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Abstracts

Shotgun proteomics for biomarker discovery in tissues

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An emerging paradigm for biomarker development begins with unbiased discovery of biomarker candidates in tissues, cell models and biofluids proximal to sites of disease. Of the existing proteomics technology platforms, none are better suited to unbiased biomarker discovery than shotgun proteomics, which has revoloutionized cell biology and biochemistry by enabling identification of the protein components of multiprotein complexes, complex subcellular proteomes and even whole cell, tissue and biofluid proteomes. In shotgun analyses, protein mixtures are digested to peptides, which then are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS), usually with multidimensional peptide separations to identify peptide and protein sequences. Despite the power of the approach, commonly used shotgun proteomics platforms are limited in sample throughput and reproducibility, particularly for detection of low abundance proteins. Shotgun proteomics platforms also tend to be poorly standardized, both in data acquisition and in data analysis. Shotgun proteomics platforms for biomarker discovery also are constrained by the need to accommodate relatively small samples ($<$1 mg wet weight or $<$100 μg protein). With support from the NCI Clinical Proteomics Technologies Assessment for Cancer (CPTAC) program, we have undertaken a systematic evaluation and redevelopment of the shotgun proteomics platform based on multidimensional LC-MS-MS using Thermo LTQ and LTQ-Orbitrap instruments. A major effort in the first project year was to integrate elements of a standard data analysis pipeline called IDPicker, which is based on the Myrimatch algorithm, label-free quantification by spectral counting and parsimony-based protein assembly. We also have evaluated the performance of multidimensional peptide fractionation using strong cation exchange (SCX) and isoelectric focusing (IEF) methods in a shotgun proteomics platform. We used cell lysate and tumor tissue samples corresponding to protein inputs of 10–100 μg, which is typical of protein amounts present in small, macrodissected tissue biospecimens. To compare the performance of these multidimensional separations, our analyses evaluated resolution of peptides by fractionation, numbers of peptide and protein identifications and cumulative identifications with replicate analyses. The data illustrate the advantages and limitations of SCX and IEF fractionation in shotgun proteomics and suggest that IEF-based platforms offer clear advantages in reproducibility for analysis of tissue samples. We have implemented a standardized shotgun proteomics platform based on IEF fractionation and LC-MS-MS on an LTQ-Orbitrap instrument and applied the platform to the analysis of pooled tissues samples from lung and colon cancers and precancers and drug-treated and untreated tumors. Application of this platform generated approximately 2,500–3,500 confident, parsimonious protein identifications from 200 μg of tissue proteins. Replicate analyses indicated a high degree of reproducibility ($>$70%) between replicate analyses. Statistical comparisons of shotgun datasets present novel challenges, but dozens to hundreds of proteins typically distinguish phenotypically different samples. Other work in the Vanderbilt CPTAC program has focused on targeted liquid chromatography-multiple reaction monitoring MS (LC-MRM-MS) analysis of tissue samples for verification of the presence and quantitative comparisons of candidate markers from shotgun proteomics or other discovery platforms. (Supported by NIH Grants CA126479 and CA126218.)

Autoantibody screening using protein microarrays

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The humoral immune response is a highly specific and adaptive sensor for changes in the body's protein

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milieu, which responds to novel structures of both foreign and self antigens. Although immunoglobulins represent a major component of human serum and are vital to survival, little is known about the response specificity and determinants that govern the human immunome. Historically, antigen-specific humoral immunity has been investigated using individually-produced and purified target proteins, a labor-intensive process that has limited the number of antigens that have been studied. We previously demonstrated that functional proteins can be produced and captured *in situ* from bound DNA templates. We describe a next generation self assembling protein microarray that displays thousands of proteins. This method relies on a novel high-yield, high quality DNA miniprep and a new printing chemistry. It was used to display proteins for over 1500 unique cDNAs with >90% success. Minimal sample variation was observed (CV = 6%) along with good day to day reproducibility (R^2 >0.95). Moreover, the displayed proteins revealed selective protein interactions. We also describe a related method for producing 96-well formatted macroarrays for monitoring the humoral response at the proteome scale. Using plasmids encoding full-length cDNAs for over 850 human proteins and 1700 pathogen proteins, we demonstrate that the microarrays are highly sensitive, specific, reproducible, and can simultaneously measure immunity to thousands of proteins without *a priori* protein purification. The microarrays were also used to examine sera from patients who were identified as responders to p53. Using various deletion fragments and tiling peptides we mapped the epitope response domains of the p53 autoantigen and found response clusters in patients. Using this approach, we demonstrate the detection of humoral immunity to known and novel self-antigens, cancer antigens, autoimmune antigens, as well as pathogen-derived antigens. This represents a powerful and versatile tool for monitoring the immunome in health and disease.

ELISA microarray platform

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We are developing ELISA microarray technology for the discovery and validation of breast cancer biomarkers. This platform is designed for the high-throughput, quantitative analysis of protein panels. Using a prototype chip, we examined 24 candidate biomarkers in human blood plasma. These age-matched samples (58 total) were collected at the time of biopsy from women that had previously tested positive by mammography. Cancer cases were from women with either ER+/Her2− or Her2+/ER− breast cancer. Preliminary results identified five circulating proteins that were statistically different in at least one of the two cancer groups. These markers appear to fall into two independent groups. Area under the ROC curves for individual proteins varied from 0.63 to 0.79. In order to find additional markers of breast cancer, we conducted a proteomics analysis of 80 samples from controls or from women with either DCIS, early ductal cancer or early lobular cancer. Samples were matched across groups based on menopausal status, age and body mass index. Initial analysis of the proteomic data has identified a number of proteins that are significantly altered in breast cancer. As such, our research has identified a number of circulating proteins that have potential for detecting breast cancer.

Targeted glycoproteomic identification of potential cancer markers

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Glycosylation is a dynamic post-translational modification that changes during the development and progression of many malignancies. Several laboratories in the Alliance for Tumor Glycomics Laboratories have focused on developing experimental approaches to exploit these post-translational changes for the identification of potential cancer biomarkers. During the oncogenesis of breast carcinoma, for example, the transcript levels and expression of the glycosyltransferase N-acetylglucosaminyltransferase Va (GnT-Va) are activated due to oncogenic signaling pathways. Elevated GnT-Va levels lead to increased levels of specific, branched N-linked glycan structures on glycoproteins, which can be detected using a carbohydrate binding protein (lectin) known as L-PHA. L-PHA does not bind to non-diseased breast epithelial cells, but late adenoma stage cells bind the lectin, which increases during the progression to invasive carcinoma. We have developed a procedure for intact protein L-PHA-affinity enrichment, followed by nanospray ionization mass spectrometry (NSI-MS/MS), to identify potential biomark-

ers for breast carcinoma. We identified L-PHA-reactive glycoproteins from matched normal (non-diseased) and malignant tissue isolated from patients with invasive ductal breast carcinoma. Comparative analysis showed 12 L-PHA-reactive glycoproteins were common to all 4 matched cases analyzed, and several were predicted to be secreted or GPI-anchored. Antibody and lectin binding analysis have validated one glycoprotein as showing expression and L-PHA binding in breast carcinoma; screening of sera for this glycoprotein has been begun. A similar methodology is now being applied to pancreatic ductal fluid from patients with pancreatic carcinoma, pancreatitis, and other gastrointestinal diseases to identify potential pancreatic cancer biomarkers. Other laboratories in the Alliance have focused directly on serum from patients with breast and other cancers, applying novel glycoproteomic analysis strategies to identify specific N-glycans and glycoproteins that express particular glycan structures. Results from these studies are promising and validate the hypothesis that glycoprotein glycan changes can be used effectively in the discovery of potential diagnostic markers.

Applications of imaging mass spectrometry to clinical diagnosis

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Direct profiling of proteins in tissue sections using mass spectrometry imaging has the power to link molecular detail to morphological changes observed by the clinical pathologist. Diagnostic decision making by pathologists in prostate cancer revolves around tissue morphology. The ability to localize disease-specific molecular changes in tissue would help improve this critical decision making process. We will present our results using prostate and renal cancer as paradigms for which MALDI-MSI can be used to discriminate cancer from non-cancer tissue and identify lethal/metastatic disease. In addition, the ability to sequence identify novel peptides directly from tissue will be presented, demonstrating the power of this approach for biomarker discovery. Combining MALDI-MSI with mirrored pathological histology results following biopsy could be a new approach to guide the clinical decision making process for urologic cancers.

Airway gene expression as a diagnostic biomarker for lung cancer

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Lung cancer is the leading cause of death from cancer in the U.S. and the world [1]. The high mortality (80–85% within five years) results, in part, from lack of effective tools to diagnose the disease at an early stage [2]. Given that cigarette smoke creates a field of injury throughout the airway [3–7], we sought to determine if gene expression in cytologically normal large airway epithelial cells obtained at bronchoscopy from smokers with suspicion of lung cancer could be used as a lung cancer biomarker. Using a training set ($n = 77$) and gene expression profiles from Affymetrix HG-U133A microarrays, we identified an 80-gene biomarker that distinguishes smokers with and without lung cancer [8]. The biomarker was tested on an independent test set ($n = 52$), with an accuracy of 83% (80% sensitive, 84% specific), and an additional validation set independently obtained from five medical centers ($n = 35$). Our biomarker had $\sim$90% sensitivity for stage-1 cancer across all subjects. Combining cytopathology of lower airway cells obtained at bronchoscopy with the biomarker yields 95% sensitivity and a 95% negative predictive value. Additionally, we found that the gene expression biomarker provides information about the likelihood of lung cancer not captured by clinical factors and that a clinicogenomic model has the highest prediction accuracy. We further show that the airway epithelial field of injury involves a number of genes that are differentially expressed in lung cancer tissue, providing potential information about pathways that may be involved in lung carcinogenesis. We have also begun to explore how this cancer-specific molecular field of injury reflects information about the perturbation of specific oncogenic pathways within an individual, potentially allowing personalized genomic approaches to chemoprophylaxis and therapy. Our findings indicate that gene expression in cytologically normal large-airway epithelial cells can serve as a lung cancer biomarker, potentially due to a cancer-specific airway-wide response to cigarette smoke.

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Translational research on biomarkers: Crossing the divide from discovery to delivery/somatic DNA biomarkers for cancer risk prediction

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The National Cancer Institute Translational Research Working Group defined translational research as research that "transforms scientific discoveries arising from laboratory, clinical or population studies into clinical applications to reduce cancer incidence, morbidity and mortality." In the case of translational biomarker research, this frequently involves adapting markers that have been studied mechanistically in low variance models, such as knockout mice differing by a single gene in a controlled environment, to human population studies, where the biomarkers enter the domain of observational epidemiology because of increased variance in genetic background and environmental exposures. The EDRN phases of biomarker validation have been very helpful in guiding research design. However, if the EDRN phases of biomarker validation are used as a guide for clinical validation, then validation will be a prolonged process that could take more than two decades for cancer risk prediction biomarkers even assuming successful and smooth funding of each transition. In this setting, advancing technology will tend to overwhelm the process. The seminar will provide a more detailed discussion of clinical assay development (presently in EDRN phase 2) from a laboratory perspective based on the NCI TRWG's Developmental Pathway for a Biospecimen Risk Assessment Device and the author's 25 years of translational research experience.

The Seattle Barrett's Esophagus Study began its translational research program in the 1980s. We developed the recommended endoscopic biopsy protocol for surveillance of Barrett's esophagus; the criteria for dysplasia in Barrett's esophagus and use of DNA content flow cytometry in Barrett's esophagus. The biopsy protocol (referred to as "the Seattle Protocol") and the dysplasia criteria ("the Seattle criteria") were widely adopted in academic and Barrett's specialty centers. Endoscopy and pathology were readily disseminated and adopted at least in part because there was an existing infrastructure, including billing mechanisms, that was already anchored in the clinic. Over time, it has become clear that endoscopy, pathology and surgery in Barrett's esophagus are all operator dependent and the methods and outcomes reported from specialty centers are not generalizable to the community. Overdiagnosis of risk and overtreatment are threatening to become as or more important than the risk of cancer in Barrett's esophagus. There are a number of challenges for translational biomarker research in Barrett's esophagus. These include (1) developing biomarkers that require fewer endoscopic biopsies than used in the Seattle Protocol, (2) identifying patient subsets whose risk is so low that surveillance intervals can be lengthened from 2 years to 8–10 years or longer, (3) identifying a high-risk population whose 5-year cumulative incidence of esophageal adenocarcinoma is 80% or greater, and (4) identifying low toxicity interventions to prevent cancer in high-risk patients. However, the greatest challenge is identifying a DNA biomarker platform(s) that can be anchored in clinical practice.

We have conducted four prospective cohort studies (consistent with EDRN phase 4) of cancer risk prediction biomarkers, including (1) the dysplasia classification system, (2) proliferation/cell cycle, (3) tumor suppressor genes (CDKN2A, TP53) and (4) chromosomal instability (LOH, tetraploidy, aneuploidy). The chromosome instability panel provided the most robust outcomes. However, in the prospective validation studies this panel used two detection platforms: LOH by STR polymorphisms and DNA content flow cytometry.

It seems likely that optimum cancer risk prediction in many diseases may be best achieved by a panel of different types of DNA biomarkers, including LOH, copy change, methylation, mutation or aneuploidy. Design of the laboratory component as well as goals and opportunities for multicenter validation trials in Barrett's esophagus will be discussed. Well designed prospective cohort studies (EDRN phase 4) of biomarkers for cancer risk prediction may also identify candidate interventions for cancer prevention.

Phase 2 validation of AFP, DCP and AFP-L3 in early stage hepatocellular carcinoma

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Introduction: Hepatocellular carcinoma (HCC) is the fifth most common tumor and the third most common cause of cancer-related deaths worldwide. Patients with small tumors have 5-yr survivals $>$60%. Cirrhosis is the most important risk factor for the development of HCC, and HCV an HBV are the major etiological agents that lead to the development of HCC through the development of cirrhosis. Alpha-fetoprotein (AFP) has been utilized as the main tumor marker in patients with HCC, with an optimal cut-off to maximize sensitivity and specificity of 20 ng/mL. However, this cut off value was done in advanced stage HCC. Des-gamma carboxy-prothrombin (DCP) and *lens culinaris*-agglutinin reactive fraction of AFP (AFP-L3) are promising markers in HCC. The aims of this study were to determine the sensitivity and specificity of DCP for the diagnosis of early HCC, and to determine whether demographic or etiology of underlying liver disease alter the expression of DCP or AFP.

Methods: We performed a large phase 2 biomarker case-control study. Controls were matched to cases according to age, gender and viral etiology (viral vs. non-viral). HCC was defined by the histological examination or by the appropriate imaging characteristics as defined by current guidelines. Controls had liver cirrhosis defined by histology or non-histologically by evidence of portal hypertension in the presence of a chronic liver disease. Assays for AFP, DCP and AFP-L3 were performed at and EDRN Biomarker Reference Laboratory at UCLA.

Results: A total of 846 patients were enrolled, of which 424 were cirrhotic controls without cancer and 422 were HCC cases. Of the cases, 208 were early stage HCC. There was a male predominance in all groups as well as Caucasian ethnicity in cirrhotic controls and early stage HCC. The majority of cases and controls had a viral etiology of their liver disease. There were 248 (58%) HCV-related controls and 215 (50%) HCV-related HCC of which 120 (58%) had early stage HCC, in contrast there were only 22 (5%) HBV-related cirrhotic controls and 67 (15%) HBV-related HCC of which 33 (15%) were early stage tumors. The hepatic function as measured by the MELD score was similar among all the groups.

The serum levels of AFP, DCP and AFP-L3 were significantly elevated in cases (both early and late stages) when compared to controls. ROC curves were plotted to identify the area under the curve (AUC) and optimal cutoffs between cirrhotic controls and early stage HCC cases. AFP had the best AUC followed by DCP and AFP-L3. AFP had the best performance for early stage HCC. The combination of AFP, DCP and AFP-L3 was the investigated. When only early stage HCC was studied, the AUC for the combination of AFP and DCP improved.

Conclusion: In the largest study ever done of biomarkers for early stage HCC, AFP had the best performance characteristics. The combination of AFP and DCP improved the detection of early stage HCC especially in patients with viral hepatitis. Testing AFP in a phase 3 study is warranted. New markers that complement AFP are needed.

AACR–NCI-FDA collaborative: Focus on biomarker informatics

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Modern research in biomedicine relies on an ever widening range of heterogeneous data types collect-

ed from increasingly high content technologies. Researchers in bioinformatics – the intellectual fusion of biology and information technology (IT) – are spearheading efforts to turn this enormous influx of data into medically useful knowledge. In cancer, a key goal is to develop and deploy new intervention strategies in prevention, detection, treatment and control that will transform cancer into a chronic, manageable disease. The FDA, in the formulation of the Critical Path Initiative, identified the development of predictive biomarkers as a key gap in current strategies to achieve this goal, both for effective treatment as well as for developing new innovative medicines. This talk addresses the role of bioinformatics in addressing these gaps on the Critical Path. Bioinformatics plays a central role in managing the deluge of data from modern data collection platforms, and in deciphering the data to extract knowledge that will facilitate biomarker discovery. In this talk, we focus on the role of bioinformatics in cancer biomarker discovery, validation, qualification and deployment into the Clinic. We outline current thinking and practice from academia, regulatory agencies and industry. The goal is to ensure that the informatics platforms underpinning the flow of data, information, and knowledge across each component of the pipeline (including data sharing within and beyond institutions from academia and industry) are developed and deployed in a manner that enhances our ability to translate the discovery of cancer biomarkers to the clinic.

EDRN knowledge environment: Design and demonstration

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EDRN's core mission is the discovery and validation of biomarkers. Critical to that mission is having an informatics infrastructure that is engineered to support biomarker research. The EDRN Knowledge Environment provides integrated access to biomarker data across the EDRN enterprise. This data and information is captured during the biomarker discovery process. The informatics team has a goal to make it available to both EDRN and non-EDRN researchers. At the heart of the EDRN Knowledge Environment is an ontology model which allows for the information across multiple systems to be interrelated based on identifying the relationships between biomarker research data. The vision is to provide scientists an integrated portal using state-of-the-art search mechanisms which allows them to find and access information resulting from EDRN studies.

Over the past year, the EDRN informatics team has made tremendous progress in unifying biospecimens, scientific data, study specific data, and biomarker data into the EDRN public portal, providing virtual access to information repositories through the EDRN grid infrastructure and realizing the vision of building the EDRN Knowledge Environment. This presentation will provide an overview of the progress, present a specific case study, and provide a demonstration of existing capabilities. It will also discuss the plans for curating and managing the biomarker data.

Somatic DNA methylation changes as molecular biomarkers for prostate cancer

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Can somatic changes in DNA methylation patterns serve as useful prostate cancer (PCA) molecular biomarkers? Clearly, with polymerase chain reaction (PCR) technologies, nucleic acid markers are detectable with extraordinary sensitivity, often in the range of a single molecule, and of the nucleic acids, DNA may be superior to RNA in terms of stability through specimen collection and sample handling. DNA methylation changes are particularly attractive for this purpose for most human cancers, because unlike point mutations, for example, CpG island (CGI) hypermethylation changes appear more consistent from case-to-case, permitting a single assay to be used to detect all cases. PCA is no exception: there have not been any common point mutations in any genes yet described, yet several consistent CGI hypermethylation changes have been reported [1,2]. Even the recently reported fusions between *TMPRSS2* and ETS family transcription factor genes are not as common as CGI hypermethylation changes [3–5].

There are now three major strategies for the detection of CpG dinucleotide methylation changes in genomic DNA from cancer cells. The first approach features the use of restriction endonucleases that cut recognition sites differently if the sites contain $^{5-\mathrm{me}}$CpG. Such enzymes have been used along with Southern blot analysis and with PCR to discriminate DNA methylation changes at particular genome sites [6,7]. Assays using $^{5-\mathrm{me}}$CpG-sensitive restriction enzymes and PCR (RE-PCR) have proven spectacularly sensitive, capable of detecting single hypermethylated CGI sequences, but appear prone to false-positive results, arising from incomplete cutting of unmethylated sequences and insufficient suppression of PCR amplification of unmethylated CGI alleles [7]. The second strategy uses sodium bisulfite modification to facilitate the selective deamination of C, but not of $^{5-\mathrm{me}}$C, to U, creating a DNA sequence difference at C versus $^{5-\mathrm{me}}$C after PCR amplification. This approach has been used for mapping and sequencing of $^{5-\mathrm{me}}$C at specific genome sites, and serves as the basis for a PCR assay in which primers specific for bisulfite/deamination converted sequences containing $^{5-\mathrm{me}}$C versus C are used to detect hypermethylated CGIs [8,9]. The bisulfite modification and PCR (MS-PCR) assays, though specific, can be less sensitive than RE-PCR assays because the bisulfite modification procedure can damage target DNA sequences [2]. A third approach involves selective capture of $^{5-\mathrm{me}}$C-containing sequences with $^{5-\mathrm{me}}$C-binding proteins or anti-$^{5-\mathrm{me}}$C antibodies [10–14]. Capture assays for $^{5-\mathrm{me}}$C-containing DNA appear sensitive, specific, easily adapted to high-throughput analysis platforms, and able to be used in association with RE-PCR and/or MS-PCR methods [7]. A new capture approach we have developed, COMPARE-MS, exploits the binding properties of a fragment of one of the $^{5-\mathrm{me}}$CpG DNA binding proteins for the specific capture of $^{5-\mathrm{me}}$C-containing DNA [7]. This capture strategy not only permits sensitive and specific detection of CGI methylation changes, but allows genome-wide mapping of methylated regions.

For PCA, the greatest amount of attention has been afforded *GSTP1* CGI hypermethylation, a change present in >90% of cases that have been carefully evaluated [6,15,16]. The somatic genome change has proven remarkably robust as a candidate molecular biomarker. Using a variety of different detection strategies, in some 51 studies with thousands cases, *GSTP1* CGI hypermethylation has been detected in DNA from prostate tissues in more than 80% of cases analyzed, with the sensitivity of detection varying somewhat depending on assay [2]. *GSTP1* CGI methylation changes are conspicuously absent from all normal human tissues, including normal prostatic epithelial cells isolated by laser capture microdissection, but are commonly present in PCA precursor lesions, such as proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) [17–19]. Thus, assays capable of detecting *GSTP1* CGI hypermethylation in DNA from prostate tissues have the potential of discriminating PCA and its precursors from other prostate abnormalities, and of detecting neoplastic cells even when not readily evident by microscopy [20]. Furthermore, such assays have been found to detect PCA DNA in prostatic secretions, permitting the use of urine specimens for prostate detection and diagnosis [21–23].

In addition to the *GSTP1* CGI, CGI sequences at more than 40 other gene sites have been evaluated for hypermethylation changes in PCA DNA. By examining many CGIs for each PCA case, CGI hypermethylation "profiles," distinct from other cancers, have emerged [1, 2]. Using the quantitative MS-PCR technology, the detection of CGI hypermethylation at combinations of sites, including *GSTP1*, *APC*, *RASSF1a*, *PTGS2*, and *MDR1*, has been reported to distinguish PCA from non-cancerous tissue with sensitivities of 97.3%–100% and specificities of 92%–100% [1]. It is likely, that more somatic targets of CGI hypermethylation will be discovered in the future; many such genes may offer new opportunities for molecular biomarkers that can be used in PCA detection and diagnosis. Will some sort of DNA methylation assay become a PCA screening tool? The best opportunity appears to be sensitive detection of somatic DNA methylation changes in urine, if the urine can be collected in such a way that it contains prostate secretions [21].

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Markers to detect a 'true precancerous breast lesion'

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Surveys show that about a million women develop benign breast lesions per year and 80,000 to 90,000 of them will subsequently develop breast cancer in USA. We can expect a dramatic reduction in incidence and deaths from breast cancer if we can detect a 'True Precancerous lesion' among benign patients and treat them with prophylactic therapies. However, currently there are no markers to screen benign tissues and identify a 'True Precancerous Lesion'. We have been working on identification and validation of such markers. We hypothesized that benign tissues from patients who developed cancer are the 'True Precancerous Lesions' and those have elevated expression of cancer promoting molecules. We have tested this by comparing global gene expression of benign tissues from patients with and without the history of developing cancer and identified over 300 potential markers. We validated three known cancer promoting markers, MMP-1, CEACAM6 and HYAL1, among the 300 by IHC in 160 archival precancerous tissues retrospectively and established that expression of the above markers is strongly associated with subsequent development of breast cancer irrespective of histology. The Sensitivity, Specificity, PPV, NPV and areas under ROC curves were 0.8 to 0.9 for individual markers and the values reached 0.95 to 0.97 when the markers were combined. We believe that the above markers could be applied to screen and identify very high risk benign patients for prophylactic therapies and prevent them from developing cancer. Refs: Poola et al, The Lancet Oncology (accept-

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*p*300 (histone acetyltransferase) Biomarker predicts prostate cancer biochemical recurrence and correlates with changes in epithelia nuclear size and shape

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Background: p300 impacts the transcription of several genes involved in key pathways critical to PCa progression. Therefore, we evaluated the prognostic value of *p300* expression and its correlation with nuclear alterations seen in tumor cells in men with long term follow-up after radical prostatectomy (RP).

Methods: NCI Cooperative Prostate Cancer Tissue Resource tissue microarray cores of 92 RP cases (56 non-recurrences and 36 PSA recurrences) were utilized for the study. *p300* expression was assessed by quantitative immunohistochemistry and nuclear alterations in Feulgen-stained nuclei were evaluated by digital image analysis using the AutoCyte$^{\mathrm{TM}}$ Pathology Workstation. Cox proportional hazards regression, Spearman's rank correlation, and Kaplan-Meier plots were employed to analyze the data.

Results: p300 expression significantly correlated with nuclear alterations seen in tumor cells; specifically with circular form factor ($p = 0.012$) and minimum feret ($p = 0.048$). *p300* expression in high grade tumors (Gleason score $\geqslant$7) was significantly higher compared to low grade tumors (Gleason score $<$7) [17.7% vs. 13.7%, respectively, $p = 0.03$]. TNM stage, Gleason score, and *p300* expression were univariately significant in the prediction of PCa biochemical recurrence free survival ($p \leqslant 0.05$). *p300* expression remained significant in the multivariate model ($p = 0.03$) while Gleason score showed a trend toward significance ($p = 0.06$). Patients with a Gleason score $\geqslant$7 and *p300* expression $>$24% showed the highest risk for PCa biochemical recurrence ($p = 0.002$).

Conclusions: p300 expression correlates with nuclear alterations seen in tumor cells and has prognostic value in predicting long-term PCa biochemical recurrence free survival.

Methylation markers for lung cancer risk prediction

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Lung cancer is the leading cause of cancer-related death in the U.S. and will soon reach epidemic levels worldwide. Mortality from this disease could be reduced through the development of an effective screening strategy for identifying persons with early stage disease and the implementation of chemopreventive strategies that can reverse or impede the progression of premalignant disease. Studies by our laboratory have evaluated genes inactivated by aberrant cytosine-guanosine (CpG) island methylation as candidate biomarkers for early detection of lung cancer. The specific hypothesis being evaluated is that methylation of genes detected in sputum can be used to identify early lung cancer in asymptomatic persons. We conducted the first study in collaboration with the Colorado Lung SPORE to prospectively evaluate a large panel of genes for their ability to predict lung cancer. This nested, case-control study of persons from the Colorado cohort revealed that a panel of genes could predict incident lung cancer 3–18 months prior to clinical diagnosis. Specifically, concomitant methylation of three or more of a six-gene panel was associated with a 6.5-fold risk and a sensitivity and specificity of 64%. We have extended our initial case-control studies with the Colorado cohort for the purpose of improving the sensitivity and specificity of the original gene panel. There were two goals for these studies: to increase power by increasing the number of cases and controls and to screen an additional 40 genes. Five genes (DAL1, PCDH20, KIF1A, P16, and DAPK) have now been identified that show significantly increased odds for methylation in cases compared to controls. In addition, we have identified 14 genes associated with a 2-fold or more increased lung cancer risk. Gene panels are being assembled to determine sensitivity and specificity. In addition, to better refine our gene panel for prospective studies, gene methylation is being assessed in sputum obtained from stage I lung cancer patients who are generally asymptomatic for disease. Results with an 8-gene panel revealed that the prevalence for methylation was similar (e.g. p16, MGMT) or strikingly exceeded (e.g., GATA4, GATA5) that seen

in the Colorado lung cancer cases. This finding parallels our observation in the Colorado case-control study where the prevalence for methylation of several genes increased as the time between sputum collection and cancer diagnosis decreased.

Gene haplotypes associated with lung cancer could integrate with a gene methylation panel to improve the sensitivitiy and specificity for early lung cancer detection. We assessed the distribution of variants in 66 genes from the DNA repair, cell cycle, methylation, and apoptotic pathways in lung cancer cases and controls from the Colorado cohort by the Illumina Goldengate assay. Principal component analysis identified several genes that appear to be associated with lung cancer. Validation studies are planned that will lead to the identification of specific gene variant alleles and haplotypes that can be integrated into an early detection panel for screening high-risk smokers. (Supported by P50 CA 58184 and U01 CA09735697)

The American-Australian Mesothelioma Consortium: EDRN Mesothelioma Biomarker Discovery Laboratory

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Mesothelioma (MM) is an orphan disease which is asbestos related, presently has a median survival of 8–18 months from diagnosis and for which therapeutic options remain problematic. Nevertheless, there is justification for pursuing early biomarkers for MM in well-characterized, asbestos-exposed subjects since (1) cytotoxic chemotherapy is associated with a 41% partial response rate (2) multimodal approaches involving surgery, chemotherapy with or without radiation therapy can have median survivals of 32 months or greater for Stage I patients. The American Australian Mesothelioma Consortium, centered at NYU, has been the funded EDRN Biomarker Discovery Laboratory for Mesothelioma with Fujirebio Diagnostics, Inc. as an industrial partner since 2006. Preliminary data regarding two specific biomarkers, SMRP and Osteopontin (OPN) were originally validated at NYU using sera and plasma from specimens which originated from clinical trials at the National Cancer Institute and the Karmanos Cancer Institute. This abstract summarizes the status of studies since September 2006.

SMRP

At NYU, we evaluated SMRP in serum from MM patients ($n = 90$), lung cancer (LC) patients ($n = 174$), age and tobacco-matched AE individuals ($n = 66$), and in MM pleural effusions ($n = 45$), benign effusions ($n = 30$), and non-MM effusions ($n = 20$) using the MesoMark$^{\mathrm{TM}}$ ELISA kit (Fujirebio Diagnostics). Receiver operating characteristic curves (ROC) were used to define true and false positive rates at various cut-offs. *RESULTS:* Mean serum SMRP levels were higher in MM compared to LC (9.47 ± 3.39 nM [mean $+/-$ SEM] vs 1.95 ± 0.44 nM, $p = 0.029$), and Stage I MM SMRP levels ($n = 12$; 2.09 ± 0.41 nM) were significantly higher than those in AE individuals (0.99 ± 0.09 nM, $p = 0.02$, respectively). Stage 2–4 SMRP serum levels were significantly higher (10.61 ± 3.89 nM, $p = 0.03$) than those for Stage 1. The area under the ROC (AUC) for serum SMRP was 0.805 for differentiating MM and AE, cut-off = 1.2 nM (sensitivity = 76.7%, specificity = 72.7%). The positive predictive value was 69% and negative predictive value was 79.8% for serum. MM pleural effusion SMRP was significantly higher than benign or other non-MM pleural effusions (65.57 ± 11.33 nM vs 18.99 ± 7.48 nM [$p = 0.001$] and 27.46 ± 11.25 nM [$p = 0.021$] respectively). *These SMRP data are compatible with results from other smaller cohorts of MM and AE patients, and confirmed the data of Robinson et al in a North American cohort.*

Osteopontin (*OPN*)

Our group previously revealed that OPN could also discriminate AE from MM, and that serum OPN levels were influenced by asbestos exposure and degree of radiographic changes. We demonstrated that in the same cohort of patients, the sensitivities of serum SMRP and serum OPN were improved when both markers were used. We also investigated the plasma concentrations of OPN to see if there was improvement in differentiating AE from MM and whether plasma OPN was a prognostic marker for MM. Plasma OPN from 39 MM (mean age 63+ 8.4 years;9 females, 30 males; 11 Stage I/II, 28 Stage III/IV; 21 having surgical cytoreduction) and from 79 asbestos-exposed (AE) individuals (mean age 63+ 10.6 years; 9 females, 70 males) was measured with the Research and Diagnostics (R&D, Minneapolis MN) and Immuno- Biological laboratories(IBL,Minneapolis, MN) kits. Differences in OPN levels in MM and AE individuals were com-

pared using ROC curves and sensitivity and false positive rates based on logistic models for each of the test kits. Survival for MM was estimated using Kaplan-Meier curves; comparisons between groups are based on log rank chi-square tests. Hazard ratios and 95% confidence intervals were estimated from Cox proportional hazards models. A formal cut point analysis was performed using the maximum chi-square with p value adjustment method to determine the OPN values that were most strongly associated with survival. *RESULTS:* The area under the ROC curve was 0.93 (R&D, cutpoint corresponding to a sensitivity of 0.91 and false positive rate of 0.23 = 59.6 ng/ml) and 0.96 (IBL, cutpoint corresponding to a sensitivity of 0.91 and false positive rate of 0.10 = 132.6 ng/ml). The median overall survival for all 39 MM was 11 months (95% CI: 5, 13) with a 37 month median overall follow up for survivors. Patients with OPN levels greater than or equal to 212.6 ng/ml had 5.7 (95% CI: 2.4, 13.3) times the mortality risk of patients with lower levels (adjusted p value = 0.007). Additional multivariable analyses indicate that lower stage (HR: 5.0; 95% CI: 1.7, 15.0; $p = 0.004$) and OPN level from the R&D kit less than the cutpoint of 212.6 ng/ml (HR: 3.5; 95% CI: 1.5, 8.4; $p = 0.004$) were significantly associated with improved survival. *These data confirm that plasma OPN may be a sensitive discriminator for the development of MM in high risk AE cohorts, and also that OPN levels in MM patients may be of prognostic importance.*

Our group has further characterized OPN as having three distinct isoforms in MM, and that each isoforms has different functional characteristics with regard to proliferation, migration, and invasion.

Validation trials for SMRP and OPN

In collaboration with the DMCC, a validation trial to establish ranges for measuring SMRP and OPN has been written. Using cohorts from Karmanos, NCI, Libby Montana, Australia, and Mt. Sinai Hospital of NYC, over 680 serum specimens representing either high risk AE or MM patients are presently housed at the EDRN Biomarker Facility at Bellevue Hospital in NYC. Blinded validation of these specimens will be performed at three validation sites. Pending the results of this validation, a prospective trial examining these markers in the serum of villagers in the epidemic sites for MM in Cappadochia Turkey will be performed. Preliminary data from these villages regarding the sensitivity and specificity of SMRP and OPN are gratifyingly consistent with the data from the EDRN Biomarker Discovery Laboratory.

New potential biomarkers

MMP9 was found in preliminary experiments to have increased expression in mesothelial cells subjected to asbestos. We measured plasma MMP9 from MM, AE, LC, and BD individuals and found that MMP9 was elevated significantly in AE but decreased in MM. Paradoxically, this was not the case with BD and LC where BD individuals had significantly lower levels of plasma MMP9. Further investigations regarding the utility of MMP9 to differentiate LC from MM as part of a combinatorial analysis of markers are ongoing.

Hyalluronic Acid Proteoglycan Link Protein 1 (*HAPLN1, CRTL1*): originally was predicted to be 23 fold elevated in the extracellular matrix of MM compared to AE cohorts from our genomic pathway analyses. Our laboratory has validated these data in matched normal peritoneum and MM specimens. Due to the lack of reagents for protein measurement, we generated a rabbit polyclonal antibody which revealed that (1) MM cell lines stained for the antibody as opposed to mesothelial cell lines or mesothelial short term cultures (2) paraffin embedded MM of any histology had cellular but not stromal staining (3)transfection of HAPLN1 into low expressing MM cell lines remarkedly increased invasion, proliferation, and migration of those cell lines compared to empty vector transfection. Attempts at the production of an ELISA are ongoing at this time.

Identification of biomarkers from glycans released from serum glycoproteins using a printed glycan array (*Collaboration with Cellexicon, Inc.*): is a new initiative that was started in 2007. Using a preliminary set of 20 specimens each representing AE, benign disease but smoker (BD), adenocarcinoma of the lung (AD), MM, and squamous cell carcinoma of lung (SC), comparative analyses of the serum glycan status was determined for AE versus MM, BD versus AD, and BD versus SC. Using six ranked glycans, an AUC of 0.93 was determined for AE versus MM. similarly, an AUC of 0.96 was recorded comparing BD to AD using 4 ranked glycans, and an AUC of 0.94 determined between BD and SC. These numbers were increased to approximately 70 AE and 70 MM in a subsequent analysis. Using 20 glycans, an AUC of 0.94 ws determined for AE versus MM with a sensitivity of 0.87 and a specificity of 0.94. These investigations are continuing.

MicroRNA profiles between MM and normal peritoneum, and between MM and AD (*Collaboration with Rosetta Genomics*) is another new initiative begun in 2007. 36 snap frozen MM (8F,28M) with 20 matching peritoneum from the time of their resections were used

for mir analysis. There were 10 Stage I/II (MS = 24 months) and 26 Stage III/IV patients (MS = 8 months). Samples were hybridized to Rosetta Genomics microRNA microarray. Stepwise Cox regression allowed the evaluation of the significance of individual and combined mirs on patient survival, alone or in tandem with clinical risk factors, such as stage, age, smoking and gender. Kaplan Meier plots and logrank analysis were used to compare survival and time to progress profiles of discrete groups. *RESULTS:* For the 20 matched tumor/normal pairs, there were 136 miRs mirs expressed in at least one set of samples. Of these, 66 mirs were significantly differentially expressed ($p < 0.005$) between MM and normal abdominal mesothelium. 34 mirs were upregulated in tumor vs. normal, while 32 were downregulated respectively. No differences in mir expression were seen with regards to gender, age, or the histology of the MM. In a univariate analysis, lower stage, epithelial histology, absence of lymph node involvement, minimal asbestos exposure and female gender were predictive of longer survival. The presence of 4 mirs significantly improved ($p = 9 \times 10^{-6}$) survival in MM patients (MS >38 months) compared to those who did not express the mirs (MS = 5 months). These data represent the first use of mirs for the discovery of potentially novel biomarkers as well as the prognostication of MM. In a separate series of experiments, Rosetta also profiled 7 MM formalin-fixed paraffin-embedded (FFPE) samples and 85 adenocarcinoma FFPE samples from multiple origins, including breast, colon, pancreas and others using the Rosetta Genomics microRNA microarray. Expression levels of over 700 microRNAs were measured on the microarray and compared between the two sample groups. Differentially expressed microRNAs were identified based on their expression on the microarray. The company further developed a microRNA-based assay using qRT-PCR and tested it on 22 MM samples and 44 adenocarcinoma samples from the following origins: lung, colon, pancreas, bladder, kidney, ovary and breast. *RESULTS:* Three microRNAs were significantly differentially expressed between MM and adenocarcinoma. One microRNA was over-expressed and two microRNAs were under-expressed in MM relative to adenocarcinoma. MM primary tumors could be separated from lung adenocarcinoma tumors with sensitivity and specificity exceeding 95%. MM could be separated from the full set of adenocarcinoma samples with sensitivity and specificity above 90%. These data imply that a small number of microRNAs is sufficient to discriminate MM from adenocarcinoma from multiple origins, and mir expression profiles in serum of MM and AE patients is beginning.

Early Detection Research Network (EDRN)-SPORE-PLCO ovarian cancer biomarker validation study

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In 2005, NCI began accepting applications for use of Prostate, Lung, Colon and Ovarian Cancer (PLCO) specimens. Dan Cramer, representing EDRN and Nicole Urban, representing Ovarian SPORES, proposed to identify the current best ovarian cancer biomarkers in a pre-validation set of case-control specimens and then apply those markers to pre-diagnostic specimens from PLCO. Boston, Fox Chase, Fred Hutchison Cancer Center, and MD Anderson provided samples to construct the set which included 160 ovarian cancer cases (blood collected prior to surgery), 480 general population and 160 benign disease controls, and quality control specimens. Case or control status was masked. Fred Hutchinson performed singleplex Luminex assays on 5 markers; MD Anderson performed Ciphergen (Vermilion)-based assays on 7 markers, Boston performed platform or plate-based assays on 13 markers; and Pittsburgh performed multiplexed Luminex bead assays on 34 markers including cancer antigen, hormone/endocrine, cell adhesion, protease, and cytokine panels.

In the prevalidation set, the top ten performing biomarkers were CA 125, HE4, CA 15.3, CA72.4, B7-H4, HK6, transthyretin, Mesothelin, IGFBP-2, and Cytokeratin 19 with sensitivities ranging from 67% to 27% at 98% specificity against general population controls. Coefficients of variation were greatest for Multiplex Luminex assays and least for plate- or platform-based assays. The PLCO has accepted our report and shipped specimens to the 4 assays sites including 119 ovarian cancer cases with specimens obtained months to years before diagnosis. Assays will be completed in March and results returned to the PLCO for unblinding. Possible implications for ovarian cancer screening will be discussed.

Diagnostic markers for early detection of ovarian cancer

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Purpose: Early detection would significantly decrease the mortality of ovarian cancer (OC). In this study we characterize and validate the combination of six serum biomarkers that discriminate between disease-free and OC patients with high efficiency.

Experimental design: 362 healthy controls and 156 newly diagnosed OC patients were analyzed. Concentrations of leptin, prolactin, osteopontin, insulin-like growth factor-II, macrophage inhibitory factor, and CA-125 were determined using a multiplex, bead-based, immunoassay system. All 6 markers were evaluated in: 1) Training set: 181 samples from the control group and 113 samples from OC patients 2) Test set: 181 sample control group and 43 ovarian cancer.

Results: Multiplex and ELISA exhibited the same pattern of expression for all the biomarkers. None of the biomarkers by themselves were good enough to differentiate healthy vs cancer. However, the combination of the six markers provided a better differentiation than CA-125. Four models with, $<$2% classification error in training sets all had significant improvement (sensitivity 84–98% at specificity 95%) over CA-125 (sensitivity 72% at specificity 95%) in test set. The chosen model correctly classified 221 out of 224 specimens in the test set, with a classification accuracy of 98.7%.

Conclusion: We describe the first blood biomarker test with sensitivity 95.3% and specificity of 99.4 % for the detection of ovarian cancer. Six markers provided a significant improvement over CA-125 alone for ovarian cancer detection. Validation was performed with a blinded cohort. This novel Multiplex platform has the potential for efficient screening in high-risk patients for ovarian cancer.

Proliferating macrophages predict worse outcome in breast cancer patients in US and West Africa using immunohistochemistry and peripheral blood analysis

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Background: Macrophages (M$\varnothing$) may play a vital role in the progression and metastasis of some tumors. Tumor cells and M$\varnothing$ interact through a CSF-1 paracrine signaling loop resulting in invasion of blood vessels and penetration of the extracellular matrix. We have found proliferating macrophages (*pm*) to be abundant in clinically palpable DCIS, and used retrospective data sets to determine their presence in invasive breast cancer (BC), and a prospective data set to determine the presence of M$\varnothing$ in peripheral blood (PB).

Methods: 110 cases were selected from UCSF tumor bank and 43 cases were from West Africa (WA). Slides were double-stained for *pm* markers, CD 68 and PCNA using IHC techniques. A subset of pts (66) had tissue available for MAC387 staining. Stage, lyph node, grade, hormone status, and treatment modalities were assessed. Prospectively, a battery of monocyte markers including CSF-1R+ was used to evaluate M$\varnothing$ from PB of 44 pts with stage I-III BC using FACS.

Results: Frequency of *pm* and MAC387+ M$\varnothing$ correlate with poor outcome in patients with long-term follow-up. These markers are more prevalent in high grade disease, 54% with *pm* $\geqslant$5 per HPF versus 34% in low grade disease ($p = 0.04$) and in tumor from WA, where the tumors are predominantly ER negative (78%). Double staining with PCNA and CD 68 is not reliable or practical due to variable staining of PCNA and time-consuming aspect of counting PCNA+/CD68+ M$\varnothing$ manually. However, MAC 387 is simpler. We are in the process of evaluating all candidate M$\varnothing$ markers: CD 68, MAC 387, CSF-1R and CD11a.

Prospectively, we are assessing M$\varnothing$ markers in the peripheral blood (PB). CSF-1R+ monocytes seem to best differentiate patients with invasive tumor, where the mean was 24.4% of total monocytes (range 0–85%). Higher levels of CSF-1R+ monocytes correlated with higher estimated 10 yr recurrence and 10 yr mortality ($p = 0.049$, $p = 0.004$, respectively) using

Adjuvant!Online® program. The mean CSF-1R values were significantly lower for the control group, 7.9% in pts without invasive cancer ($p = 0.003$).

Conclusions: Proliferating macrophages are associated with higher recurrence and worse survival in BC and may be a target for therapy and early detection. We are prospectively evaluating and correlating levels of CSF-1R M∅ in both PB and tissue.

Beyond PSA 2008 – Novel biomarkers for prostate cancer under clinical evaluation

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The utility of tPSA for early detection of prostate cancer has been questioned, especially following a negative biopsy. Our group has investigated novel tissue (methylation markers – GSTP1, APC), urine (PCA3) and serum markers (proPSA, EPCA-2) for detection of prostate cancer in retrospective and prospective clinical studies. GSTP1 and APC methylation ($N = 86$ – Veridex study group) showed SENS 95% – NPV 96%, PCA3 (N = 570 – GenProbe study group) ROC-AUC 0.7, proPSA (N = 123 – EDRN study group) ROC-AUC 0.69, EPCA-2 (N = 385 – Hopkins data) with SPEC 97% – SENS 94%. The results of these preliminary trials show great promise for these markers in the future.