4th Principal Investigators Meeting Abstracts

4th Principal Investigators Meeting
Innovative Molecular Analysis Technologies Program
June 16-18, 2003
Hilton San Diego Resort
San Diego, California

Abstracts

Daniel Kenan, M.D., Ph.D.
Duke University

Differential Gene Expression in Endothelial Cells by RNP-tag Analysis

Daniel J. Kenan,[1] Barbara D. Lipes,[1] Luiz Penalva,[2] Christopher D. Kontos,[3] and Jack D. Keene[2]

[1]Department of Pathology, [2]Department of Molecular Genetics and Microbiology, [3]Department of Cardiology, Duke University Medical Center

Despite the central role of endothelial cells (ECs) in cancer biology, relatively little is known about the molecular perturbations that occur in tumor endothelium and their relationship to disease. Tumor ECs are particularly difficult to study because their behavior is influenced by characteristics of the tumor microvascular environment that cannot be recapitulated ex vivo, including EC functional interactions with adjacent stromal and tumor cells and their dependence on blood flow and extracellular matrix for stable differentiation. This project seeks to develop a robust technology platform enabling analysis of tumor EC gene expression within native tissues. The technology is based on the ability to co-immunoprecipitate RNA binding proteins together with their associated mRNA species from cell and tissue extracts. The primary implementation of the technology will engineer a transgenic mouse expressing an epitope-tagged poly(A) binding protein under control of an endothelium-specific promoter. Anti-epitope tag antibodies can then be used to recover endothelium mRNAs from both normal and tumor transgenic tissue extracts. Recovered mRNAs can be detected and quantitated in high-throughput fashion using microarray technologies. In addition, the mRNAs will be used as input for construction of SAGE and EST libraries that will serve to validate the microarray data as well as to guide the construction of EC-targeted custom microarrays. Human homologues will be identified for any genes that are differentially expressed in murine tumor endothelium, and the homologues will be validated for specificity of expression in human tumor endothelium. The technology will be used to study murine and human EC gene expression in a variety of tumors and normal tissues across a range of anatomic sites. Detected alterations in tumor EC gene expression that may affect disease progression or that can serve as diagnostic markers or therapeutic targets will be validated in ECs from different tumor and tissue types. This project will generate reagents, information, and technologies that are likely to lead to improved detection and treatment of cancer.

Gerard McGarrity, Ph.D.
Intronn, Inc.

Real-Time *in vivo* Imaging of Gene Expression by SMaRTTM (Spliceosome Mediated RNA Trans-Splicing)

M. Puttaraju,[1] S. Bhaumik,[2] L.G. Mitchell,[1] G. McGarrity,[1] G. Temple,[1] and S.S. Gambhir[2]

[1]Intronn Inc., and [2]The Crump Institute for Molecular Imaging, Department of Molecular and Medical Pharmacology, UCLA School of Medicine

Objectives: Spliceosome-mediated RNA trans-splicing (SMaRT) is a novel technology to reprogram genes at the level of mRNA splicing. We have previously reported imaging of gene expression in live cells and animals by trans-splicing of a bioluminescence reporter gene, synthetic Renilla luciferase (hRluc) (AMI, 4:S17, 2002). Here, we report our recent developments to improve the efficiency of SMaRTTM for real-time imaging.

Methods: To measure the maximal level of signal generated through RNA trans-splicing, different sizes of hemi-reporter "mini-gene targets" consisting of a part of the hRluc sequence as "5' exon" coupled to the coding sequence for human papilloma virus (HPV16) E7 and the intronic sequences immediately upstream were constructed, here referred to as HPV-LucT. Two pre-trans-splicing molecules (PTMs) were engineered, consisting of a "binding domain" complementary to the HPV-LucT followed by 3' splice elements (branch point sequence, polypyrimidine tract, and acceptor AG dinucleotide) and two different lengths (255nt and 50nt) of hRluc sequence as a "3' exon." Transient transfections of N2a/293T cells were done with appropriate combinations of HPV-LucT and PTMs capable of regenerating, via trans-splicing of 5' and 3' hRluc exons, the active luciferase. Recovered luciferase (hRL) signal was measured using cell extracts and a luminometer. Confirmation of intact hRluc mRNA and protein was further assessed with RT-PCR and Western blotting.

in vivo delivery of different PTMs were performed by tail vein injection of transferrin-polyethylineamine (Tf-PEI)-PTM complexes (Mol. Ther. In Press) into nude mice (N=2) bearing transiently transfected HPV-LucTN2a cells (5x106 cells) implanted subcutaneously. The mice were imaged 24 hours later, using a cooled charge-coupled device (CCD) camera (PNAS 99:377-382, 2002) after tail vein injection of the substrate coelenterazine (60 µg/mouse). Control tumors consisting of N2a cells (5x106 cells) were implanted in the same mice.

Results: Cis-splicing of HPV-LucT3 produced no detectable luciferase signal in cell culture. Depending on the PTM exon size, we obtained ~5-15% restoration of hRluc. Smaller PTMs repaired HPV-LucT more efficiently and showed significantly (P0‹.05) higher hRL activity than larger PTMs. Statistically significant (P‹0.05) signals were also generated *in vivo* from HPV-T3N2a-implanted cells (6.9E+03 p/s/cm2/sr), in comparison to control N2a implanted cells (3.0E+03 p/s/cm2/sr) in the same mouse, when imaged under cooled CCD camera.

Conclusions: These results indicate that variations in size of the 3'-hemireporter gene within a PTM can significantly influence its trans-splicing efficiency, both *in vitro* and *in vivo* . We have shown previously that variations in size and sequence of PTM binding domains, as well as in choice of PTM 3' splice elements, can markedly affect the efficiency and specificity of the trans-splicing reaction (Mol. Ther. 4:105-114, 2001). These results taken together indicate that multiple features of a PTM are potentially important in trans-splicing reactions. We are currently developing a high-throughput genetic screen to discover and define the most optimal combinations of PTM elements for molecular imaging and therapy.

Further, this study demonstrates the feasibility of imaging of mRNA repair, both *in vitro* and *in vivo* This approach should eventually allow imaging of endogenous mRNA in living subjects, potentially imaging the expression of a wide variety of endogenous genes.

Alvin Beitz, Ph.D.
University of Minnesota

Use of 2-D Gel/Mass Spectrometry and Oligonucleotide DNA Microarrays To Study Tumor Protein Production *in vivo* and *in vitro*

Alvin J. Beitz, Mary Baddorf, and Daryl Hoskins Department of Veterinary Pathobiology, University of Minnesota

Our lab is interested in determining what factors are secreted by tumors *in vivo* and how tumor cells change when they grow in an *in situ* environment as compared to an *in vitro* culture. To study this we have developed an *in vivo* microperfusion procedure to directly sample tumor secreted proteins and peptides in a mouse hindpaw fibrosarcoma model. We are comparing samples of tumor secreted proteins isolated *in vivo* with those found in tumor homogenates and with those isolated from tumor cells *in vitro* . Proteins in microperfusion or homogenate samples are isolated on 2-D gels and subsequently trypsinized and identified using mass spectrometry. Early results on homogenates of the foot tumor show that while the 2-D gel electrophoresis is going very well, the MALDI-TOF identification of proteins remains unreliable at the present time. Because of the limitations we have encountered with MALDI-TOF, we are currently starting to utilize MALDI-MS/MS and ESI-MS/MS procedures rather than MALDI-TOF in order to increase the reliability of protein identification.

In addition mRNA is being isolated from tumor cells *in vivo* and *in vitro* and message expression analyzed using Affymetrix microarrays. We have found a number of tumor cell mRNAs that are significantly up-regulated *in vivo* compared to the cells in culture. These include P lysozyme; cathepsin S; chemokine receptor 5; small inducible cytokine B subfamily, member 9; guanylate nucleotide binding protein 1, Fc receptor, IgG low affinity Iib; and transforming growth factor, beta-induced, 68 kDa; and chemokine receptor 1. Additional genes that were up- or down-regulated will be reported at the meeting. By comparing mRNA expression with protein expression, we hope to elucidate

the effects of the tumor environment on protein production and secretion.

David Morris, Ph.D.
University of Washington

Translating a Transcriptome: High-Throughput Analysis of mRNA Translation

David R. Morris,[1] Vivian L. MacKay,[1] Xiaohong Li,[2] Kyle A. Serikawa,[1] Xie L. Xu,[2] G. Lynn Law,[1] Lue Ping Zhao,[2] and Roger Bumgarner[3]

Departments of [1]Biochemistry and 3Microbiology, University of Washington; and [2]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center

A transcriptome provides the database from which a cell assembles its unique collection of proteins. However, translation of individual mRNA species into their encoded proteins can be regulated, sometimes resulting in discordance between the level of an mRNA and the abundance of its protein product. We have developed a methodology, translation state array analysis or TSAA, that simultaneously assesses the level of an mRNA and its association with the translational machinery. Detailed descriptions through TSAA of the translational behavior of 4931 transcripts of Saccharomyces cerevisiae show that, indeed, there is a striking diversity across the constellation of mRNA molecules. Transcripts of different genes can vary from complete engagement with polysomes to total sequestration in mRNP particles. Of those mRNAs primarily located in polysomes, the average ribosome spacing on the message can vary by more than an order of magnitude. Thus, this spectrum of translational behavior provides an extraordinary dynamic range of regulation, exclusive of actual transcript levels. These diverse translational properties do not show a strong correlation with global mRNA properties such as transcript abundance, codon bias, initiator AUG context, or estimated lengths of the untranslated regions. For example, abundant mRNAs tend on average to be better translated than the population as a whole, but some representatives of this class, such as the mRNA products of GCN4 and HAC1, are located only to a minor extent in polysomes. On the other hand, low-abundance mRNAs often localize greater than 90% in polysomes, examples being the CDC28, CLB3, and ADE1 products. These results are consistent with each mRNA having its own set of unique structural properties that confer its specific translation activity. The information garnered from this analysis allows one to estimate relative rates of protein production across the transcriptome and provides new insights into this additional level of gene expression control.

Zuyi Wang, Ph.D.
Catholic University of America

Machine Learning for Molecular Analysis of Cancer

Yue Wang,[1] Zuyi Wang,[2] Javed Khan,[3] Robert Clarke,[4] Junying Zhang,[5] and Jianhua Xuan[5]

1Virginia Polytechnic Institute and State University, [2]Children's National Medical Center, [3]National Cancer Institute, [4]Georgetown University Medical Center, [5]The Catholic University of America

The challenge of cancer treatment has been to target specific therapies to pathogenetically distinct cancer types, to maximize efficacy, and to minimize toxicity. However, cancers with similar histopathological appearance can follow significantly different clinical courses and show different responses to therapy. The recent development of gene microarrays provides an opportunity to take a genome-wide approach to predict clinical heterogeneity in cancer treatment and potentially discover new diagnostic and therapeutic targets. Such global views are also likely to reveal previously unrecognized patterns of gene regulation and generate new hypotheses warranting further study. This talk will first present our comprehensive framework aimed at developing a statistically principled, model-supported, and visually insightful machine-learning approach for microarray gene expression data analysis. The discussion will then focus on pheno/gene cluster discovery by VISual Data Analyzer (VISDA) and pheno-classification/ prediction by adaptive multilayer perceptron neural networks (wFC-DCA-MLP).

Dale Larson, M.S.
Harvard Medical School

Surface Plasmon Enhanced Microscopy; A New Technology with Optical Resolution of 10 nm

Dale Larson Harvard Medical School

SPEM is a new scanning microscopy technology with optical resolution that rivals electron microscopy. SPEM is based on a novel photonics device invented in the Technology and Engineering Center at Harvard Medical School that produces a small, bright source of propagating light. The device acts as a "photonic funnel"; light is collected over a large area (relative to the aperture) and is emitted through the aperture with an effective concentration of approximately 3X (300%). The device can be produced to provide either a single light source or an array of independent light sources. Devices are currently being fabricated with 35-nm to 150-nm apertures, and apertures around 5-nm to 10-nm are expected in the near future.

This photonics device forms the basis for a new scanning microscopy technology, SPEM, where an array of nanometric light sources illuminate a sample to excite fluorescently labeled molecules. The coordinates of the fluorescence are recorded and the device is stepped (step size on the order of the aperture size) over one position and fluorescence is excited and recorded again. When the scan is completed the coordinates where fluorescence was measured are used to assemble an ultra-high-resolution image. The first application of SPEM will be for tissue samples embedded in either OCT or paraffin. Multi-spectral imaging using either quantum dots or fluorophores is enhanced with SPEM because the small excitation area dramatically simplifies

deconvolution of emission spectra from multiple sources by limiting the number of labeled molecules that can be in the small area.

Advantages of SPEM include:
- Resolution of <=50nm, and possibly down to 5-nm
- Fast scanning due to the array format (using a hexagonal array with ~1,000 to 10,000 apertures)
- Fast scanning due to increased irradiance
- Reduced background fluorescence because only a fraction of the sample is illuminated for each step
- Compatibility with current immunohistochemical practices
- Can be designed as an accessory to existing inverted microscopes

Ion image (50kX magnification) of a three-aperture linear array (150-nm apertures with center-to-center spacing of 350 nm). The 2-μm square is used to normalize the emission from the apertures in the array.
Emission from the three-aperture linear array (1.4 numerical aperture oil immersion lens) showing partially collimated light.

Bruce Mayer, Ph.D.
University of Connecticut

SH2 Profiling: Classifying Tumor Cells on the Basis of Global Tyrosine Phosphorylation State

Kazuya Machida[1], Gonzalo Rivera[1], Irina Nurieyeva[1], Peter Nollau[2], and Bruce J. Mayer[1]

[1]University of Connecticut Health Center, and [2]Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany

Signals that regulate cell proliferation, differentiation, and motility are often transmitted via activation of tyrosine kinases, and amplification or mutational activation of tyrosine kinases is often seen in human cancers. Src Homology 2 (SH2) and PhosphoTyrosine Binding (PTB) domains are small, modular protein domains that bind specifically to tyrosine-phosphorylated peptides. Over a hundred different SH2 and PTB domains are known in humans; each of these domains binds to a different spectrum of tyrosine-phosphorylated peptides. We are developing methods that use SH2 and PTB domain binding to probe the global tyrosine phosphorylation state of cells and are exploring whether such patterns can provide the basis for classification of tumor cells.

We have used a far-Western blot-based SH2 profiling method to analyze both tumor-derived cell lines and primary human cancer samples. The method provides a wealth of information and has the potential to serve as the basis for both class discovery and class assignment. It can also highlight specific tyrosine-phosphorylated proteins that correlate with clinically relevant parameters and which can subsequently be identified. Prospective studies are currently under way to profile tumor samples from patients with

hematological malignancies and carcinoma of the breast. Over 30 SH2 or PTB domain probes are currently available in the laboratory, and ongoing studies are aimed at further improving the sensitivity of the SH2 domain probes and decreasing background. We have also found that SH2 domains can be used to probe sections of paraffin-embedded, formalin-fixed tumor specimens, opening the door to large-scale retrospective studies to establish whether patterns of SH2 binding significantly correlate with clinical outcomes.

We have also been seeking to develop high-throughput, quantitative SH2 profiling assays that can be used for the rapid analysis of small amounts of tissue. Toward this end we have tested reverse-phase protein arrays, which show promise for quantitating relative levels of SH2-binding sites in large number of samples simultaneously. We have also begun development of a one-tube multiplexed SH2 profiling assay, in which the binding of tens or hundreds of SH2 domains to a protein sample could be quantitated at one time. For such assays, each protein binding domain must bear a unique, quantifiable tag; current efforts involve *in vivo* biotinylation of probes and their subsequent labeling with unique oligonucleotides tags coupled to streptavidin. Quantitation of DNA-tagged probes can be performed by established PCR-based methods with high throughput. SH2 profiling of tumor samples is expected to allow meaningful classification that will aid in development of therapies tailored to the individual patient.

Martin Stanton, Ph.D.
Archemix Corporation

Nucleic Acid-Based Biosensors as Tools for Cancer Research and Drug Discovery

Dilara Grate, Anthony Keefe, Chunhua Wang, Jeff Kurz, Thomas McCauley, Scott Lewis, Markus Kurz, Ryan Boomer, Stefan Gross, Sharon Cload, Alicia Ferguson, Nobuko Hamaguchi, and Martin Stanton Archemix Corp., Cambridge, MA

Current methods that profile genome-wide RNA or protein levels typically do not provide information concerning levels of the actual bioactive forms of proteins in biological samples. The ability to detect post-translational protein modifications, such a protein phosphorylation, represents a significant advance over existing techniques for expression profiling. Archemix has developed a variety of nucleic acid-based biosensors, including aptamers and allosteric ribozymes (RiboReportersTM) that are capable of monitoring the post-translational state of proteins important in the development and progression of disease. We have created both solution-phase and chip-based biosensors for specific, multiplex detection of isoforms of cancer-associated proteins (e.g., MAP kinases, VEGF, IMPDH, bFGF) in complex biological matrices such as serum and cell extracts.

Dan Jay, Ph.D.
Tufts University

Functional Proteomics To Identify Proteins in Cancer Cell Invasion

Brenda K. Eustace[1], Takashi Sakurai[1], Jean K. Stewart[1],Leodovico Ilag[2], and Daniel G. Jay[1]

[1]Department of Physiology, Tufts University School of Medicine, and [2]Xerion Pharmaceuticals AG, Martinsried, Germany

We have developed a novel functional proteomic screen to identify proteins involved in cancer cell invasion. The screen uses a direct protein inactivation technique named fluorophore-assisted light inactivation (FALI). We have used FALI with both monoclonal (mAb) and recombinant single-chain variable fragment antibodies (scFv) directed randomly against components of the surface proteome of HT-1080 fibrosarcoma cells. These binders were used with FALI to directly inactivate the bound proteins in cells. The cells were then tested for their ability to invade a thin Matrigel layer in an *in vitro* invasion assay. Antibodies that showed a significant effect on invasion with FALI were used for immunoprecipitation and mass spectrometry to identify the bound protein.

Several interesting proteins have been identified in this screen. These targets include proteins with previously characterized roles in invasion, which act as a validation of our screen, as well as those with uncharacterized roles. One of these proteins that had not been previously shown to function in invasion has been further validated using FALI with commercial antibodies and pharmacological inhibition. We have also begun to elucidate a potential mechanism of action for this validated protein target.

Our findings show novel and important functions for specific surface proteins in cancer cell invasion. Surface proteins are particularly amenable to therapeutic intervention using function blocking antibodies or pharmacological agents, and thus the information from this screen can be directly used to suggest new therapies. This FALI screening strategy also has wide applicability as a method to identify functionally important proteins in a variety of disease processes.

Bernard Futscher, Ph.D.
Univeristy of Arizona

Epigenomic Changes During Leukemia Cell Differentiation: Analysis of Histone Acetylation and Cytosine Methylation using CpG Island Microarrays

Marcela Nouzova, Nicholas Holtan, Jose Munoz-Rodriguez, Nirav Merchant, Susan Millar, R. Ben Isett, and Bernard Futscher

Arizona Cancer Center, College of Pharmacy, University of Arizona

Aberrations to the normal levels and patterns of 5-methylcytosine and histone acetylation are a common feature of human cancers. These aberrations are known to

disrupt the orderly expression of genes and participate in the transformed phenotype. Microarray technology offers an opportunity to measure and analyze these epigenetic changes on a genome-wide scale. To this end, we have sequence-validated a human CpG island library containing 6,800 genomic clones. These sequenced clones were first processed through FAKtory, a configurable, GUI-based software that provides a configurable DNA fragment pipeline of processing stages through which DNA traces must pass. Afterward, finished CpG island sequences were subjected to BLAST analysis to the HTGs, nr, and UCSC DNA-sequence databases. Approximately 5,000 unique clones were identified a vast majority with characteristics consistent with CpG islands. Almost all redundant clones were of mitochondrial genome, or either interspersed or satellite repetitive sequence origin. All clones were arrayed onto glass slides for 2-color fluorescence hybridization experiments that assessed cytosine methylation and histone acetylation states.

To test and validate these CpG island arrays, we used a well-described model of all-trans-retinoic acid (ATRA)-induced differentiation of human acute promyelocytic leukemia cells (NB4). Cytosine methylation and histone acetylation states were determined for normal peripheral blood mononuclear cells, untreated, undifferentiated NB4 cells, and ATRA-differentiated NB4 cells. CpG island microarray hybridization results show that the untreated NB4 cells have increased methylation of their CpG islands compared to PBLs, but ATRA had no detectable effect on NB4 cytosine methylation levels during the differentiation process. Cytosine methylation differences between NB4 and normal peripheral blood mononuclear cells detected by microarray profiling were confirmed by bisulfite sequencing. To assess changes in chromatin structure, DNA isolated from chromatin immunoprecipitations from the various cells were used to probe the CpG island microarrays. While ATRA did not induce changes in genomic methylation levels, it did induce complex changes in histone acetylation throughout the genome of ATRA-treated NB4 cells. First, a number of single copy CpG islands associated with genes displayed increased levels of histone acetylation including the known target, RAR beta. Second, increased levels of histone H4 acetylation were seen in the high copy alpha satellite sequences, suggesting that high copy elements localized to centromeric regions become preferentially acetylated during leukemic cell differentiation. Third, histone acetylation changes observed occurred independently of changes in 5-methylcytosine. Histone acetylation changes detected by microarray profiling were confirmed by ChIP -PCR analysis. In summary, CpG island microarrays can be used to provide a genome-wide assessment of epigenetic changes in cancer and normal cells.

Vincent Gau, Ph.D.
Genefluidics, Inc.

Rapid Molecular Detection of PML/RAR Using Electrochemical Sensorarray

Vincent Gau

GeneFluidics, Inc., Monterey Park, CA

Overview:
We propose a series of pilot experiments to test the hypothesis that a totally integrated, rapid detection system for the screening of the PML/RARalpha translocation in raw blood samples using a MEMS-based electrochemical sensor array, which is superior in some applications over existing technology. The ultimate goal of these studies is the development of an inexpensive, user-friendly system capable of diagnosing PML/RARalpha translocation cases in less than one hour. The challenge of this task is in modifying the present sensor design and protocol so minor alterations in genetic code can be detected using the ultra sensitive, highly specific sensor array.

Significance:
Acute promyelocytic leukemia (APL) comprises a distinct subset of acute myeloid leukemia (AML), and represents 10-15% of all AML cases (Stone and Mayer, 1990). In general, patients with APL tend to be younger than patients with other types of AML, with 90 percent of cases occurring between the ages of 15 and 60 (Avvisati et al, 1991). In addition, patients with APL are much more likely to suffer from disseminated intravascular coagulation (DIC) at the time of presentation, which increases the risk of life-threatening bleeding (reviewed in Tallman and Kwaan, 1992). Treatment in the past consisted of chemotherapy, which unfortunately worsened the DIC frequently, and early hemorrhagic death was common. Within the last fifteen years a vitamin A derivative, all-trans retinoic acid (ATRA), has been documented to promptly resolve DIC, and to significantly increase the complete remission rate and overall survival of APL patients (Huang et. al., 1988, Sun et. al., 1992, Castaigne et. al., 1990, Warrell et. al., 1991, Chen et. al., 1991). Additionally, there is evidence that administration of ATRA decreases the incidence of early hemorrhagic death (Visani et. al., 2000).

John Welsh, Ph.D.
Sidney Kimmel Cancer Center.

Vertical Coverage Arrays in Analyzing Transcription

Rosana Risques, Gaelle Rondeau, Martin Judex, Michael McClelland, and John Welsh

Sidney Kimmel Cancer Center, San Diego, CA

Vertical arrays are microarrays whose spotted elements consist of low-complexity representations (LCRs) of mRNA populations from different sources. For example, these LCRs can be made from control and treatment groups, normal and tumor samples, etc. The concept behind the invention is to be able to place thousands of experimental samples on a single chip so that the expression behavior of genes can be explored in response to many variables. LCRs are used to limit the sequence complexity in any single spot, thereby increasing the representation of its member sequences and decreasing background hybridization. LCRs can be made in various ways. We have focused on LCR preparation using RNA arbitrarily primed polymerase chain reaction (RAP-PCR). RAP-PCR works either by subjecting RNA to reverse transcription using oligo-d(T)20, followed by arbitrary priming where arbitrarily chosen

primers are used in low-stringency PCR, or by subjecting RNA to reverse transcription with initial use of an arbitrary primer followed by low-stringency PCR. Either strategy samples the mRNA population according to the sequence of the arbitrary primer. With 30 arbitrary primers or primer pairs, it is possible to detect most of the transcripts in a cell. When LCRs prepared in this way are immobilized on a glass slide, they can be hybridized with a fluorescently labeled probe and the resulting fluorescent signal reports the relative abundance of the transcript in the mRNA population from which the LCR was derived. We devised this approach in order to be able to determine the response of several hundred exemplar genes to several hundred drugs, so that we can choose those drugs that are most likely to perturb other genes regulated by the same transcription factor as the exemplars. These drugs will then be used in standard microarray experiments to identify genes that are likely to be controlled by the transcription factor. We have demonstrated the efficacy of the vertical array approach.

Jean-Pierre Issa, M.D.
University of Texas at M. D. Anderson Cancer Center

Methylated CpG Island Amplification for Methylation Profiling of Cancer

Jean-Pierre Issa, Rajan Mannari, and Xuqi Chen

Department of Leukemia, and The University of Texas at M. D. Anderson Cancer Center

DNA methylation within promoter-associated CpG islands is a common mechanism of gene silencing in cancer and targets many critical processes involved in tumor development and invasion as well as DNA repair. Methylation profiling refers to the assessment of the methylation status of multiple genes in cancer. Such profiling is increasingly recognized as useful in tumor classification and identifies biologically and clinically distinct subgroups. In practice, methylation profiling is limited by available technology, and most large-scale studies have focused on a small number of genes. Methylated CpG island amplification (MCA) was developed to overcome such limitations. In this technique, amplicons specific for methylated CpG islands are generated by methylation-sensitive restriction enzyme digestion and ligation of PCR adaptors in the vicinity of methylated islands. This allows amplification of a large number of CpG islands simultaneously (theoretically, 70% of all islands). These amplicons can either be spotted on slides and probed with gene-specific probes, or can be labeled and used to probe filters or slides spotted with a large number of target CpG islands. A limiting step, then, is the identification of specific targets of hypermethylation in cancer.

Using MCA coupled with representational difference analysis, we have been cloning promoter-associated CpG islands in various cancer cell lines. In an initial study, we had isolated 33 clones differentially methylated in colon cancer, but the number and identity of associated genes were unknown. Reanalysis of these using BLAT searches of the most recent release of the human genome sequence identifies 19 sequences that are

located in the 5' promoter area of genes. In current MCA/RDA experiments using leukemia and colon cancer cell lines, we have identified an additional 27 promoter-associated CpG islands hypermethylated in cancer. Finally, a preliminary review of genes known to be hypermethylated in cancer revealed 30 commonly inactivated genes, the methylation of which could be detected by MCA, bringing the total of testable targets to over 75. These studies then provide the tools for initiating MCA-based methylation profiling of human cancers.

Jan Schnitzer, M.D.
Sidney Kimmel Cancer Center

Vascular Proteomics: Mapping Targets on Endothelia and Caveolae *in vivo* for Organ- and Tumor-Directed Delivery and Transcytosis

Jan E. Schnitzer

Sidney Kimmel Cancer Center, San Diego, CA

The future of cancer detection and treatment lies in the development of technologies to facilitate the targeted delivery of drugs and genes specifically to their intended sites of action inside tumors in order to enhance efficacy by creating higher drug concentrations in the tumor tissue while reducing drug toxicity by minimizing exposure to other organs. Unfortunately, the effectiveness of modern anticancer immunotherapy using antibodies against tumor cell surface antigens coupled to toxins has been disappointing because the tumor endothelium effectively blocks access to their intended target sites on the tumor cells. An alternative strategy for the therapy of solid tumors is to target agents to the endothelium of tumor blood vessels rather than to the tumor cells themselves to cause infarction of the tumor by rapid and selective destruction of the vasculature supplying the tumor. A new addition to the vascular targeting strategy that has become apparent from our recent work is the potential utility of a vesicular transport pathway (caveolae) discovered in endothelium for selectively overcoming this key cell barrier to facilitate directed delivery to underlying tumor tissue cells.

We have developed a novel identification technology platform and targeting strategy for discovering accessible *in vivo* targets in normal and neoplastic tissues. Using novel subfractionation techniques for purifying luminal endothelial cell plasma membrane and caveolae directly from tissue, we have produced in different tumors and tissues high-resolution proteomic maps of the endothelial cell surface directly in contact with the circulating blood. Multiple protein separation techniques coupled with MS and database analysis directly establishes the molecular heterogeneity of this interface among tissues and has produced an extensive database including organ-, tumor- and caveolae-specific protein targets. Antibodies targeting caveolae in a specific vascular bed rapidly accumulate in a single tissue at levels up to 89% of IV dose in 30 min. Immunoconjugates increase tissue-specific drug delivery by 200-fold and allow transcellular delivery to improve localized bio-efficacy.

Subfractionation coupled with MS analysis also unmasks 5 key tumor-induced targets

expressed at the endothelial cell surface that are not readily detected by standard proteomic or genomic analysis. Tissue staining with antibodies raised to corresponding peptides confirms tumor induction at the endothelial cell surface of various rat, rabbit, monkey, and human tumors, including lung, prostate, liver, brain, and breast. One target, TE3, concentrates in caveolae and appears quite tumor specific. Biodistribution studies reveals significant accumulation of IV-injected TE3 antibody in solid tumors within 1-2 hours. Gamma scintigraphy using radiolabeled TE3 antibodies injected IV permits rapid visualization of chemically induced primary tumors in rats as well as metastatic rat lung tumors *in vivo* . Thus, tumors can affect their vasculature to express a distinct molecular signature that can be identified using our novel profiling strategy. Profiling accessible functional targets is an important logical step not only for achieving site-directed pharmacodelivery *in vivo* but also for overcoming the normally restrictive endothelial cell barrier to transport drugs and possibly even genes to their intended targets, the underlying tissue cells. Such targeting vectors may improve molecular imaging of tumors and help re-engineer past therapies showing considerable promise *in vitro* but failing clinically because of poor delivery and systemic side effects.

Ie-Ming Shih, M.D., Ph.D.
Johns Hopkins University

Assessment of Plasma DNA Levels, Allelic Imbalance and CA-125 as Diagnostic Tests for Cancer

Hsueh-Wei Chang[1],Shing M. Lee[3], Steven N. Goodman[3], Gad Singer[1], Sarah K. R. Cho1, Lori J. Sokoll1, Fredrick J. Montz[2], Richard Roden[1], Zhen Zhang[1], Daniel W. Chan1,[3], Robert J. Kurman1,[2], and Ie-Ming Shih1,[2]

[1]Department of Pathology, [2]Department of Gynecology and Obstetrics, and [3]the Oncology Center, The Johns Hopkins Medical Institutions

Background: Tumor-released DNA in blood represents a promising molecular marker for cancer diagnosis.

Methods: We first addressed whether measurement of plasma DNA concentration was a feasible screening method for ovarian and other cancers. Second, using ovarian cancer as an example, we performed digital single-nucleotide polymorphism (SNP) analysis to precisely determine allelic status in plasma DNA and to evaluate the potential of this new technology for detection of ovarian cancers. We contrasted this with the combination of plasma DNA concentration and serum CA125 levels.

Results: Based on data from 330 patients, the area under the receiver-operating characteristic (ROC) curve for plasma DNA concentration was 0.90 for neoplastic versus healthy patients and 0.74 for neoplastic versus non-neoplastic patients. Given 100% specificity, the highest sensitivity achieved was 57%. Of the 330 patients, digital SNP analysis was performed on 54 ovarian cancer patients and 31 non-neoplastic disease controls. Allelic imbalance (AI) in at least one SNP in plasma DNA was found in

87% (95% CI: 60%-98%) of stage I/II and 95% (95% CI: 83%-99%) of stage III/IV patients and none in 31 patients without neoplastic disease (specificity 100%, CI: 89% to 100%). For the 63 patients with serum CA125 data, DNA plasma concentration added information to serum CA125 levels by increasing the area under the ROC curve from 0.78 to 0.84.

Conclusion: Measurement of plasma DNA levels may not be sensitive or specific enough for use as a cancer screening or diagnostic tool, even in conjunction with CA125, but detection of allelic imbalance in plasma DNA using the digital SNP analysis holds a great promise for the detection of cancer.

Branimir Sikic, M.D.
Stanford University

Gene Expression Profiling of Unknown Primary Cancers

No Abstract Available.