTAP:cholesterol nanoparticle encapsulating an optimized FUS1 expression plasmid that showed selective uptake by fresh cancer cells compared to normal cells and mediated tumor regression in orthotopic xenograft mouse models. In this clinical trial, a FUS1 expression plasmid in a DOTAP:cholesterol nanoparticle was injected intravenously in stage 4 lung cancer patients who had received cisplatin combination chemotherapy and showed tumor progression at the time of entry into the study. Nanoparticle-DNA complexes were manufactured in GMP facilities to meet specifications of OD400, size, appearance, and transfection efficiency. Patients received doses ranging from 0.01 to 0.03 mg/kg at 3-week intervals for a maximum of six doses. Dexamethasone and diphenhydramine premedications were added and eliminated the only clinically significant toxicity of fever. To date, 13 patients have been entered on the study at 3 different doses (0.1, 0.02, 0.03 mg DNA/kg). All patients could be evaluated for the primary endpoint of toxicity, and with pretreatment, there was no significant drug-related toxicity. Four patients received only one dose because of rapidly progressing disease at a site requiring local treatment. Eight patients received two or more doses and can be evaluated for response, with three patients achieving stable disease (3-7+ months) and five patients progressing. Median survival time for all patients is 14.6 months, which compares favorably to the published 7 months median survival for second-line chemotherapy and 4 months for best supportive care for non-small cell lung cancer. Actuarial survival at 12 months was 55%. A maximum tolerated dose (MTD) has not been reached. Pre- and 24-hour post-treatment tumor biopsies were obtained from three patients by percutaneous computed tomographic guidance from a central tumor location. A quantitative real-time reverse transcriptase PCR (RT-PCR) analysis using a plasmid FUS1 sequence-specific probe was performed on samples blinded to time of biopsy. A high level of plasmid FUS1 expression was detected in all three post-treatment samples but not in three pretreatment samples and negative controls by RT-PCR. An estimated four copies of the FUS1 gene per cell were detected in a specimen from a patient receiving 0.01 mg DNA/kg. When the dose was increased to 0.02 mg/kg, the copy number per cell increased to 20 and 28 copies per cell in two patients. DOTAP:cholesterol FUS1 nanoparticles can be safely administered intravenously in patients with stage 4 lung cancer. Gene expression is detected in tumors, and there is an indication of antitumor activity.

Keywords: nanoparticle therapy; tumor suppressor gene; tyrosine kinases

139 Identification of Melanoma Cells With Resistance to BRAF and MEK Inhibitors

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One of the problems facing the clinical development of small-molecule kinase inhibitors is selecting the patient group with the greatest likelihood of response. Although most melanomas harbor activating BRAF mutations, this does not always predict a good response to drugs that inhibit the MAP kinase pathway. We recently demonstrated that there is considerable heterogeneity in the antiproliferative response (IC_{50} from 200 nM to 10 μM) seen to MEK inhibitors in V600E mutated melanoma cell lines [1]. Although the reasons for this MEK/BRAF inhibitor resistance are not clear, it is known that amplifications in components of the cell cycle machinery can overcome the need for high BRAF/MEK/ERK activity. To investigate the mechanism for this resistance,

we searched our microarray database of more than 30 melanoma cell lines and found 2 lines with extremely high expression of cyclin-dependent kinase 4 (CDK4). Mutational analysis of these CDK4 overexpressing melanomas showed them to be wild-type for both N-Ras and BRAF mutations. In Western blotting studies, we found that the CDK4 overexpressing melanomas had very low levels of phospho-ERK but high expression of cyclin D1 compared to our standard panel of melanoma lines. The role of CDK4/cyclin D1 overexpression in BRAF/MEK inhibitor resistance was confirmed in both cell proliferation assays and three-dimensional spheroid studies. In agreement with our cell line data, recent pathological studies have also identified a similar subgroup of melanomas (3 of 53) that have CDK4 overexpression [2]. In summary, we have identified a novel subgroup of melanomas in which overexpression of CDK4 and cyclin D1 compensates for the presence of either a BRAF or an N-Ras mutation. We suggest that this subgroup of melanomas will likely require pharmacological strategies different from those being developed for BRAF V600E mutated melanomas.

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Keywords: melanoma; resistance; therapy

274 Activation of LMP1- and LMP2-Specific T Cells for the Immunotherapy of EBV-Positive Lymphomas With an Adenoviral Vector Encoding Full-Length LMP1 and LMP2

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Background: LMP1 and LMP2 are potential targets for the immunotherapy of EBV-positive malignancies such as EBV-positive lymphomas. We have shown in the past that LMP1- and LMP2-specific T cells can be activated from EBV-seropositive individuals, with adenoviral vectors encoding either an inactive form of LMP1 (dLMP1) or LMP2. The aim of this study was to construct and characterize an adenoviral vector containing dLMP1 and LMP2 (Ad_dLMP1-I-LMP2) for the generation of LMP1- and LMP2-specific T cells.

Methods: To evaluate the activity of Ad-dLMP1-I-LMP2 to reactivate LMP1- and LMP2-specific CTL, we took advantage of the tropism of Ad5F35 adenoviral vectors, which preferentially transduce monocytes after incubation of peripheral blood mononuclear cells (PBMC). Transduced monocytes expressed LMP1 and LMP2, and reactivated LMP1- and LMP2-specific T cells, that could be expanded further with autologous LCL transduced with Ad-dLMP1-I-LMP2. The presence of LMP1- and LMP2-specific CTL was determined by tetramer analysis, IFN-γ ELISpot assays, and cytotoxicity assays.

Results: Stimulation of PBMC with Ad-dLMP1-I-LMP2 transduced monocytes from 10 HLA-A*2, EBV-seropositive donors resulted in an at least 30-fold to 100-fold expansion of LMP1- or LMP2-specific T cells, as judged by the presence of HLA-A*2-restricted, tetramer-positive T cells. LMP2-specific responses were detected in all donors, whereas LMP1-specific responses were found in 7 of 10 donors. The presence of LMP1- and LMP2-specific T cells was confirmed in a subset of donors with IFN-γ ELISpot assays. In addition, generated LMP1- and LMP2-CTL killed autologous fibroblasts expressing LMP1 and LMP2, as well as LCL.

Conclusion: The constructed adenoviral vector, Ad-dLMP1-I-LMP2, is a promising candidate for the ex vivo generation of LMP1- and LMP2-specific CTL and may also be used as a vaccine for the in vivo boosting of adoptively transferred T cells. Targeting the subdominant LMP antigens expressed in EBV-positive lymphomas may improve the efficacy of adoptive immunotherapy approaches.

Keywords: antigen-specific T cells; EBV-positive lymphomas; immunotherapy

187 Design and Evaluation of Novel Small-Molecule Inhibitors of the MDM2-p53 Interaction as a New Anticancer Therapy

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Pharmacological reactivation of the tumor suppressor p53 in tumor cells using a nonpeptide, cell-permeable small-molecule MDM2 inhibitor to target the MDM2-p53 interaction is being pursued as a novel cancer therapeutic strategy. Despite promising preclinical in vitro and in vivo results reported using Nutlin-3, no small-molecule MDM2 inhibitor has advanced into human clinical trial. Recent genetic studies using a switchable endogenous p53 mouse model has shown that p53 is spontaneously active in all tested tissues of mdm2-deficient mice, triggering fatal pathologies, which raises a serious concern that an MDM2 inhibitor may be highly toxic to animals if it can activate p53 in normal tissues. In this study, we present the design of a new, highly potent, extremely selective and orally bioavailable small-molecule MDM2 inhibitor (MI-219). MI-219 binds to MDM2 with a K_i value of 5 nM and displays >10,000-fold selectivity over MDMx. MI-219 potently inhibits cell growth and effectively induces cell cycle arrest and apoptosis in prostate cancer and other cancer cell lines through activation of p53. While MI-219 induces cell cycle arrest in normal prostate epithelial cells, it fails to induce cell death. MI-219 is extensively distributed in tumor and normal tissues in mice and activates p53 in both tumor and normal tissues. MI-219 is highly effective in inhibition of tumor growth as a single, oral agent in LNCaP and other xenograft models of human cancer with wild-type p53, while causing no tissue damages in radio-sensitive and resistant tissues. Taken together, our studies provide compelling evidence that pharmacological activation of p53 by an MDM2 inhibitor in the presence of MDM2 has a very different pathological effect on normal tissues as compared to genetic activation of p53 in the absence of MDM2 gene. MI-219 or its analogues warrant extensive preclinical testing and clinical evaluations as a new therapy for the treatment of human prostate cancer and other types of tumors.

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Keywords: MDM2; p53; small-molecule inhibitors

140 Anti-CS1 Humanized Monoclonal Antibody HuLuc63 Inhibits Myeloma Cell Adhesion and Growth in the Bone Marrow Milieu and Targets the Cells for Killing via Antibody-Dependent Cellular Cytotoxicity

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Current monoclonal antibody (mAb) therapies for multiple myeloma (MM) have had limited success because of narrow target expression across MM patient samples. A preferred strategy is to develop cytotoxic human mAbs against novel antigens that are highly expressed in MM cells yet have limited expression in other cell types. In this study, we first characterized cell surface CS1 as a novel MM antigen and further investigated the potential therapeutic utility of HuLuc63, a humanized anti-CS1 mAb, for treating human MM. CS1 (CD2 subset 1, CRACC, SLAMF7), a member of the CD2 family of cell surface glycoproteins, was found to be highly expressed in myeloma cells. CS1 mRNA and protein were universally expressed in CD138-purified primary tumor cells from the majority of MM patients (>95%) using microarray expression profiling and flow cytometric analysis, respectively. CSI is also present in the majority of MM cell lines that are either drug-sensitive or resistant. Specifically, CS1 was expressed at adhesion-promoting uropod membranes of polarized MM cell lines and patient MM cells, and short interfering RNA (siRNA) targeted to CS1 inhibited MM cell adhesion to bone marrow stromal cells (BMSCs). HuLuc63 inhibited MM cell binding to BMSCs in a CS1-specific manner. Significantly, HuLuc63 induced antibody-dependent cellular cytotoxicity (ADCC) against MM cells in CS1- and dose-dependent manners. The ADCC occurred even against MM cells adherent to BMSCs, which confer resistance to conventional therapies. In addition, HuLuc63 triggered autologous ADCC against primary MM cells resistant to conventional or novel therapies including bortezomib (Velcade®) and HSP90 inhibitor; and pretreatment with conventional or novel anti-MM drugs markedly enhanced HuLuc63-induced MM cell lysis. Pretreatment with lenalidomide, which augments NK cell function, significantly increased NK cell-mediated lysis of autologous patient MM cells triggered by HuLuc63. Importantly,

administration of HuLuc63 resulted in significant tumor regression in a xenograft model of human MM. Finally, circulating CS1 is only detected in MM patient sera, but not in normal donors. These results, in aggregate, define the functional significance of CS1 in MM and provide the preclinical rationale for clinical trials of HuLuc63, either alone or in combination, to improve patient outcome in MM.

Keywords: ADCC; monoclonal antibody immunotherapy; multiple myeloma

238 Dysfunctional KEAP1–NRF2 Interaction in Non-Small Cell Lung Cancer as a Critical Factor for Chemoresistance

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Lung carcinomas are the leading cause of cancer deaths in the United States and worldwide in both men and women. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases, with 65% of these patients presenting with unresectable stages III and IV disease. Chemotherapy for advanced, inoperable NSCLC is generally palliative. The major factor contributing to the failure of chemotherapy in lung cancer is the development of drug resistance. Nuclear factor erythroid-2-related factor 2 (NRF2) is a redox-sensitive transcription factor that positively regulates the expression of genes encoding antioxidants, electrophile detoxification enzymes, and drug efflux pumps, and confers cytoprotection against oxidative stress and xenobiotics in normal cells. Kelch-like ECH-associated protein 1 (KEAP1) negatively regulates NRF2 activity by targeting it to proteasomal degradation. Increased expression of cellular antioxidants and electrophile detoxification enzymes has been implicated in resistance of tumor cells against chemotherapeutic drugs.

Systematic analysis of the *KEAP1* genomic locus in lung cancer patients and cell lines revealed deletion, insertion, and missense mutations in functionally important domains of *KEAP1* and a very high percentage of loss of heterozygosity at 19p13.2, suggesting that biallelic inactivation of KEAP1 in lung cancer is a common event. Sequencing of *KEAP1* in cell lines and NSCLC samples revealed somatic mutations in *KEAP1*. All the mutations were within highly conserved amino acid residues located in the Kelch or intervening region domain of the KEAP1 protein, suggesting that these mutations abolish KEAP1 repressor activity. Evaluation of loss of heterozygosity at 19p13.2 revealed allelic losses in the NSCLC cell lines and tumor samples. Constitutive activation of NRF2, as a result of loss of KEAP1 function, enhances the expression of antioxidants, electrophile detoxification enzymes, and drug efflux pumps and confers chemoresistance and radioresistance. Silencing of NRF2 expression by shRNA in cancer cells enhanced the intracellular accumulation of drugs and caused chemosensitization as well as radiosensitization. These studies indicate that increased NRF2 activity is a critical factor for therapeutic resistance.

Keywords: chemoresistance; lung cancer; NRF2

203 Bim Mediates Tyrosine Kinase Inhibitor-Induced Apoptosis in Lung Cancers With Activating EGFR Mutations and Its Upregulation Is Attenuated by Secondary Resistant Mutations, T790M and a Novel L747S

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Sequencing of the epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC) has identified somatic activating mutations in the majority of patients responsive to reversible EGFR tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib. These EGFR-dependent tumors eventually become TKI-resistant and the common secondary T790M mutation accounts for half the tumors with acquired resistance to gefitinib. However, the key pro-apoptotic proteins involved in TKI-induced cell death and other mechanisms involved in resistance remain unclear. To explore key pro-apoptotic mediators of TKI-induced apoptosis, we screened NSCLC cell lines that were either sensitive or resistant to reversible TKIs. Gefitinib-induced apoptosis was linked to upregulation of the "BH3-only" polypeptide Bim, which was observed only in gefitinib-sensitive EGFR mutant lung cancer cells. To determine whether Bim was also involved in secondary resistance conferred by acquired EGFR mutations, we analyzed the common T790M

and a novel acquired EGFR mutation in exon 19 (a leucine to serine substitution at position 747 - L747S) in *cis* to the activating L858R mutation identified in an NSCLC patient with a 40-month response to gefitinib prior to progression. Using the Ba/F3 system, we show that significant attenuation of apoptosis occurs in constructs containing L858R-L747S and L858R-T790M upon gefitinib exposure, with the latter being the most resistant mutation. The gefitinib-induced upregulation of Bim and apoptosis were inhibited by the presence of T790M and, to a lesser extent, L747S. In the cell line NCI-H1975, which harbors the L858R-T790M mutation, upregulation of Bim and apoptosis were seen only after exposure to the irreversible EGFR inhibitor CL-387,785 and not gefitinib. Knockdown of Bim by an siRNA strategy was able to attenuate significantly apoptosis induced by TKIs in EGFR-mutant cell lines HCC827 (carries an exon 19 deletion and is highly sensitive to gefitinib) and NCI-H1975. These results not only describe a novel secondary gefitinib-resistant mutation, but also provide insights into a key effector of TKI-induced apoptosis and suggest that induction of Bim may have a role in the treatment of TKI-resistant NSCLCs.

Keywords: apoptosis; Bim; EGFR mutations

037 HRQOL and Satisfaction With Cancer Treatment Outcome From the Perspective of Early-Stage Prostate Cancer Patients and their Spouse/Partners: Results From a Prospective, Multicenter Study

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Background: Localized prostate cancer treatment modifications are commonplace, despite the paucity of multicenter evidence regarding their impact on patient-reported outcomes. We sought to identify determinants of health-related quality of life (HRQOL) after contemporary primary prostate cancer treatment, and to measure effects of such changes on patient or spouse satisfaction with treatment outcome.

Methods: HRQOL was measured prospectively in a multicenter study of 1,205 prostate cancer patients before and after radical prostatectomy, brachytherapy, or external radiotherapy, 90% of whom completed 2.6 years of median followup; satisfaction with treatment outcome was evaluated among these men and 614 eligible spouses. Associations of patient characteristics and treatment factors with patient or spouse-reported outcomes were evaluated by GEE.

Results: Adverse sexual effects of prostatectomy were mitigated by nerve sparing ($p = .02$). Although urinary incontinence was evident after radical prostatectomy, significant benefit in urinary irritative/obstructive HRQOL was also observed ($p<.001$). Neoadjuvant hormone therapy worsened adverse effects of radiotherapy on sexuality and vitality ($p<.001$). Brachytherapy patients reported durable adverse urinary irritative, bowel, or sexual effects, and transient incontinence or vitality/hormonal effects ($p<.001$ for each); NHT compounded the latter ($p<.001$). Obesity ($p = .001$), prostate size ($p<.001$), co-morbidity ($p = .005$), and age ($p = .02$) affected HRQOL outcomes, whereas African American race was associated with worse treatment outcome satisfaction ($p = .02$). HRQOL changes were independently associated with outcome satisfaction among patients and their spouse-partner ($p<.001$).

Conclusions: Obesity, prostate size, and African American race impacted HRQOL and satisfaction with outcome, respectively, after primary prostate cancer treatment. Nerve sparing mitigated adverse surgery effects, while neoadjuvant hormone therapy magnified adverse radiotherapy or brachytherapy effects. Patient HRQOL changes impacted treatment satisfaction among patients and their spouses.

Keywords: health-related quality of life; outcomes; prostate cancer

Organ Site Breakout Sessions

The following abstracts were submitted to the Organ Site Breakout Sessions.

Please refer to the Program Book for session times and locations.

Recipient of Avon-NCI Progress for Patients (PFP) Awards Program: A Public-Private Partnership

282 Early Assessment of Response to Targeted Therapy in Breast Cancer

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Rationale: The ability to identify molecular targets (ER, HER2/neu) in order to tailor treatment to tumor phenotype provides a major advance in breast cancer treatment. Potent targeted agents with low toxicity, aromatase inhibitors (AI), and/or trastuzumab significantly improve outcomes. However, patients are usually subjected to months of treatment before efficacy can be adequately assessed. Early measurement of response is therefore a clinically important need for targeted therapy. Studies assaying material from serial biopsy have shown that a drop in proliferative index as early as 2 weeks predicts a favorable outcome. We have complemented biopsy-based measures of response using positron emission tomography (PET) imaging to measure response after 2 weeks of targeted therapy. Early assessment of response of the tumor may help refine targeting strategies and avoid prolonged futile therapy.

Preliminary Data: Breast cancer imaging with PET [F18]-fluorodeoxyglucose (FDG) 2 months into neoadjuvant therapy correlates with pathologic response and survival. Early PET FDG imaging of small cell lung cancer, sarcoma, and GIST tumors has been shown to predict response. In addition, our group and others have used imaging to measure therapeutic target expression using molecular imaging more specific than FDG PET: we used [F18]-fluoroestradiol (FES) PET to measure ER function and observed that whole-body FES PET predicts response to hormone therapy. Preliminary data from our center and others support the ability of a combination of FES and FDG PET to measure early response to targeted therapy.

Methods: Patients initiating treatment on either AI or trastuzumab will undergo standard baseline staging imaging with FDG PET. Following 2 weeks of single-agent treatment, repeat core biopsy and FDG PET will assess early response. Patients on single-agent trastuzumab may then have additional hormonal therapy or chemotherapy added to their treatment. Core biopsies will be assayed for proliferation index (Ki-67 or MIB-1), hormone receptor expression, HER2 level expression, and quantitative hormone receptor levels by RTPCR. Quantitative changes in regional tracer uptake from imaging at 2 weeks will be compared to biopsy results and to subsequent standard measures of response. Patients with ER-positive tumors co-enrolled in FES imaging trials also have serial FES imaging performed.

Preliminary Results: Patients have found the procedures tolerable and agreed to participate in the study undergoing neoadjuvant and palliative therapy on and off clinical protocols. We have seen a dramatic drop in proliferation rate, which correlates with PET FDG findings and response in some patients, but long-term followup is limited. One patient with anthracycline refractory HER2-positive inflammatory breast cancer, undergoing single-agent trastuzumab followed by trastuzumab plus paclitaxel, showed a decline in proliferative index from 40% to 20% and an SUV drop from 7.9 to 3.4, and a robust clinical response. Serial PET FES imaging shows a drop in FES uptake in patients early on AI therapy, but this observation has been variable.

Discussion: At least in some patients, early dramatic changes are apparent by biologic imaging and tissue assay to denote a response to targeted therapy. Further analysis and patient numbers are needed to determine the significance of our early observations. Results from this work would establish a role for early PET to identify tumors destined to fail to respond to therapy and would indicate a need to change treatment for tumors lacking initial signs of response.

Keywords: breast cancer; PET FDG; targeted

283 The Angiogenic Factor Cyr61: $\alpha_6\beta_1$ Integrin and Estrogen Receptor Connection in the Control of Hormonal Response and the Antiestrogen Resistance in Breast Cancer

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We have shown that the pro-angiogenic factor CCN1 is overexpressed in invasive breast cancer cells. The expression of CCN1 is sufficient to promote breast cancer cell proliferation, cell survival, chemosensitivity, and the acquisition of estrogen-independent and antiestrogen-resistant phenotype, apparently through $\alpha_v\beta_3$ integrin-activated ERK1/ERK2 MAPK signaling. However, integrin $\alpha_6\beta_1$ is associated with invasive carcinoma, but the specific participation of this integrin in the hormonal response is unknown. Here, we investigated the following: (1) whether expression of a CCN1 mutant with substitutions in the $\alpha_6\beta_1$- binding domains (TM) impedes CCN1's ability to induce an E_2-independent and/or the antiestrogen-resistant phenotype and (2) whether CCN1 participates in the regulation of ER-transcriptional activity in MCF-7 cells.

MCF-7 cell lines stably expressing either the CCN1 wild-type (wt), a CCN1-TM mutant, or the MCF-7/pBabe (empty vector) were grown in E_2 depleted conditions and subjected to anchorage-independent growth assays in the presence of estrogen (E_2 [10^{-9} M]) and antiestrogens (tamoxifen [10^{-7} M] and ICI 182,782 [10^{-7} M]). Our results showed CCN1 induced anchorage-independent growth in the absence of E_2 and in the presence of antiestrogens. In contrast, MCF-7/TM mutation impaired the ability of CCN1 to induce an E_2-independent and the antiestrogen-resistant phenotype. These data indicate that the CCN1-$\alpha_6\beta_1$ interaction contributes to the CCN1-mediated induction of E_2-independent and antiestrogen-resistant phenotype.

To determine whether CCN1 regulates the ER transcriptional activity and whether the blockage of CNN1 and $\alpha_6\beta_1$-integrin interaction modifies this activity, we used estrogen-response-element (ERE)-reporter assays. E_2-deprived cells were transfected with ERE-luciferase and the control vector pRL-CMV for 24 hours. Then cells were incubated with E_2, antiestrogens, or E_2 plus antiestrogens. Luciferase activity was detected using a TD20-20 luminometer. Our results showed that MCF-7/CCN1 cells exhibited a higher ERE-Luc activity in response to E_2 and antiestrogens than did MCF-7 and MCF-7/TM cells. In addition, the basal level of ERE-Luc activity in MCF-7/CCN1 was the highest, when compared to that of MCF-7 and MCF-7/TM cells.

These data show that blockage of the CCN1-$\alpha_6\beta_1$ interaction abolishes the transcriptional activation of ERE-genes induced by CCN-wt, suggesting a participation by integrin and CCN1 in the transcriptional activity of ER. In parallel, we demonstrated that CCN1 and ER were localized in the nucleus and co-immunoprecipitated from nuclear extracts. These results support a possible nuclear role for CCN1 as a coactivator of ER, involved in the transcriptional activation of proliferative and survival ERE-genes in breast cancer cells.

Keywords: antiestrogen resistance; cyr61- angiogenic factor; integrin receptors

284 Interference of the HER2 Extracellular Domain at Subdomain I and III Interaction Promotes Growth Advantage and Modulates Hormonal Response

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The responsiveness of estrogen receptor (ER)-positive breast cancer to endocrine therapy is frequently reduced in cells overexpressing HER2. We have demonstrated that stimulation of ER suppresses HER2, indicating that estrogen controls the activity of HER2. We have also demonstrated that cells expressing both ER and HER2 have a growth advantage, because both pathways effectively promote cell growth, proliferation, and tumor development. Once the ER is blocked, cells take advantage of the HER2 pathway and vice versa. Clinically, patients whose tumors overexpress HER2 and are ER-positive respond poorly to both endocrine therapy and trastuzumab, a monoclonal antibody targeting specifically $p185^{HER2/neu}$. Their tumors are characterized as aggressive de novo, anti-estrogen-resistant, ER-positive. A number of strategies are currently being used to target the HER2, including trastuzumab against its ECD and small-molecule inhibitors blocking its tyrosine kinase activity. Yet, there is a need to develop novel therapies inhibiting HER2 dimerization with other HER family members or to interfere with ER. To understand the latter, we developed a series of deletion mutations into subdomain III of HER2 ECD, which contributes most of the determinants involved in ligand binding and signal transduction. ECD deletion mutants of HER2 were retrovirally expressed in BT-474 (HER2+ and ER+) breast cancer cells. We have demonstrated that disruption of subdomain III (Δ8-HER2) impacts the interaction between subdomains I and III, resulting in alterations in receptor heterodimerization. Interestingly, blockage of subdomain I and III

interaction promotes anchorage-independent growth in the absence of estrogen, and cells develop an exquisite sensitivity to estradiol. On the other hand, the presence of anti-estrogens (4OH-tamoxifen and ICI-182780) does not inhibit the growth of Δ8-HER2 cells. The data identify the "functional site" of HER2 responsible for allowing HER2 overexpressing cells to bypass their hormone dependence for growth and anti-estrogen sensitivity. These findings reveal for the first time that an essential activating sequence exists on subdomain III of the HER2 ECD, as its disruption disables the HER2 heterodimerization loop and blocks subsequent activation of the HER2-driven oncogenic signaling and ER activation. High-affinity peptides and/or antibodies with specificity for this molecular-activation switch may represent a novel targeted approach for the management of HER2+/ER+ breast cancer.

Keywords: Herceptin resistance; HER2 therapy; hormonal response

285 Barrett's Esophagus, Oxidative Damage, and Apoptosis Resistance: The Role of Gastric Acid and Bile Acids

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Background: Barrett's esophagus (BE) is a premalignant condition associated with elevated risk for the development of esophageal adenocarcinoma (ADCA). Previous studies indicated that apoptosis resistance and oxidative damage are associated with the development of BE and ADCA. However, the factors and molecular pathways involved in this process are unclear. In this study, we tested the hypothesis that bile acids and gastric acid, two components of gastroesophageal refluxate, can induce oxidative stress, oxidative DNA damage, and activation of antiapoptotic STAT3 pathway. We also evaluated whether the effects of bile acids and gastric acid may be inhibited by treatment with the cytoprotective bile acid ursodeoxycholic acid (UDCA).

Methods: Oxidative DNA damage was evaluated by staining with an antibody to 8-hydroxy-deoxyguanosine (8-OH-dG) in BE tissues with different degrees of dysplasia. Furthermore, three esophageal cell lines (BE cells, normal squamous esophageal HET-1A cells, and adenocarcinoma Seg-1 cells) were exposed for 1-10 minutes to control media, media containing bile acid cocktail, media acidified to pH 4, and media at pH 4 supplemented with 0.1 mM bile acid cocktail, and evaluated for induction of reactive oxygen species (ROS). In addition, the cells were treated as above but with 0.1 mM UDCA added to the media. Immunohistochemistry was also used to assess levels of phosphorylated STAT3 in esophageal tissue samples from BE patients with different grades of dysplasia. In addition, the esophageal cells were evaluated for STAT3 activation, and IL-6 and Bcl-x_L expression.

Results: Immunohistochemical analysis showed that 8-OH-dG and phosphorylated STAT3 are present at increased levels in epithelial cells of dysplastic BE. An increase in ROS occurs after exposure to pH 4 and a bile acid cocktail in all three cell lines. These increases in ROS were inhibited by treatment with 0.1 mM UDCA. Treatment of Seg-1 cells with media containing bile acid cocktail and acidified to pH 4 induced increased activation of STAT3, IL-6 secretion, and increased expression of Bcl-x_L. Inhibition of the STAT3 pathway using STAT3 siRNA or a JAK inhibitor resulted in increased apoptosis.

Conclusion: STAT3 activation and oxidative DNA damage are increased in dysplastic BE tissue. Oxidative stress and aberrant IL-6/STAT3 signaling are induced in esophageal tissues and cells by short exposures to bile acids and low pH. These alterations, if persistent in vivo, may underlie the development of BE and tumor progression. UDCA treatment may inhibit oxidative damage induced by cytotoxic bile acids and gastric acid.

Keywords: Barrett's esophagus; bile acids; reactive oxygen

286 Molecular Imaging and Drug Discovery in Gastrointestinal Cancer

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Optical imaging technologies provide unprecedented opportunities to visualize molecular events in living cells or tumors. This includes monitoring tumorigenic conversions of normal human cells, as well as effects of in vivo gene silencing on tumor growth and progression in mouse models. Optical bioluminescence or fluorescence imaging is easily adaptable to high-throughput cell-based chemical library screening to identify small molecules with molecularly targeted antitumor effects. This includes modulation of transcriptional events, protein-protein interactions, post-translational modifications, protein stability, or subcellular localization. Molecular imaging approaches therefore offer important opportunities for new drug discovery and development targeting a number of problem areas in gastrointestinal cancer including (1) drug resistance, (2) oncogene-driven tumors, (3) mutations in tumor suppressor genes such as p53, and (4) therapeutic resistance under hypoxic conditions. Approaches include screening, structure-activity relationships (SAR by imaging), target validation in vivo, and antitumor effects in vivo. There are opportunities for collaboration between the Network for Translational Research: Optical Imaging (NTROI) program and the SPORE program to accelerate the pace of drug development. These emerging strategies provide a paradigm for accelerating drug discovery and development through use of optical imaging at multiple stages up to and including clinical evaluation.

Keywords: apoptosis; imaging; therapeutics

287 Marked Efficacy of Difluoromethylornithine Plus Sulindac in Reducing Recurrent Colorectal Adenomas in Patients With Prior Adenomas: Results of a Randomized, Placebo-Controlled Double-Blind Trial

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Epidemiologic and experimental data suggest that colon cancer should be largely preventable through dietary or chemical intervention. Polyamines are actively involved in proliferation and differentiation of many tissues. Lowering intracellular levels of polyamines by inhibiting the initial step (ornithine decarboxylase) in the polyamine synthesis pathway by the specific enzyme-activated inhibitor difluoromethylornithine (DFMO) results in a decrease of adenomas and cancers in many preclinical organ-site models, most notably the colon.

From 1989 to 1997 we conducted a series of pilot and Phase IIa and Phase IIb trials in which we determined the lowest dose at which polyamine content of the colonic flat mucosa would be lowered, with the goal of finding a nontoxic dose. We combined DFMO with the NSAID sulindac at low dose for a variety of mechanistic, experimental, and clinical reasons. In the Phase IIb trial, recurrence of adenomas was correlated with polyamine changes in the flat mucosa. Several years later, we expanded to a full Phase III trial in which recurrence of adenomas became the primary endpoint.

Based on the recommendation of the Data Safety and Monitoring Board, an accelerated second analysis was done after 70% of the patients had completed their 3-year or off-study colonoscopies. It was determined that the study could be stopped for efficacy because the study goals had been met. Adenomas ($p<.0001$) and advanced adenomas ($p<.015$) were reduced in the active arm in an amount comparable to that seen in animal models. Both adjacent rectal mucosal biopsy putrescine and spermidine levels were also markedly reduced in the active intervention arm, after both 12 and 36 months. PGE2 levels were unaffected.

Toxicity was carefully monitored. All toxic side effects of any nature and hematologic, gastrointestinal, and cardiovascular toxicities were analyzed and found to be not significantly different ($p>.05$) between the two arms. Clinical audiologic toxicity was also not different. Serial audiograms also did not show changes across the normal hearing range threshold, although more patients had a frequency decrease of 10 dB or greater across the whole range of frequencies tested.

These results confirm the value of conducting de-escalation studies before a full Phase III trial in identifying a dose and regimen for the preventive therapy of adenomas that are both efficacious and nontoxic. Larger trials will be needed to determine the absolute risk of cardiovascular events and audiologic changes from this regimen. Longer term studies will be needed to determine whether the absolute endpoint of colorectal cancer incidence can be reduced either in patients with low-stage prior colorectal cancers or very high-risk individuals.

Keywords: colon polyps; NSAIDS; polyamines

288 A Novel Nontoxic Multitargeted Approach for Pancreatic Cancer Therapy

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Among many cell signaling pathways that are critical for cancer development and progression, which ultimately lead to invasion, angiogenesis, and metastasis, EGFR signaling and activation of nuclear transcription factors are considered very important. Therefore, targeting EGFR has been a focal point in cancer therapy. Using xenograft models, we have recently reported a novel approach by which EGFR signaling could easily be attenuated, thereby killing cancer cells in vitro and inhibiting tumor growth in vivo. We have found that the isoflavone (genistein) could attenuate EGFR signaling via inactivation of the PI3K/Akt signaling pathway. Since activation of the EGFR and PI3K/Akt signaling pathway is often found in association with activation of NF-κB, it is important to delineate the role of molecular cross talk among these signaling pathways for cancer therapy. Recently, we found that NF-κB activation is affected by Notch-1 and FOXOM1, which, as such, are considered important targets in cancer therapy. Using multiple cellular and molecular approaches such as MTT assay, gene transfection, flow cytometry, real-time RT-PCR,

Western blotting, tumor cell migration, invasion, and angiogenesis assays, we found that the isoflavone genistein is a potent multitargeted agent that interrupts the cross talks between NF-κB, Notch-1, and FOXOM1, thereby killing pancreatic cancer cells in vitro and causing potent antitumor activity in vivo. Moreover, we found that inactivation of these signaling pathways by isoflavone could lead to enhanced killing of pancreatic cancer cells by conventional chemotherapeutic agents. On the basis of our results, we believe that isoflavone could be an ideal nontoxic multitargeted agent for the treatment of pancreatic cancer either alone or in combination with conventional chemotherapeutic agents.

Keywords: EGFR; isoflavone; NF-κB

289 Mouse Model of Endometrial Tumorigenesis Is Useful for Identification of Potential Molecular Marker of Invasive Disease

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Mutations in the *PTEN* tumor suppressor gene play a central and early role in the development of uterine endometrioid carcinoma (UEC), with mutations found in 30%-80% of invasive tumors and 20% of the hyperplastic precursor lesions. In addition, several studies have shown an association between *PTEN* mutations and DNA mismatch repair (MMR) deficiency in UEC, but the consequences of this association have not been thoroughly studied. Furthermore, little is known about the genetic alterations associated with transition from the noninvasive, hyperplastic precursor lesions to invasive carcinoma, a transition with diagnostic relevance. To address these issues, we used the previously described *Pten*$^{+/-}$/*Mlh1*$^{-/-}$ mouse model. The majority of complex atypical hyperplasias (CAHs), as well as carcinomas, showed biallelic inactivation of *Pten* mediated through LOH or intragenic mutation of the wild-type allele. A higher frequency of *Pten* mutations at coding repeat sequences was present in invasive lesions of *Pten*$^{+/-}$/*Mlh1*$^{-/-}$ mice compared with CAHs in the *Pten*$^{+/-}$ mice, while the frequency of LOH was similar. These findings indicate that specific mutations in *Pten* are a consequence of MMR deficiency in this setting. To identify genes differentially expressed between CAH and invasive carcinoma, global gene expression profiles were compared from noninvasive and invasive lesions in the *Pten*$^{+/-}$/*Mlh1*$^{-/-}$ mice. In the invasive lesions, oviduct-specific glycoprotein (OGP) levels were significantly elevated, as compared to CAHs. This finding was validated by real-time RT-PCR and were further substantiated by immunohistochemical staining of primary human UECs and CAHs. Our studies highlight the usefulness of this mouse model not only in understanding the genetic underpinnings of endometrial carcinoma, but also as a tool to develop diagnostic markers for endometrial carcinoma.

Keywords: endometrial carcinoma; mouse model; PTEN

290 *Mig-6* Modulates Uterine Steroid Hormone Responsiveness and Exhibits Altered Expression in Endometrial Disease

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Diseases of the female reproductive tract represent a significant women's health problem. Endometrial cancer is the most common gynecologic malignancy and the fourth most common cancer in women. Additionally, endometriosis affects an estimated 5 million women in the United States and is commonly associated with infertility and pelvic pain. One of the major hallmarks of uterine diseases is disruption of steroid hormone control of uterine cell proliferation and differentiation. Using high-density DNA microarray analysis, we identified mitogen-inducible gene 6 *(Mig-6)* as a gene regulated by progesterone (P4) and the coactivator SRC-1. Here we show that ablation of *Mig-6* displayed defects in P4 and estrogen (E2) signaling. Using homologous recombination in embryonic stem (ES) cells we have generated a conditional allele for *Mig-6*. This mouse was crossed with the PR-Cre mouse model to an ablate of *Mig-6* in the reproductive tract. Conditional ablation of *Mig-6* in the uterus resulted in sterility and the development of severe endometrial hyperplasia at 5 months of age. Female mice exhibited increased sensitivity in response to P4 and E2 treatment. Treatment of these mice with E2 resulted in the development of endometrial cancer. Analysis of *Mig-6* expression in the human endometrial samples taken from patients with endometrial cancer showed a significant reduction in *Mig-6* expression. Together, these results suggest that *Mig-6* is a critical regulator of uterine function in both the mouse and human.

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Keywords: endometrial cancer; *Mig-6*; steroid hormone

291 Development of a Preventive Pan-HPV Vaccine

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Human papillomavirus (HPV) is a causal agent for ~5.3% of cancers worldwide, including cervical cancer and subsets of genital and head and neck cancer. Persistent HPV infection is a necessary, but not sufficient, cause of cervical cancer. Of the >100 HPV genotypes, only about 12, termed "high-risk," are associated with cancer. HPV16 is present in ~50% of all cervical cancers and HPV16, HPV18, HPV31, and HPV45 together account for ~80%. Most high-risk HPV infections are subclinical and cleared by the host's immune system. The remainder produces low- or high-grade squamous intraepithelial lesions (SILs), also called cervical intraepithelial neoplasia (CIN), which also may regress spontaneously. However, persistent high-grade SIL represents the precursor lesion of cervical cancer, and carcinogenic progression is associated with integration of the viral DNA, loss of E2, and upregulation of viral oncogene expression, and chromosomal rearrangements like 3q gain. Cytologic screening of the cervix for SIL and intervention have reduced the incidence of cervical cancer in the United States by an estimated 80%, and HPV viral DNA and other molecular tests may improve screening further. The licensure of a preventive HPV vaccine based upon L1 virus-like particles ushers in a new era, but issues remain, including protection restricted to a few oncogenic HPV types, access in low resource settings, and impact on current cytologic screening protocols. Importantly, preventive HPV vaccination does not help with current HPV infection or disease. I will examine the potential of second-generation L2-based preventive HPV vaccines and a combination preventive and therapeutic HPV vaccine to address these outstanding issues.

Keywords: cervical cancer; HPV; vaccine

292 Interdisciplinary Approaches to Head and Neck Cancer Research by a Specialized Program of Research Excellence (SPORE) at Emory University Winship Cancer Institute

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The overall goal of the Emory University Winship Cancer Institute Head and Neck Cancer (HNC) SPORE is to improve prevention and treatment of head and neck cancer with a strong emphasis on new discoveries, and rapid translation to patients who are suffering from disability and morbidity caused by this disease. The SPORE program consists of four major translational research projects, three supportive cores, a developmental research program, and a career development program. The WCI HNC SPORE uses an interdisciplinary approach to meet its objectives by carrying out projects with co-investigators in basic, translational, and clinical science. All projects are hypothesis driven and explore biology, prevention, and molecular-driven and nanotechnology-driven novel therapeutic approaches in HNC. The main projects are (1) Chemoprevention with Green Tea Polyphenon E and EGFR-TKIs in HNC, (2) Targeting Death Receptors-Mediated Apoptosis for HNC, (3) Development of Novel Curcumin Analogs for the Treatment of Head and Neck Cancer, and (4) Biodegradable Nanoparticle Formulated Taxol for Targeted Therapy of HNC. All projects in our SPORE program are centered on clearly defined translational objectives including pilot clinical trials (project 1, Phase I study of chemoprevention; project 2, biomarker studies; project 3, Phase 0 trial of EF24; project 4, Phase 1b clinical study) that have close interaction with the cores. The two research cores (Specimen Resources and Pathology Core and the Biostatistics and Data Management Core) assist the main research projects, developmental research projects, and career developmental investigators in performing HNC translational research. The Administrative Core provides scientific and financial oversight for the SPORE program. We also have a strong commitment of support, including space recruitment, shared resources, and matching funds from the Emory Winship Cancer Institute, Emory University School of Medicine, Woodruff Health Science Center at Emory University, Georgia Cancer Coalition (GCC), and the Georgia Research Alliance (GRA). The Emory WCI HNC SPORE is designed as a highly integrated team approach to achieve the goals of the program and will also interact with investigators from HNC SPORES at other institutions and other research programs in order to improve outcomes for HNC patients.

This research is supported in part by P50 CA128613.

Keywords: head and neck cancer; prevention; targeted therapy

293 Bronchoalveolar Lavage Fluid as a Proximal Biofluid for Lung Cancer Biomarker Discovery: Paired Sample Analysis of Ipsilateral vs. Contralateral Lung BALF Samples by "MuD-Sep" Mass Spectrometry Profiling

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Introduction: Paired (ipsilateral, contralateral) bronchoalveolar lavage fluid (BALF) samples from 16 stage IA-IB and 12 stage II, III, and IV NSCLC lung cancer patients were subjected to multidimensional separation and proteomic profiling by MALDI-TOF mass spectrometry.

Methods: Pre-surgical BALF samples were collected under anesthesia in the operating room by injecting 100 mL saline through a bronchoscope to the tumor-containing lobe and the healthy contralateral lung. The samples were recovered under vacuum into a mucus trap, then frozen at -80 °C. Samples were concentrated by ultrafiltration >3 kDa, normalized for total protein concentration, and subjected to three dimensions of separation: denaturing depletion of albumin & IgG (Enchant Multi-Protein Affinity Separation Kit; Pall Corp.); anion exchange (AE) fractionation in six pH steps (Q HyperD F resin; Pall Corp.); and magnetic bead-based affinity purification (HIC8, WAX-pH9, WCX; Bruker Daltonics). Resulting fractions were profiled using CHCA matrix by linear MALDI-TOF MS on a Bruker Ultraflex II in the 1-20 kDa m/z range. For evaluation of higher molecular weight components, AE fractions without bead purification were neat-spotted with SPA matrix on gold targets and read in a Ciphergen PBSIIc ChipReader (Bio-Rad). Preprocessing of MALDI spectra was performed using ClinProToolsTM v2.1 software (Bruker Daltonics). Evaluation of extracted feature information was performed using paired statistical analysis before creation of classification training models.

Preliminary Data: The BALF sample depletion and AE fractionation strategy, adopted from serum profiling protocols, has resulted in a diverse assortment of spectral profile features for comparative statistical analysis by a variety of classification models. Individual patient profiles are quite distinct, with a substantial number of features showing correlated expression patterns in both lungs of certain patients. However, differentially expressed spectral features are also observed when comparing ipsilateral vs. contralateral lung within a patient. Paired-statistical evaluation of these features for significance, as well for correlation to suspected levels of blood/serum protein contamination, or the observation of significant sample subtypes, will be reported. The spectral features and classification models with utility in distinguishing Stage IA-IB ipsilateral from contralateral lung will be evaluated on comparable spectral data generated from the later stage cancer patient BALF samples.

A further assessment of the protein content of these samples after the MuD-Sep preparations will be made by both one-dimensional and two-dimensional PAGE. Combined profiling results from fractionated BALF samples may inform the selection of subproteomic samples (AE fractions, and/or magnetic bead purifications derived from them) of individual or pooled BALF samples for further characterization and protein identification by LC-MS/MS in support of biomarker discovery, identification, and validation.

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Keywords: bronchoalveolar lavage fluid; lung cancer biomarker discovery; MALDI-TOF MS proteomic profiling

294 Genetic Variations in the CYP2C9, UGT1A6, and PTGIS Genes and Lung Cancer Risk

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Regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been reported to be associated with a reduced risk of lung cancer in a few epidemiologic studies. The results from previous studies, however, have been inconsistent. Recent studies suggest that prostacyclin (PGI_2) may exert cancer-inhibitory effects in the development of lung cancer. We hypothesize that genetic polymorphisms in the genes encoding prostacyclin synthase (*PTGIS*) and enzymes involved in NSAID metabolism (*UGT1A6* and *CYP2C9*) may be associated with lung cancer risk. We evaluated this hypothesis using data from the Danish Diet, Cancer, and Health cohort and a population-based case-control study conducted in North Jutland County, Denmark, including a total of 573 lung cancer cases and 857 controls. Genotyping assays for polymorphisms in the *UGT1A6* (Thr181Ala, Arg187Ser), *CYP2C9* (Arg144Cys, Ile359Len), and *PTGIS* (T-192G [rs5580], Arg373Arg [G/T, rs5629]) genes were performed using the TaqMan

genotyping assays. For the *PTGIS* Arg373Arg (rs5629) polymorphism, the T allele was associated with a reduced risk of lung cancer with OR of 0.73 (95% CI, 0.55-0.96) and 0.72 (95% CI, 0.42-1.25) for G/T and T/T genotype, respectively, when compared to G/G genotype. No apparent interaction between NSAID use and *PTGIS* Arg373Arg polymorphism was found. No significant association of *UGT1A6* and *CYP2C9* gene polymorphisms with lung cancer risk was found. Results of this study raise the possibility that genetic polymorphisms in the *PTGIS* gene may contribute to the susceptibility of lung cancer. Further research on genetic variability in the *PTGIS* gene in relation to risk of lung cancer is needed to determine the etiologic significance of the association.

Keywords: eicosanoids; lung cancer risk; SNP analysis

295 Strategy To Identify Genes Responsible for EGFR-inhibitor Resistance and Novel Biomarkers in Lung Cancer

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The invasive/metastatic nature of epithelial cancers is linked to the epithelial-mesenchymal transition (EMT), typified by loss of epithelial markers (e.g., E-cadherin) and gain of mesenchymal markers and a motile phenotype. EMT is a normal developmental process regulated by E-box binding transcriptional repressors, including ZEB-1/2, Snail, Slug, E12/E47, and Twist. We previously reported that E-cadherin loss in lung cancer correlates with poor outcome, and we discovered that ZEB1 was responsible for this loss. Recently, we demonstrated that E-cadherin confers sensitivity to the EGFR inhibitor, gefitinib. As predicted, not only does ZEB1 expression correlate with gefitinib resistance, but treatment with HDAC inhibitors reverses the resistance and induces E-cadherin. Affymetrix array analyses of 30 non-small cell lung cancer (NSCLC) cell lines have identified a gene expression signature that corrrelates with gefitinib resistance. Among these are genes associated with EMT.

We have developed a model system for identifying ZEB1 target genes that contribute to the pathogenesis/progression of lung cancer and resistance to EGFR inhibitors. Among these, we expect to identify new targets engaged by ZEB1 along with potential biomarkers for invasive/metastatic phenotypes and ZEB1 activity. The gefitinib-sensitive (E-cadherin-positive) NSCLC cell line, NCI-H358, was stably transfected with plasmids carrying an FRT homologous recombination site and CMV-driven tetracycline repressor. MYC-tagged ZEB1, HA-tagged SNAIL, or empty vector was inserted into the FRT site by FLP recombinase-mediated homologous recombination and transfectants selected with hygromycin. Western blot verified inducible expression for both proteins. Microarray analysis (in triplicate) of gene expression changes, performed after 2 and 12 days of induction, identified a large number of suppressed and induced genes.

Initial high-interest genes were identified as those with concordant changes upon ZEB and SNAIL induction. These were compared to the 30 NSCLC cel line database. ZEB/SNAIL induced 1,075 and suppressed 1,287 genes in common. A total of 34 induced genes were upregulated and 135 suppressed genes were downregulated in gefitinib-resistant NSCLC cell lines. Similar comparisons identified 28 induced and 170 genes repressed concordantly with NSCLC lines expressing high ZEB1. We identified a number of secreted proteins induced by ZEB, of which some were induced to high levels. In vitro, normal H358 cells grow as compact epithelial sheets, but ZEB1 induction caused proliferation in multiple cell layers and detachment from substrate. By MTT assay, both ZEB and SNAIL increased resistance to gefitinib. In vivo, ZEB1-expressing cells formed rapidly growing, highly invasive tumors that were heterogeneous and poorly differentiated.

Our results confirm the role of ZEB1 in modulating response to EGFR inhibitors and demonstrate the important contribution these transcription factors can make to invasive behavior of lung cancers. Similar studies, including shRNA-mediated ZEB1 knockdowns, are now being performed in additional cell lines to establish a consensus set of ZEB1 target genes. We anticipate that these will result in novel biomarkers and new therapeutic interventions.

Keywords: invasion; metastasis; ZEB1

296 Shotgun Proteomic Analysis of Lung Adenocarcinoma

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We explored the utility of the shotgun proteomics approach using multidimensional liquid chromatography-tandem mass spectrometry (LC-MS-MS) to discover proteins differentially expressed between normal lung and adenocarcinoma of the lung.

Methods: Three lung adenocarcinomas and three adjacent normal samples were lysed with 50% 10 mM Tris HCl, 50% trifluoroethanol, followed by overnight trypsin digestion. Strong cation exchange (SCX) and/or isoelectric focusing (IEF) peptide separation methods were used to fractionate the tissue lysates before reversed-phase LC-MS-MS (Thermo LTQ ion trap mass spectrometer). Each pair of samples was analyzed in triplicate. Tandem mass spectra were searched in a data-dependent scanning mode using the SEQUEST algorithm against the International Protein Index 3.151 human database containing both forward and reverse sequences for all proteins. Search outputs were filtered with the HUPO high-confidence peptide identification criteria.

Results: We identified 1,369 proteins with at least 2 peptide hits from 3 tumors and adjacent normal samples. Of these, 764 proteins were observed only in tumor tissues and 64 proteins were observed only in normal tissues. We identified 200 proteins with 10 or more peptide hits. Of these, 93 proteins were found only in tumor tissues, including alpha tubulin, 14-3-3 proteins, and p63. Approximately 55% of the identified proteins were found in replicate SCX-LC-MS-MS analyses. Of the proteins with subcellular localization information in the Gene Ontology database, there were 131 membrane proteins (36%), 93 cytoplasmic proteins (25%), 69 nuclear proteins (19%), and 75 extracellular proteins (20%). IEF combined with LC-MS-MS analysis identified 681 proteins from one paired normal and tumor tissue, and 81% (553) of these proteins were also observed in 3 paired SCX separation experiments.

Conclusions: We identified hundreds of proteins differentially expressed between adenocarcinomas and noninvolved adjacent lung tissues using shotgun proteomic analysis. All subcellular compartments were represented, showing the suitability of this approach as a profiling method. IEF peptide separation yielded results comparable to SCX separation. Although optimization of this tissue shotgun proteomics platform is needed, these results show the potential utility of this approach for characterizing tumor proteomes and providing candidate diagnostic biomarkers.

This research was supported by the Lung SPORE CA90949.

Keywords: diagnostic biomarkers; proteomics

297 Verification of Lung Cancer Biomarker Candidates by Liquid Chromatography-Tandem Mass Spectrometry With Multiple Reaction Monitoring

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Proteomics, transcriptomics, and other high-dimensional analyses yield many potential protein biomarkers for cancer, but validation of large numbers of candidates is impractical. Here we describe the use of liquid chromatography-tandem mass spectrometry with multiple reaction monitoring (MRM) to verify protein biomarker candidates in tumor and control tissues. In this approach, MRM is used to detect MS-MS fragmentations of specific tryptic peptides derived from the proteins of interest. We used both Thermo LTQ linear ion trap and Thermo TSQ Quantum triple quadrupole instruments to monitor up to 10 (LTQ) and 240 (Quantum) MRM transitions to detect up to 10 (LTQ) and 60 (Quantum) proteins in a single run. Each peptide is monitored by four transitions corresponding to major b- or y-ion fragmentations. The integrated chromatographic peak areas for the transitions were summed and compared to summed peak areas for beta-actin, which was used as a normalization standard. We applied this approach to screen 25 lung cancer biomarker candidates in a test set of 10 pairs of tumor tissues and control tissues (5 adenocarcinoma and 5 squamous lung tumors). The MRM analyses detected 9 candidate proteins, of which 2 were downregulated and 3 were upregulated in tumor tissues. These 9 candidates were detected in unfractionated tissue lysates, which indicates that they are relatively abundant proteins. Fractionation of tissue protein extracts or peptides derived from tissue protein tryptic digests can enable detection of lower abundance candidates. Refinements of the method will address the optimum number of peptides and MRM transitions to monitor to balance sensitivity, specificity, and throughput. This method provides a generally

applicable means of verifying and screening candidate markers before application of rigorous quantitation with stable isotope-labeled peptide standards.

Keywords: diagnostic biomarkers; proteomics

298 Serum Shotgun Proteomic Analysis of Pools of Patients With Lung Cancer or Matched Controls

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Introduction: At present no diagnostic or screening tool is available for the early detection of lung cancer. Our goal was to select candidate biomarkers from the serum proteome for the detection of lung cancer.

Methods: Proteomic analysis was performed on triplicates of three pools of serum samples, with each pool consisting of 20 patients with squamous, adenocarcinoma of the lung, or control subjects matched for age, sex, and smoking history. Sera were depleted of albumin and immunoglobulins using the HiTrap Cibacorn Blue and HiTrap Protein G columns, respectively, and the flow-through (Fraction 1) and the albumin-bound (Fraction 2) fractions were analyzed separately. Following trypsin digestion, each fraction was fractionated by strong-cation exchange chromatography into 15 fractions and analyzed using reverse-phase liquid chromatography coupled to a Thermo LTQ ion trap mass spectrometer. Tandem mass spectra were collected using data-dependent scanning mode on the top nine most intense ions and searched using the SEQUEST algorithm against the International Protein Index 3.151 human database containing both forward and reverse sequences for all proteins. HUPO filtering criteria were applied to the data using an in-house software tool (CHIPS). For each of these peptides or proteins, spectral counts were obtained from each of the replicate runs and these were compared among the three groups using a Poisson-based general linear model with adjustment for false discovery rate.

Results: From the triplicates of the 3 pools analyzed, a total of 1,784 unique peptides were identified from both Fraction 1 (1,116 peptides) and Fraction 2 (668 peptides). The peptide counts were similar among the pools: 1,606 for the controls, 1,518 for the squamous, and 1,572 for the adenocarcinomas. Based on peptide frequency, we found statistical difference in the counts of 30 peptides between adenocarcinoma and controls and of 62 peptides between squamous and controls.

Conclusions: Shotgun proteomic analysis of pooled serum samples resulted in a list of differentially expressed serum proteins that may facilitate lung cancer biomarker discovery. Although methodological and analytical tool refinements as well as validation in an independent set of serum samples are needed to confirm the clinical significance of the peptide biomarkers identified in this study, direct shotgun proteomic analysis of the serum was proven to have the potential of identifying biomarkers of lung cancer.

This work is supported by the Lung SPORE CA90949.

Keywords: diagnosis; proteomics; serum

299 Human Bronchial Epithelial Cells for Studying Multistep Lung Cancer Pathogenesis and Identifying Cytokine Biomarkers and Therapeutic Targets

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Purpose: (1) To identify a minimal set of changes required for normal human bronchial epithelial cells to transform to lung cancer. (2) To identify cytokines induced by defined oncogenes as biomarkers and therapeutic targets of lung cancer.

Introduction: We established a series of cdk4/hTERT-immortalized human bronchial epithelial cell (HBEC) lines, which are able to differentiate into mature airway cells in organotypic cultures but do not form colonies in soft agar or tumors in nude mice. We observed that combinations of p53 knockdown with mutant *EGFR* or with physiological levels of oncogenic *KRAS*^{V12} progressed HBEC3s part but not all of the way toward malignancy. These cells form colonies in soft agar, but fail to form tumors in nude or

SCID mice. To transform HBECs to full malignancy we have explored three additional steps: (1) expression of higher levels of oncogenic KRAS^{V12}, (2) exogenous expression of *c-Myc*, and (3) biologic selection for the subset of cells generating colonies in soft agar colony. Also, to identify cytokines induced by defined oncogenes as biomarkers and therapeutic targets of lung cancer, we performed Illumina microarray gene expression analysis of HBEC with *KRAS*^{V12} or mutant *EGFRs*.

Methods: HBEC3, HBEC15, and HBEC17 (from three different people) were engineered with various vector systems to have stable p53 knockdown (with shRNA), express oncogenic *KRAS*^{V12} at physiologic or tenfold higher, and exogenously overexpress c-Myc. These changes were introduced alone and in combination, and the manipulated cells were tested for soft agar colony formation efficiency (SACFE) and subcutaneous tumor formation in NOD/SCID mice. Illumina array gene expression analysis and array-CGH were performed to determine genomic changes associated with malignant progression as well as to identify cytokines induced by defined oncogenes.

Results: With higher expression of *KRAS*^{V12} alone, SACFEs were ~3%, and the combination of higher *KRAS*^{V12} and p53 knockdown increased SACFE to 10%. Importantly, the combination of higher *KRAS*^{V12} and p53 knockdown led to occasional tumor formation in NOD/SCID mice (2 tumors/8 mice) as well as development of a subset of very large (10 times larger) soft agar colonies (at a rate of ~0.1%-0.2% of plated cells). Furthermore, addition of *c-Myc* to this combination led to 100% tumor formation in NOD/SCID mice (7 tumors/7 mice). This combination (p53 knockdown, *KRAS*^{V12}, and *c-Myc*) was also tested for another HBEC, HBEC17, and led to three tumor formations of eight mice injected. Large colonies formed by HBEC3 with p53 knockdown and *KRAS*^{V12} were isolated and found to have SACFE of ~10%-15% with the large colony phenotype breeding true. Seven large colonies were expanded and three formed tumors. Of interest, two were adenocarcinomas and one was a squamous cell carcinoma. Gene expression analysis of HEBC3 with *KRAS*^{V12} or mutant *EGFR* identified several cytokines upregulated by these oncogenes, including VEGF and IL8.

Conclusions: (1) The combination of five oncogenic changes occurring in human lung cancer (telomerase expression, p16/Rb bypass, p53 ablation, oncogenic *KRAS*^{V12}, and c-Myc) transforms HBECs to full malignancy, with differences in tumor formation frequency between HBECs derived from different individuals (interindividual variation in the susceptibility to oncogenic transformation). (2) One HBEC strain can give rise to lung cancers with different histologies. (3) Defined oncogene changes lead to specific new cytokine expression and secretion patterns that are candidate biomarkers and therapeutic targets for lung cancer.

Keywords: cytokines; oncogenes; tumor suppressor genes

300 Interaction Between the COX-2 Pathway and HGF/c-Met Pathway in Non-Small Cell Lung Cancer

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Hepatocyte growth factor (HGF) and its receptor c-Met have been shown to play a role in non-small cell lung cancer (NSCLC) development and progression, and high expression levels of both HGF and c-Met are correlated with poor survival. Some of the oncogenic actions of HGF are thought to be mediated by cyclooxygenase-2 (COX-2), resulting in the production of prostaglandin E2 (PGE2). We have previously examined five c-Met-positive NSCLC cell lines (201T, 273T, 784T, A549, and H23) and have shown that 50 ng/mL HGF can increase COX-2 protein expression by up to fivefold, and that this induction occurred through both the Erk1/2 and p38 pathways. This increase in COX-2 protein was accompanied by the secretion of PGE2, and, in turn, 50 μM PGE2 treatment resulted in increased lung tumor cell proliferation. We have confirmed these findings and have further shown by Northern analysis that 50 ng/mL HGF can also increase COX-2 mRNA expression to a maximum 3.5-fold over basal levels following serum deprivation. Since COX-2 mRNA stability is a known mechanism of COX-2 gene regulation, we are currently measuring the half-life of COX-2 mRNA in the presence and absence of HGF in NSCLC cells. Additionally, HGF induction of COX-2 was accompanied by activation of the AP-1, c/ebp, and creb transcription factors detected by TranSignal Protein/DNA arrays; recognition sequences for these transcription factors are found in the COX-2 promoter. AP-1, c/ebp, and creb transcription factor activation in NSCLC nuclear extracts treated with 50 ng/mL HGF for 1 hour was also confirmed using electrophoretic mobility shift assays. HGF-induced binding was reduced in the presence of Erk1/2 (U0126) or p38 (SB202190) inhibitors and was eliminated by unlabeled competition reactions. Addition of 50 μM PGE2 to NSCLC cells can also rapidly phosphorylate c-Met by a maximum 3.3-fold, while significantly promoting secretion of TGFα into the culture media. This suggests that HGF/c-Met pathway activation resulting in PGE2 secretion can further activate the c-Met pathway in a reinforcing loop, establishing a rationale for dual therapeutic targeting. HGF (50 ng/mL) and PGE2 (10 μM) can stimulate invasion in NSCLC cell lines by 1.3-fold and 1.8-fold ($p = .0420$ and $p = .0042$), respectively. Cells transiently transfected with a c-Met antisense U6 expression plasmid, or treated

with a COX-2 inhibitor (celecoxib), could significantly decrease the HGF- or PGE2-induced invasion. The PGE2-induced invasion was also Src-dependent. Combined targeting of both pathways resulted in enhanced anti-invasion effects versus either c-Met antisense or celecoxib treatment alone. These findings suggest that targeting the HGF/c-Met pathway in conjunction with inhibiting the COX-2 pathway is a potentially effective therapeutic strategy for lung cancer.

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Keywords: c-Met; COX-2; lung cancer

301 Germline Genetic Variations in Drug Action Pathways Predict Clinical Outcomes in Advanced Lung Cancer Treated With Platinum-Based Chemotherapy

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Purpose: Genetic polymorphisms contribute to inter-individual variation in drug response. However, a single polymorphism is likely to exhibit a modest effect. Therefore, we applied a pathway-based approach to evaluate the cumulative effect of multiple polymorphisms on clinical outcome of patients with non-small cell lung cancer (NSCLC).

Methods: We genotyped 25 functional polymorphisms in 16 key genes involved in cisplatin metabolism and action and evaluated their associations with overall survival in 229 NSCLC patients receiving first-line cisplatin-based chemotherapy.

Results: Several biologically plausible main effects were identified in individual analysis. More importantly, when six polymorphisms in nucleotide excision repair genes were analyzed jointly, a significant trend of reduced risk of death with decreasing number of putative unfavorable genotypes was observed (p for trend <.001; log-rank p<.001). Survival tree analysis revealed potential higher-order gene-gene interactions and categorized subgroups with dramatically different survival experiences, based on distinct genotype profiles. The median survival time was 78.5 months for terminal node 1 in the low-risk group, 15.1 months for terminal node 10 in the medium-risk group, and 6.7 months for terminal node 9 in the high-risk group (log rank p<.001). We also constructed a prediction hazard model. The area under the curve (AUC) increased from 0.71 (using clinical variables only) to 0.84 (using clinical, epidemiological, and genetic variations from survival tree analysis).

Conclusions: Our results highlight the clinical potential of taking a pathway-based approach and using a survival tree analytic approach to identify subgroups of patients with distinctly differing outcomes.

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Keywords: chemotherapy; pharmacogenetics; prediction model

302 Function of PIK3CA Mutations and Gene Copy Number in Human Lung Cancers

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Background: Somatic mutations mainly in the helical or kinase domains of PIK3CA have been reported in various human cancers including lung cancer, but the functional effects of these mutations in lung cancer are still unclear. Chromosome 3q, where PIK3CA is located, is known to be frequently amplified in lung cancer.

Methods: We analyzed the mutation status of exons 9 (helical domain) and 20 (kinase domain) of PIK3CA using 86 non-small cell lung cancer (NSCLC) cell lines, 47 small cell lung cancer (SCLC) cell lines, and 691 resected NSCLC tumors from patients in Japan (n = 323), Taiwan (n = 148), United States (n = 150), and Australia (n = 70). We also searched for PIK3CA gene copy number in the same cell lines and 356 of 691 NSCLC tumors using quantitative real-time PCR. We also measured PI3-kinase activity in

PIK3CA mutant and wild-type cell lines including one cell line with increased PIK3CA copy number to assess the effects of PIK3CA gene alteration. We investigated protein expression of PI3K/Akt pathway in PIK3CA-mutant and amplified cell lines by Western blotting. For functional analyses, we performed knockdown of PIK3CA using siRNA and verified the effects of inhibition of PIK3CA on cell proliferation status in PIK3CA mutant cell lines.

Results: We identified mutations in NSCLC cell lines (4 of 86, 4.7%), SCLC cell lines (3 of 47, 6.4%) and NSCLC tumors (11 of 691, 1.6%). The mutations in NSCLC were more frequent in exon 9 (3 of 4 in cell lines and 7 of 11 in tumors) than in exon 20 while all the mutations in SCLC cell lines were in exon 20. PIK3CA mutation status was not associated with patient age, gender, smoking history, clinical stage, or ethnicity in resected NSCLC tumors. We detected increased PIK3CA copy number in NSCLC cell lines (9 of 86, 10.5%), SCLC cell lines (2 of 47, 4.3%), and NSCLC tumors (61 of 356, 17.1%). Increased PIK3CA copy number was more frequent in squamous cell carcinoma (5 of 14 cell lines, 35.7%, and 46 of 139 tumors, 33.1%) than in adenocarcinoma (4 of 51 cell lines, 7.8%, and 12 of 195 tumors, 6.2%). Mutations and highly increased gene copy number in PIK3CA were mutually exclusive. While the mutation status of EGFR, KRAS, BRAF, and HER2 genes were mutually exclusive, one cell line and three clinical samples with PIK3CA mutation also had KRAS or EGFR mutations. Therefore, mutation status of PIK3CA is not mutually exclusive to the other genes. All the PIK3CA mutant cell lines (4 NSCLC, 3 SCLC) had significantly increased activity of PI3-kinase compared to PIK3CA wild-type cell lines. One wild-type cell line with increased PIK3CA copy number also had increased PI3-Kinase activity. Phospho-Akt was expressed in PIK3CA-altered cell lines indicating that mutant or amplified PIK3CA has increased kinase activity. Knockdown of PIK3CA by siRNA resulted in absent expression of phosphorylated Akt, which is downstream of PIK3CA, and inhibited cell growth of PIK3CA mutant cells.

Conclusions: PIK3CA mutations are present in a subset of NSCLC and SCLC cancers and amplifications target squamous cell carcinomas. Mutations and amplifications in PIK3CA are mutually exclusive, whereas mutations in PIK3CA are not mutually exclusive of mutations in KRAS or EGFR. PIK3CA alterations are associated with increased PI3-kinase activity, and knockdown of PIK3CA results in inhibition of cell growth of PIK3CA mutant cells. These findings suggest that PIK3CA may be a candidate for targeted therapy in lung cancer.

Keywords: gene amplification; siRNA; targeted therapy

303 Circulating Tumor Cells as Biomarkers in Patients With Metastatic Prostate Cancer

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Background: Developing tumor-specific markers to select targeted therapies and to assess clinical outcome in patients with metastatic prostate cancer remains a significant area of unmet need, given the modest association with changes in PSA, and the limitations of imaging distant metastases. Antibody-capture technologies have been applied to isolate circulating tumor cells (CTCs) from small volumes of peripheral blood. Profiling prostate tumor specimens at Memorial Sloan-Kettering Cancer Center has shown that patients with progression, despite castrate levels of testosterone, harbor changes in the androgen receptor, such as amplification of androgen receptor (AR), rendering tumor cells more sensitive to androgen, and promoting tumor proliferation.

Methods: CTCs, positive for epithelial cellular adhesion molecule and nuclear DAPI, and negative for CD45 white blood cell marker, were isolated from 120 patients with clinical castrate metastatic disease. All patients had rising prostate-specific antigen (PSA) levels and were on stable treatment regimens at the time of CTC sampling. Bone scan index (BSI) was measured by calculating the bone marrow involvement from the region of the skeleton and the bone surface area involved by metastasis. FISH was performed in CTC for AR and myc genes, as previously described.

Results: The mean age in this patient cohort was 70 years, and median PSA at the time of CTC draw was 117 ng/mL (interquartiles range, IQR, 40.1-399). The patterns of metastatic spread included disease in soft tissue only in 12 patients (10%), in bone and soft tissue in 67 (56%), and in bone only in 41 patients (34%). Median CTC count was 9 cells per 7.5 mL of blood (IQR = 2-51). A large number of patients (57%) had five or more circulating tumor cells, while only 21% patients had one or less CTC per sample of blood. CTC counts were higher in patients with bone involvement compared with soft-tissue-only metastasis (11 vs. 2.5, $p<.01$), while only moderate correlations to BSI and PSA were noted. Patients failing only hormonal ablation showed significantly fewer CTC (2.5, IQR = 1-4), compared to patients failing first line of therapy (10, IQR = 4.25-47.75), or multiple therapies (20, IQR = 3-91). FISH analysis in CTC for AR showed more frequently gene amplification or gains in copy number in patients with higher CTC count.

Conclusions: In this report, we show that CTC frequency is related to patterns of metastatic spread and prior therapeutic history, and not simply a matter of increasing disease burden. We have shown an increased presence of AR chromosomal abnormalities in patients with more advanced disease. The results suggest that the ability to shed cells into the circulation and the molecular profiles of CTC are intrinsic properties of the tumor, providing unique information that, properly applied, can impact significantly on patient management and clinical trial design.

Keywords: biomarker; CTC (circulating cell tumor); prostate

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