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Posters

Enhancement of Biomarker Discovery by Fractionation of Human Serum Using MicroSol-IEF
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The wide range of protein concentrations found in human serum poses a considerable challenge with regard to proteomic analysis, as it restricts the detection and quantitation of low abundance proteins, including cancer biomarkers. A critical component of several alternative protein profiling strategies designed to detect lower abundance proteins in serum is a high resolution fractionation method developed in our laboratory. This micro-scale solution isoelectrofocusing (MicroSol-IEF) method is now available from Invitrogen Corp. as the Zoom-IEF Fractionator^{TM}. This simple, inexpensive and efficient device is used to separate proteins according to their isoelectric points (pI) into up to seven fractions, thereby reducing sample complexity and consequently simplifying and enhancing the detection capacities of downstream analytical methods, including top-down approaches such as 2-D gels and bottom-up LC/LC-MS/MS approaches. We previously reported the advantages of this approach for analysis of human breast cancer cell extracts. In addition we have used the device to fractionate human and mouse serum either with or without major protein depletion.

We are currently optimizing fractionation of human serum for biomarker discovery using Micro-Sol IEF by systematically evaluating the effects of different experimental conditions on serum proteome fractionation. Parameters examined include maximum sample loads with and without major protein depletion, optimal focusing conditions, and effects of various buffer formulations. Recent results show that either MicroSol IEF prefractionation or major protein depletion can substantially enhance our ability to detect lower level proteins but the combination of these two steps in tandem provide limited synergy. In contrast, a new 4-D separation method developed in our laboratory shows great promise for detection of low abundance serum proteins. This protein array-pixelation method utilizes depletion of the six most abundant serum proteins, MicroSol IEF fractionation, 1-D gel electrophoresis and LC-MS/MS. Based upon initial results, it may be advantageous to fractionate serum proteomes into more than 5 to 7 pI range fractions.

Development of Assays to Detect Cancer Specific Proliferating Cell Nuclear Antigen (csPCNA)

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The proliferating cell nuclear antigen (PCNA) is a DNA polymerase delta accessory factor, and has a role in the DNA synthetic, repair, and transcription processes of the mammalian cell. Two forms of PCNA have been previously described in human cells. Non-malignant cells express only a basic form of the PCNA molecule, while cancer cells express an additional acidic form of the protein, which is identified as a cancer specific PCNA (csPCNA). csPCNA has been shown to be present exclusively in a variety of cancer cell lines and cancer tissues tested, but has not been found in any genetically matched normal cell lines, normal tissues, or benign tumors. Thus, csPCNA may serve as an early tumor marker, if it can be detected at sufficiently low concentrations in otherwise-asymptomatic patient specimens.

Malkas and Hickey's previous data showed that a recombinant xeroderma pigmentosum G protein (XPG) selectively binds only the csPCNA, but does not bind the non-malignant, or "normal" form of PCNA. Creatv proposes a sandwich immunoassay that will provide high specificity to capture, identify, and quantify csPCNA in serum of prostate cancer patients. The assay will be performed on the inner surface of a capillary waveguide. The waveguide will be prepared as follows: (a) XPG will be immobilized to the waveguide

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via biotin/avidin interaction, (b) patient serum will be incubated in the capillary and XPG will capture the csPCNA, while the non-malignant isoform of PCNA and other serum components will be washed away, (c) PC10 antibody, a commercially available monoclonal antibody to both csPCNA and PCNA, tagged with fluorescent label, will be used for detection. A sandwich will be formed only if the analyte (csPCNA) is captured, generating a fluorescent signal.

Currently, there are no commercially available reagents for this assay. csPCNA will be obtained from human HeLa and MCF7 cell lines. Normal PCNA will be obtained from non-malignant 184A1N4 breast cell line. Creatv is preparing csPCNA and normal PCNA, and IUPUI will provide XPG to develop the sandwich assay. After developing the assay using csPCNA in buffer, assays using serum samples of prostate cancer patients and normal serum controls will be developed. Experiments are in progress and results will be presented.

Serum Proteomic Profiling Using SELDI-TOF for Prostate Cancer Diagnosis

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Intriduction and objective: The serum prostate-specific antigen (PSA) test has been extensively used in early detection of CaP; however, its low specificity for CaP detection (25–30%) has emphasized the need for better CaP-specific diagnostic biomarkers. Clinical utility of serum surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) proteomics is currently being investigated in our laboratory as well as in others. Reproducibility and data analysis issues are being addressed through robotic automation and use of multiple bioinformatic approaches. As a follow-up of our initial study on the diagnostic potential of serum proteomic profiling using a dual-chip (SELDI-TOF) assay which gave both a sensitivity and specificity of 85% for detection of CaP, we further evaluate the performance of this assay on a larger cohort of patients.

Methods: Sera from 370 CaP patients who underwent radical prostatectomy and 70 healthy male volunteers with PSA <4 ng/ml and with no evidence of prostate cancer were included in the study. Serum samples were processed in duplicate using a Biomek 2000 robotic workstation. SELDI-TOF profiles were generated using IMAC3-Cu and WCX2 arrays by Ciphergen Protein Biological System II ProteinChip Reader. Data analysis was performed using Biomarker Patterns Software. A training set consisting of 50 CaP patients and 50 controls were used for decision tree building. A blinded test set consisting of 20 controls and 320 CaP patients was used to challenge the algorithms. Additional control specimens are being collected for further analysis.

Results: A decision tree algorithm consisting of 4 discriminatory peaks with mass designations of 4067Da and 12617Da on the WCX2 array and 7782Da and 9306Da on the IMAC3-Cu array was generated by Biomarker Patterns Software. The algorithm correctly identified 255 of the 320 CaP patients and 17 of the 20 controls in the test set giving a sensitivity of 80% and specificity of 85% on validation.

Conclusions: Using a larger cohort of CaP patients and streamlined robotic processing of specimens, serum proteomic profiling using SELDI-TOF in a follow-up study continues to show promising potential for CaP detection with high sensitivity and specificity. Further evaluations/development of this new technology is underway including additional specimens and new algorithms for the analysis of the same data set.

Elevated Serum Levels of Heat Shock Proteins in Small Cell Lung Cancer

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Heat Shock Proteins (hsp) were first discovered as a cohort of proteins that are powerfully induced by heat shock and other chemical and physical stresses in a wide range of species. The hsp have been subsequently characterized as molecular chaperones, proteins which have in common the property of modifying the structures and interactions of other proteins. HSP 27, 70, 90, and 110 increases to become the dominantly expressed proteins after stress. In addition to the hsp induced by

heat, cells also contain a large number of constitutively expressed hsp. Many tumor types have been reported to contain high concentrations of heat shock proteins of the hsp28, hsp70 and hsp90 families. The role of hsp in tumor development may be related to their function in the development of tolerance to stress. Elevated hsp expression has been conjectured to participate in tumor pathogenesis through the ability of individual hsp to block the pathways of apoptosis and permit malignant cells to arise despite the triggering of apoptotic signals. Hsp expression is likewise thought to afford protection of cancer cells from treatments such as chemotherapy and hyperthermia by thwarting the pro-apoptotic influence of these modalities. The mechanisms underlying elevated hsp expression in tumor cells are not known. Hsp have been regarded as intracellular proteins. However, enough evidence has been shown that under certain circumstance they are released from cells. We have determined the circulating level of hsp70 and hsp27 in small cell lung cancer representing limited disease (LD), extensive disease (ED), no evidence of disease post therapy (NED) and relapsed (RL). Our data indicates higher level of circulating hsp 70 and hsp 27 in different groups of patients compared to normal healthy individual. The group representing ED and RL showed significant level of change. While hsp70 level remains consistently higher in all patients within a group, patient to patient variation was observed in the serum level of hsp27.

Proteomic Precision

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Background: Surface enhanced laser desorption/ ionization time-of-flight mass spectrometry (SELDI-TOF) technology provides individual mass-to-charge ratios (m/z) that correspond to individual proteins. We were interested in the precision of these m/z measurements.

Methods: We used the serum spectra of men who either had organ-confined prostate cancer ($N = 80$) or were age-matched controls ($N = 80$). We picked arbitrary m/z values across the spectra. We used SAS (Cary, NC) to assess the correlation (Pearson) between adjacent m/z ratios at each arbitrary m/z value across the patients in the prostate cancer group and the control group (Table 1). In addition, we calculated the m/z correlation between the patients in the two groups at the same m/z ratios (Table 2).

Table 1.

m/z	controls	prostate ca.
0	0.88175	0.90763
1000	0.98553	0.98722
2000	0.93872	0.99464
3000	0.99172	0.96558
4000	0.99862	0.99853
5000	0.93169	0.95180
6000	0.94084	0.96054
7000	0.99425	0.99822
8000	0.98273	0.97647
9000	0.99312	0.99397
15000	0.99997	0.99999
25000	0.99969	0.99945
35000	0.99996	0.99991
45000	0.99998	0.99997
55000	0.99832	0.99887
65000	0.99998	0.99999
75000	0.99999	0.99998
85000	0.99979	0.99952
95000	0.99999	0.99998
150000	0.99998	0.99994

Table 2.

m/z	correlation
0	0.03808
1000	0.27406
2000	0.07815
3000	0.04925
4000	0.26043
5000	−0.14705
6000	0.02760
7000	−0.11235
8000	−0.11067
9000	−0.18388
15000	0.01602
25000	0.08703
35000	0.00091
45000	−0.20117
55000	−0.05021
65000	0.19529
75000	0.06000
85000	0.08286
95000	−0.00716
150000	0.06520

Results: The adjacent m/z values were almost perfectly correlated at each arbitrary m/z value. The m/z values between cases and controls demonstrated an almost complete lack of correlation.

Discussion: Since adjacent m/z values represent different proteins we expected there to be almost no correlation between m/z values. We found the opposite, there was an almost perfect correlation between adjacent m/z values. Of course, there are isotopes for each protein but they cannot account for the observed degree of correlation. Since most circulating proteins are not related to prostate cancer we expected there to be a high correlation between the cases and controls at each m/z but we observed an almost total absence of correlation between m/z values.

Analysis of Breast Aneusomy in Four Chromosomes using Fluorescence In Situ Hybridization on Tissue Microarrays

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Through the recent advances in the development of molecular probes, Fluorescent In Situ Hybridization

(FISH) has been a standard technique that allows investigators to refine their analysis of chromosomal anomalies in the study of cancer. Previous studies have used Multi color FISH to identify aneusomic patterns in individual cells among cytology specimens from FNAs, touch preparations, ductal lavage, and other forms of bodily fluid or aspirates. Studies done on whole tissue specimens have been limited. We performed FISH on a breast tissue microarray comprised of 246 breast cancer cases, using directly labeled locus-specific and alpha-satellite DNA probes, LSI 1®, CEP® 8, CEP 11, and CEP 17 (Breast Aneusomy Probe Set, Vysis, Inc.), to assess chromosome 1, 8, 11, and 17. Representative cells were analyzed at random, measuring the number of signals or ploidy of each cell correlated with their respective histologies. Our preliminary findings show a significant difference in the ploidy of normal cases versus benign and malignant cases, and suggest that the analysis of chromosomal gain proves to be beneficial and of more significant value than analysis of chromosomal loss in tissue specimens. Our results indicate that the percent of cells with chromosomal gain among the four markers was highest in ductal carcinoma in situ (DCIS), followed by invasive ductal (IDC), then in benign cases, such as ductal hyperplasias (DH), atypical ductal hyperplasias (ADH), and other metaplasias, as compared to normal. DCIS was found to have the highest frequency of polysomy in all 4 chromosomes when compared to other histologies. Chromosome 8 was most frequently amplified, followed by chromosome 1. LSI 1 and CEP 8 exhibit a higher sensitivity and specificity than the other probes for detecting amplification. Our results suggest that these probes may have clinical diagnostic and/ or prognostic value in the detection of cancer. We will present our complete data.

HLA-DQB1 Allele Distribution and Other Risk Factors for Cervical Cancer in Vietnamese Women

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Cervical cancer is the second cause of cancer-related deaths of women in the world and the leading cause of death from cancer among women in developing countries. Persistent infection with high risk human papillomaviruses (HR-HPV) is essential but not the exclusive prerequisite for the initiation of cervical carcinogenesis. Epidemiologic factors such as commencing sexual encounters at a young age and having many sexual partners play a role in increasing the chance of exposure to HPV. In addition, cigarette smoking, nutrition, and co occurrence of other sexually transmitted infections may act as cofactors with HPV. However, since only a small percentage of HPV infected women develop cervical cancer, host genetics seem to play a key role in the whole process. The role of genetics is strongly suggested by the observation that women in Vietnam have one of the highest rates of cervical cancer in the world. Likewise, Vietnamese women residing in the United States have the highest rate of cervical cancer in the US population where the incidence rate among Vietnamese women is 5 times the Caucasian rate. Epidemiologic information and Pap smears were obtained from101 asymptomatic Vietnamese women (aged 23 to 90) attending the OB/Gyn clinic of Da Nang General Hospital in Da Nang, Vietnam. Whereas 2% of the women studied were light smokers, 60% lived in a household with a smoker. The majority of the subjects, 99%, had only one sexual partner; 82% had their first sexual experience in their 20's, 11% in their 30's and only 5% in their late teens. From the 101 Pap smears, 13% had dysplasia, 31% had ASCUS, and 56% were cytologically normal. HPV infection prevalence was 53% (54 samples): 50 carried a single HPV type while 4 harbored multiple types. HPV types 6, 11, 16, 18, 20, 23, 31, 33, 39, 52, 53, 55, 58, 59, 62, 70, 74, 84, DL416 and RTRX9 were detected. HLA-DQB1 typing was successfully performed on 96 samples. DQB1*0301 (24.5%) and DQB1*0501 (22.4%) allele frequencies were the highest among the Vietnamese population in this study. Strikingly, HLA-DQB1*0301 allele has been reported as a high risk allele for cervical cancer and DQB1*0501 allele has been described as a protective allele in other populations. When allele frequencies were cross tabulated against cytological and HPV infection status no significant differences were found. Coexistence at high frequency of two known suscepti-

ble and resistant HLA-DQB1 alleles in this Vietnamese population, strongly suggests other interacting genetic determinants besides HLA-DQB1 which deserve further research.

Stage-Specific Modifier Genes for Transition Between Early and Later Stages of Cancer – Implications for Predictive Value of Early Lesions

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Early stages of cancer development are potentially important diagnostic and prognostic markers that could facilitate choices of preventive or therapeutic measures. Their relevance is based on their potential to progress to more advanced stages of cancer.

This potential is influenced by somatic mutations in the early lesions and by the functional characteristics of their cells. However, using the mouse models of colon and lung cancer, we have shown that one of the most important factors influencing the rate of the progression from the early lesions to genuine tumors is the genotype of the host. We have demonstrated that the number of aberrant crypt foci (ACF) in colons of carcinogen treated mice is strain-dependent and does not correlate with the number of adenomas [1]. Although some strains develop large numbers of ACFs and large numbers of adenomas, others small numbers of ACFs and small numbers of adenomas, there are as many strains that develop large numbers of ACFs but small numbers of adenomas, or small numbers of ACFs and large numbers of adenomas. In fact, the ratio of the average number of ACFs and average number of adenomas per mouse in different strains ranges from 0.4 to 12 and it is not obviously dependent on size of the ACFs or the degree of dysplasia. This indicates that the "predictive" value of the ACFs varies between different mouse strains up to thirty-fold and is to a large extent determined by the host's genotype. As the differences in inducibility of ACFs are genetic, the responsible genes can be identified by genetic and molecular analysis.

The genetic variation in the "predictive value" of early lesions reflects the presence of stage-specific modifiers of tumor progression. To investigate and identify the responsible genes, we have developed models for study of such modifiers in the colon and lung cancer. They include strains with specific differences in the frequency of early, advanced, and highly progressed stages that can be used for identification of the stage specific modifiers. Identification of the genes modifying the transition from the early lesions to advanced tumors will open possibilities for a more precise assessment of the prognostic significance of the early lesions, but also for a better understanding of the molecular mechanisms underlying the early stages of tumor progression.

Reference

[1] C.J.A. Moen et al., *Cancer Res* **56** (1996), 2382–2386.

Enhancing Cancer Serological Biomarker Detection Using Major Protein Depletion Prior to Alternative Down Stream Protein Profiling Methods

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A major goal of proteome research is disease biomarker discovery in biological fluids. Disease markers, which can be key factors for early diagnosis, monitoring response to therapy, and detection of relapse, are usually present at relatively low concentrations (ng/ml or less). Serum and plasma offer a promising resource for cancer biomarker discovery because collection of these samples is minimally invasive and blood is thought to contain the majority of protein constituents found in the body. However, the complex nature of serum, and the presence of a modest number of very abundant proteins (0.1 to 40^+ mg/ml) makes detection of low abundance cancer biomarkers very challenging. We are evaluating several methods to divide the proteome into smaller subsets to identify as many proteins as possible and detect low abundance disease biomarkers.

A highly promising first step for most analysis strategies is to deplete as many of the major proteins as possible. A range of methods to deplete high abundance proteins were evaluated including dye based affinity kits, individual antibodies and multiple antibodies. The goal was to deplete as many proteins as possible to

very low levels while minimizing incidental losses of non-targeted proteins. The effects of depletion were evaluated on 1-D and 2-D gels as well as by protein array-pixelation (a combination top-down/bottom-up approach). The most effective major protein depletion method currently available is the Agilent MARS antibody column, an HPLC column containing polyclonal antibodies to 6 of the must abundant human serum proteins. A more convenient spin column version of this purification system, which can be more easily adapted to use at 4°C to minimize proteolysis, is currently being evaluated. The targeted proteins were effectively removed with minimal losses of non-targeted proteins. Due to this efficient removal of about 85% of the total serum protein content, at least 10 to 20-fold higher volumes of depleted serum could be analyzed by down-stream separation methods. This substantially improved detection sensitivity, but the next most abundant proteins became limiting and prevented detection of even lower abundance proteins. Hence, to optimize detection of low abundance cancer biomarkers, it would be highly desirable to remove approximately 18 of the most abundant proteins, which constitute about 98% of the total serum protein content. It is estimated that this would improve detection limits by most protein profiling methods by at least 200-fold or more.

Autoantibodies to Annexin XI and Other Autoantigens in the Diagnosis of Breast Cancer

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The use of SEREX was a major advancement in immunoscreening and led to the identification of a large group of candidate autoantigens recognized by cancer patient sera. However, the multiple autoantigens reported using SEREX by far outnumber the accepted tumor-restricted antigens and none have so far been proven to have definitive diagnostic value in clinical practice. Often, the identified antigens are patient-specific rather than tumor-specific or if they are tumor-specific, they may be infrequent, rare or absent in expression libraries made from cancer cell lines or other non-autologous cells. Other antigens recognized by cancer patient sera may be related to the spectrum of autoimmunity or to the aging process and may not be at all related to cancer. The skepticism of the scientific community in regard to autoantibody-based methods resides in the universal failure of enormous amount of work attempting to identify biomarkers of clinical significance. Since autoantibodies are the normal immune response, one important problem not previously solved by autoantibody-based methods of identifying tumor-associated antigens, is to consistently demonstrate their tumor relevance. As a consequence the cancer-relatedness of novel serologic markers identified by immunoscreening cDNA expression libraries must be established independently. We have introduced substantial modifications in the SEREX methodology designed to minimize the confounding effect of unrelated antibodies, that in our view have prevented the discovery of diagnostic biomarkers of definitive value for the diagnosis of cancer. The most important innovations inherent to this method are related to the selection of the cloning sera, since we have demonstrated that not all cancer patient sera are suitable for screening cDNA libraries. Sera containing high titre, recurrent IgG signals of identical molecular mass on immunoblots are selected for screening a T7 phage display cDNA library made from a non-autologous cancer cell line. In the process of screening the T7 phage library we omit steps that may prevent the identification of cancer-related molecules, such as absorption with host cells or with vector antigens and biopanning with pools of "non-cancer" sera to eliminate irrelevant clones since we have found that these procedures can also eliminate relevant clones. Immunoreactivity rather than random selection is used in each round of biopanning as the main criterion to select positive clones. The size of the clones (single or multiple) is determined by RT-PCR and the nucleotide sequence of each clone is determined. Finally, the cancer relatedness of the cloned antigens is determined by constructing an autoantigen microarray with the cloned ESTs and probing this array with cancer patient sera and suitable non-cancer control sera.

This method has been used in the discovery phase of a panel of novel autoantigens with potential value for the early diagnosis of breast cancer (Autoantibodies to annexin XI and other autoantigens in the diagnosis of breast cancer. Félix Fernández Madrid, Naimei Tang, Huda Alansari, José L. Granda, Larry Tait, Kathryn Carolin, Mihail Moroianu, Xiaoju Wang, and Robert L. Karvonen. Cancer Research, in revision, 2004).

Affymetrix Gene Expression Profiles in Premalignant Bronchial Mucosa

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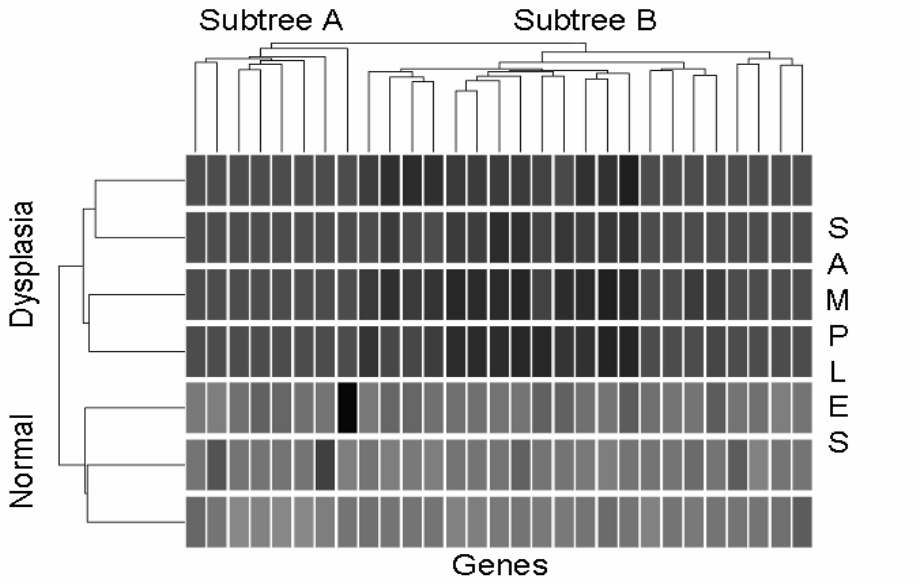

Table--Gene Clusters overexpressed in dysplastic bronchial epithelium	
Subtree A	**Subtree B**
keratin 6A	BCL2-related ovarian killer
keratin 6A	carcinoembryonic antigen-related cell adhesion molecule 8
sciellin	CD109 antigen (Gov platelet alloantigens)
small proline-rich protein 1A (SPRR1A)	cytochrome P450, family 3, subfamily A, polypeptide 5
small proline-rich protein 1B (cornifin)	epithelial membrane protein 1
small proline-rich protein 2B	GPI-anchored metastasis-associated protein homolog
small proline-rich protein 3	heparin-binding growth factor binding protein
small proline-rich protein 3	Homo sapiens cDNA: FLJ21198 fis, clone COL00220.
	Homo sapiens, clone IMAGE:4822062, mRNA
	Human tumor antigen (U6) mRNA, complete cds.
	hypothetical gene supported by BC028978
	hypothetical protein FLJ32110
	hypothetical protein MGC35033
	keratin 23 (histone deacetylase inducible)
	keratin 6B
	potassium channel, subfamily K, member 7
	ribosomal protein L18a
	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2
	sister-of-mammalian grainyhead
	transmembrane 4 superfamily member 1
	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)

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To evaluate phenotypic changes that occur during tobacco-induced lung carcinogenesis, have located central airway lesions in high-risk smokers by fluorescence bronchoscopy. Abnormal sites were biopsied for conventional light microscopic examination and two additional biopsies were flash frozen for RNA extraction and oligonucleotide microarray analysis. Affymetrix HGU133 Plus 2 microarray profiles from dysplastic biopsies were compared with profiles of normal mucosa from never smoker volunteers. A total of 7 lesions were assessed, 4 dysplasias from high risk smokers and 3 sample sets from never smokers. Initially, data from the four dysplastic biopsies was compared with that from the three normal biopsies using ANOVA assuming equal variances, a p-value cutoff of 0.05% and the multiple testing correction Benjamini and Hochberg false discovery rate. This resulted in a list of 163 genes. Since we were primarily interested in genes with high differential expression, we furthered the ANOVA list for those genes that were expressed at a level 5 to 3000

fold higher in dysplasia than in normal lung tissue. We arrived at a final list of 29 genes that are overexpressed in dysplastic bronchial biopsies in comparison to normal biopsy material. Cluster analysis using the 29 gene list clearly separated dysplastic from normal epithelium and divided the genes into categories that roughly corresponded to level of expression. In the attached figure subtree A consisted of 6 genes (two in duplicate) that are overexpressed at a high level while the genes list for subtree B contain genes expressed at an intermediate level (5–10 fold). A remarkable feature of this list is the high frequency of genes associated with squamous differentiation. The cytokeratin subtype most highly expressed is subtype 6, a finding that is confirmed by independent immunohistochemical studies. The small proline-rich region proteins cross-link structural proteins in the epidermal cornified envelope. These are proteins that are associated with epithelial cornification and epidermal differentiation. Overall the most consistently and highly expressed genes are associated with the well-documented proclivity of dysplastic cells to differentiate in the lower airways as squamous cells rather than mucociliary cells. The role of significantly overexpressed genes in subtree B is less clear at the present time.

Discovery of Aberrant Expression of Tumor-Associated Genes in Lung Cancer and Demonstration of Expression of Those Genes in Sputa of Lung Cancer Patients and High Risk Smokers

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	Pos	Neg	Totals
Tumor	38 (73%)	14 (27%)	52 (100%)
Dysplasia	27 (69%)	12 (31%)	39 (100%)
Normal	2 (22%)	7 (78%)	9 (100%)

Several obstacles have prevented the development of effective screening methods for lung cancer including 1. anatomical inaccessibility 2. unpredictability of tumor site of origin 3. confounding effects of smoking on biomarker expression and 4. incomplete basic information on lung cancer genotype and phenotype. To overcome these obstacles we have developed an algorithm for the identification of genes that are highly overexpressed in lung cancer and for testing of specimens that are accessible including blood, urine and sputum in lung cancer and high-risk patients. RNAs from 14 lung carcinoma lines including 2 small cell and 12 non-small cell lines were applied to an Affymetrix HU133A microarray and normalized against RNA from cultured normal epithelial cells. List of potential biomarker for genes that are 1.) overexpressed in tumor cells at 30 times the level of normal cells and 2.) of potential biological interest. Since we expected a high level of heterogeneity among the tumors, consistency of overexpression was not a criterion for selection of candidate biomarker genes. A list of candidates was then tested by RT-PCR against an expanded panel of cell lines, tumors homogenates and normal tissues. Five genes that were expressed at high levels in tumor but were silent in non-neoplastic tissue were identified, including MAGE A3/6, XAGE1, TM4SF4, NUP210 and PGP 9.5. We then used a sensitive nested RT-PCR method to test Saccomanno's fixed sputum from patients with lung carcinoma, from normal never smokers and from smokers at high risk for lung cancer as determined by the presence of obstructive lung disease (FEV1 $>$70% of predicted value) and sputum atypia. All specimens were tested in triplicate and were considered positive if any single RT-PCR reaction was positive. The frequencies of subjects with one or more positive test results are listed in the adjacent table. Chi square analysis indicates that differences between normal and tumor and between normal and dysplasia are significant at $p < 0.003$ and $p < 0.005$, respectively. Twenty-four (63%) of the positive tumor patients and were positive in more than one test as were 23 of the positive dysplasia subjects. Neither of the two positive normal subjects was positive in more than one test. We conclude that a sensitive nested RT-PCR method can demonstrate expression of a subset of genes that are specifically tumor-associated in lung cancer sputa and in sputa of high-risk smokers. The prognostic significance of this test in high-risk smokers is the subject of ongoing investigation.

SELDI Serum Assays that Classify Cancer Types by Measuring Variants of Host Response Proteins

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Protein expression profiling is used to discover and characterize biomarkers that can be used for diagnostic, prognostic, or therapeutic purposes. We recently discovered a multi-marker panel comprised of apolipoprotein A1, transthyretin, and inter alpha trypsin inhibitor heavy chain 4 (ITH4) that could distinguish patients with early stage ovarian cancer from healthy controls. These proteins represent a host response present at the earliest stage of cancer. We demonstrate that two of the host response proteins, transthyretin, and inter-alpha trypsin inhibitor heavy chain 4 (ITIH4), are extensively post-translationally modified. These modifications include truncation, cysteinylation, and glutathionylation. Assays using surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) may provide a means to confer clinical specificity to these proteins because of their ability to detect and quantitate multiple post-translationally modified forms of these proteins in a single assay. Quantitative measurements of these modifications using chromatographic and antibody-based ProteinChip® array assays reveal that these post-translational modifications occur to different extents in different cancers, and that multivariate analysis permits the classification of these cancers. We have termed this process host-response protein amplification cascade (HRPAC), since the process of synthesis, post-translational modification and metabolism of host response proteins amplifies the signal of potentially low-abundant biologically active disease markers such as enzymes.

Identification of Biomarkers for Gastric Pre-Neoplasia Using Gene Microarray and Proteomic Analysis of Laser Capture Microdissected Lineages

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While chronic infection with Helicobacter pylori is acknowledged as a requisite for the development of atrophic gastritis and gastric cancer, the intermediate steps between loss of parietal cells and the development of neoplasia are less well-established. Oxyntic atrophy leads alterations in fundic mucosal lineages including foveolar hyperplasia and mucous cell metaplasias. While goblet cell intestinal metaplasia has received the most attention as a putative precursor for gastric cancer in humans, in mice goblet cell metaplasia is not observed in Helicobacter infected mice that go on to develop gastric cancer. We have recently found that both human gastric cancer and murine gastric cancer are associated with mucous cell metaplasia, designated spasmolytic polypeptide-expressing metaplasia (SPEM), which establishes an antral phenotype lineage in the gastric fundus. Using both gene microarray and 2D-DIGE proteomics, we have examined SPEM cells isolated by laser capture microdissection from male Helicobacter-infected C57BL/6 mice. The microarray studies led to the identification of 9 protein gene products and 2 non-coding RNAs that were enriched in SPEM over surface cells. Ten out of 11 of the transcripts were also expressed in regions of gastritis cystica profunda, a pre-neoplastic pathology. The non-coding transcript for Xist was only observed in SPEM and gastritis cystica profunda in the infected male mice. Antibodies against prothymosin-alpha confirmed the over-expression of this anti-apoptotic protein in SPEM and gastritis cystica profunda. Proteomic analysis identified the upregulation of ER-60, an ER chaperone, in SPEM cells. Antibodies against ER-60 confirmed its expression in SPEM in mice and humans. We have further studied the expression of prothymosin-alpha and ER-60 in tissue microarrays from gastric cancers and non-cancerous mucosa. Quantitative analysis of prothymosin-alpha expression suggests the over-expression of prothymosin-alpha in the nuclei of gastric cancers. The results demonstrate that gene microarray and proteomic analysis of microdissected normal, metaplastic and neoplastic cell lineages can identify critical biomarkers for gastric pre-neoplasia.

Assessment of the Association Between the Puvii and Xbai Estrogen Receptor Alpha Gene Polymorphisms and the Risk of Prostate Cancer in a Multiethnic Population

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Introduction and objectives: Normal prostate development is an estrogen-dependent process. Recent studies suggest the possibility that polymorphisms of the estrogen receptor alpha may be associated with an increased risk for prostate cancer. The objective of this study was to evaluate the association of estrogen receptor alpha PvuII (-397 T –> C, located in intron 1) and XbaI (-351 A –> G, located in exon 2) polymorphisms and the risk of prostate cancer in a multiethnic population.

Materials and methods: A case-control study consisting of 1,969 men (1,098 Caucasians, 568 Hispanics and 303 African Americans) was conducted evaluating participants in the San Antonio center for Biomarkers Of Risk for prostate cancer (SABOR) study to assess their genotype for the *PvuII* and *XbaI* polymorphisms in the estrogen alpha receptor gene. Optimal controls were defined as those without a history of prostate cancer, normal digital rectal examination, PSA $\leqslant$2.5 ng/ml and age $\geqslant$50 years ($\geqslant$40 years if African American or if positive family history of prostate cancer).Allelic frequency determinations were made across ethnic groups. Logistic regression analysis adjusted for age was conducted to assess the association between these polymorphisms and cancer risk.

Results: Allelic frequency was significantly different across ethnic groups for both polymorphisms. However, case-control analysis performed on 503 cases and 1,097 optimal controls stratified by ethnicity demonstrated no association between the *PvuII* and *XbaI* polymorphisms and the risk of prostate cancer in our study population.

Conclusions: Based on our results, we can not confirm that there is an association between the *PvuII* and *XbaI* polymorphisms in the estrogen receptor alpha gene and the risk of prostate cancer.

SELDI MS and SELDI-Like MS Discovery of Cancer Serum Biomarkers: Does the Low Mass Sub-Proteome Have Diagnostic Value or Is the Approach Fatally Flawed?

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Conventional SELDI (surface enhanced laser desorption ionization) MS is a high throughput protein profiling method that has generated much excitement, but has substantial limitations. Chemistries used for protein simplification are general and isolate complex mixtures that include many signal-suppressing proteins that are outside the low mass range that is effectively profiled by MALDI MS. Useful mass signals are primarily limited to $<$20 kDa because sensitivity and resolution decrease rapidly as the mass range increases. Most important, it is extremely difficult to obtain definitive protein identifications for interesting features, which is particularly critical for identifying events that may appear to be associated with a cancer state but actually reflect more generalized changes such as proteolysis or oxidative damage.

We (and other groups) have recently developed improved SELDI-*like* methods that retain the advantages of high throughput, sensitivity, and complex pattern recognition, while addressing the shortcomings of conventional SELDI-MS. A promising approach is to selectively isolate the low mass sub-proteome using denaturants to disrupt non-covalent interactions followed by ultra filtration using a concentrator membrane, followed by either automated MALDI MS or LC-ESI MS. This strategy removes the high abundance large proteins outside SELDI's range of detection ($>$20 kDa) to minimize signal suppression and adequate sample remains in solution for the identification of proteins or peptides responsible for potentially diagnostic features seen on MALDI spectra. After identification of the proteins responsible for diagnostic patterns, one can establish quantitative ELISA assays or antibody array assays to reliably quantitate putative biomarker levels on large patient cohorts.

We are also developing improved computational methods to detect complex patterns of biomarkers. Current methods typically involve searching (e.g. genetic algorithms, simulated annealing) or dimensionality reduction (e.g. principle components analysis, neural networks) that sacrifice completeness of the solution for computational tractability. We have implemented a method of deterministic and complete pattern discovery that finds all patterns of biomarkers in all combinations of cases.

However, an interesting fundamental question is whether the low mass sub-proteome is the optimal place to search for cancer biomarkers. All known cancer biomarkers discovered by non-proteomic methods are larger than 25 kDa, and small proteins and peptides are rapidly removed from the blood unless they

are associated with large macromolecular complexes. In this regard, several groups have proposed that major serum proteins act as molecular sponges to protect small biomarkers. But a key question is whether sufficient disease specific biomarkers exist in the low mass range that can be distinguished from variations in proteolysis of major proteins. We are systematically addressing this problem by evaluating the complexity, concentration, and identity of small proteins and peptides in the low mass range in normal serum. These studies show the entire low mass subproteome represents a very small portion of total serum protein content and is apparently less complex than the high mass component of serum.

Antibody-Based Early Detection of Prostate Cancer Biomarkers

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Evaluation of prostate specific antigen (PSA) is the current screening method for early detection of prostate cancer. PSA is more prostate specific than cancer specific, and elevated PSA can also be found in benign conditions of the prostate. Thus, in spite of the overall success of PSA screening, there is a continued need for more sensitive and specific methods for early detection of prostate cancer.

We have developed an antibody-based approach to biomarker discovery in cancer, focusing on differential protein expression, using: i) a large collection of about 100,000 high affinity polyclonal antibodies raised against individual human proteins and their isoforms; ii) a proprietary multiplex protein array immunoassay, namely the matrix protein array technology (MPAT), coupled to a readout and data analysis system; and iii) clinical samples (tissue and serum) from normal, benign prostate hyperplasia (BPH), early and advanced prostate cancer patients. Protein extracts from specimens were printed onto a membrane, then allowed to interact with individual antibodies. Antibody-sample reaction was detected by chemiluminescence and quantified by computer analysis of a CCD-acquired image, followed by statistical analysis.

In the screening of tissue specimens we have identified three distinct panels of antibodies specific for cancer, BPH, and prostate tumor, respectively. In addition, serum screening of 150 samples (provided by EVMS) revealed antibody panels discriminating early versus late stage prostate cancer. Further characterization of the biomarkers was obtained by western blot analysis.

These biomarkers offer potential applications in the early diagnosis of prostate cancer and discrimination from benign conditions of the prostate.

Somatic Cell Sampling and Recovery (SCSR): A Noninvasive Approach to Cellular Biomarkers

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Early detection of disease processes requires access to somatic cells. This is generally obtained by invasive procedures such as biopsies or surgical intervention. A noninvasive approach for obtaining somatic cells would therefore be a major advance in our search for early detection of biomarkers. Noninvasive technology strategy is based on the fact that gastrointestinal tract sheds millions of cells per day into the fecal stream and they can be recovered in their native state for downstream studies of cancer biomarkers. The colonic cells can be isolated in a viable state (85%) from a small sample (0.5 gm–1.0 gm) of human stools noninvasively, by a proprietary procedure known as Somatic Cell Sampling and Recovery (SCSR). Exfoliated colonic cells are therefore an ideal source of cellular material for the study of tumor- associated biomarkers in patients with colonic neoplasia. The cell yields isolated by SCSR procedure generally are in excess of 10 million per gram of stool wet weight. Thus this system presented here is optimized for rapid throughput, enabling the investigator to detect the expression of several markers in cells isolated from human fecal samples e.g., markers of lineage; cytokeratin 8, 18 and 19; house keeping genes, β actin and glyceraldehyde 3 phosphate dehydrogenase (GAPDH), marker for haematopoietic origin CD34, the leukocyte common antigen CD45,

transmembrane glycoprotein CD44, carcinoembryonic antigen (CEA), colon specific antigen (CSA), Prolactin, Cox-2, Multidrug resistance (MDR), human insulin receptor (hIR), Her/neu-2, c-myc, Adenomatous Polyposis Coli (APC), β catenin and cyclin D1. Besides detecting these markers using RT-PCR and flow cytometry, the quantification of expression levels using Real Time PCR will provide further insight into the early noninvasive diagnosis of colon cancer.

Correlations Between Hormone Related Biomarkers in Ductal Lavage Samples from Women at High Risk for Breast Cancer

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Introduction: We are conducting a tamoxifen intervention study of high-risk women, with ductal lavage (DL) sampling of breast epithelium and ductal fluid, prior to and six months following initiation of tamoxifen therapy.

Methods: Asymptomatic, high-risk women undergo DL; the effluent is separated into a cell pellet and duct lavage supernatant (DLS). The cells are used for cytological evaluation and immunohistochemistry (IHC) for estrogen receptor ? (ER), COX-2, and Ki-67. The supernatant is lyophilized, reconstituted in saline, and radioimmunoassays are used to measure estradiol and its precursors, progesterone, cathepsin D, EGF, and IL-6. The DLS values are expressed per mg total protein.

Results: Of the first 60 women entered, 39 had both sufficient cells and sufficient protein for analysis in at least one duct. Their mean age was 49 years, the mean Gail score was 2.5, and cytological atypia was present in 30% of women. Women with cytological atypia had higher mean Gail scores ($p < 0.015$), higher total epithelial cells ($p < 0.002$), and significantly higher levels of DHEA, DSH, ES, and EGF. ER labelling index (LI) increased with age ($p < 0.055$) and was positively correlated with COX-2 LI ($p < 0.018$), and cell number ($p < 0.001$). Ki-67 LI was not significantly related to any other parameter, but showed a trend towards a positive correlation with DHEA levels in the DLS. The DHEA levels were also related to increasing Gail scores ($p < 0.014$).

Conclusions: Estradiol precursors show a stronger correlation with cytological atypia in DL samples than estradiol; if this finding persists, the implications for breast cancer prevention strategies will need to be considered.

Application of Novel and Traditional Serum Markers in Univariate and Multivariate Analysis to Improve the Sensitivity and Specificity of Cancer Detection

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Using cDNA database mining strategies accessing differentially expressed genes and real-time quantitative RT-PCR, we have identified four novel serum markers (DDX101C, DDX108P, DDX110B, and DDX105L) that are elevated in serum from cancer patients. DDX101C is also known in the literature as Reg IV, DDX108P as Spondin-2, DDX110B as B7x, and DDX105L as napsin A.

We developed a series of dual monoclonal antibody sandwich ELISAs to analyze the levels of these proteins in more than 2500 serum samples. The samples included 555 normal controls, 1107 cancer samples, and 1023 samples from various benign diseases. All four markers are detectable in most sera but show elevated levels in serum samples from cancer patients when compared to healthy controls. DDX101C and DDX108P were elevated in all five major cancer types (breast, colon, lung, prostate, and ovarian), whereas DDX110B and DDX105L showed tissue specificity for ovarian and lung cancer, respectively. Furthermore, a separate study with additional ovarian serum samples shows that DDX101C and DDX110B improve the sensitivity and specificity of ovarian cancer detection in CA125-negative and early stage cancer.

Receiver Operating Characteristic (ROC) curves in univariate and multivariate analyses were used to de-

termine the sensitivity and specificity of these markers to detect cancer. Furthermore, traditional cancer serum markers (CEA, CA15.3, CA19.9, CA125, PSA, and %free/total PSA) were measured in the relevant serum samples, and their performance in cancer detection was compared against the four novel markers.

The data suggest that all four novel markers, DDX101C, DDX108P, DDX110B, and DDX105L, have similar or better sensitivity than the traditional serum markers. In some cases, combination of the novel and the traditional markers significantly improved the cancer detection rate. Comprehensive evaluation of each marker in five major cancer types, with univariate and multivariate analyses, will be presented.

Duodenal Juice SELDI Profiling for the Diagnosis of Pancreatic Cancer

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Background: Pancreatic cancer is the 5th leading cause of cancer-related death in the US, causing an estimated 30,000 deaths each year. The 5-year-survival rate remains at less than 5% during the last decades. This abysmal prognosis is related to the fact, that most patients ($>$80%) are diagnosed at a late stage, when the cancer has spread and is not amenable to surgical resection anymore.

Early detection of pancreatic cancer has the potential to improve the prognosis by increasing the proportion of patients that can undergo potentially curative surgery. Early detection efforts are currently hampered by an absence of sufficiently accurate minimally invasive diagnostic tests for resectable pancreatic cancer.

Pancreatic juice, collected via gastroscopy (EGD) after iv. secretin stimulation (duodenal juice) is an attractive medium for the analysis of potential pancreatic cancer biomarkers. Markers produced locally by the tumor or its microenvironment are likely to be present in higher concentrations in duodenal juice than in plasma. This method also circumvents the need for cannulation of the pancreatic duct (as in ERCP) to collect the specimen, with its inherent risk of causing pancreatitis.

In an effort to identify new biomarker for pancreatic cancer, we analyzed duodenal juice specimens from patients with pancreatic cancer, chronic pancreatitis and other non-pancreatic gastrointestinal diseases.

Materials and methods: Duodenal juice was collected by aspiration after iv. Secretin application via EGD from 23 patients with pancreatic cancer, 17 with chronic pancreatitis and 23 with non-pancreatic gastrointestinal diseases (controls). The patients were part of a prospective screening study for pancreatic cancer at the Johns Hopkins Medical Institutions (CAPS study). Unfractionated duodenal juice was analyzed by surface-enhanced laser desorption and ionization (SELDI) on IMAC3-Cu^{++} ProteinChips (Ciphergen Biosystems Inc.) by a standard protocol. Chips were read on a PBS IIC ProteinChip reader and data were analyzed using the ProPeak software package (3Z Informatics). Internal quality control samples consisting of pooled serum and pooled duodenal juice samples were run on each chip. All experiments were performed in duplicate.

Results: For the differentiation of pancreatic cancer samples from control samples (other GI disease), a consistent set of 3 peaks (m/z values: 3370, 3441, 10055 Da) which retained most of the discriminating value of the total set of 173 peaks was identified on both replicates of the data set. Markers 3370 Da and 3441 Da were upregulated in pancreatic cancer samples; marker 10055 Da downregulated.

For the differential diagnosis of pancreatic cancer from chronic pancreatitis, a larger number of peaks (m/z values: 3510, 9120, 7294, 8866, 9401, 8754) exhibited diagnostic potential.

In the overall differentiation of cancer and non-cancer (chronic pancreatitis and other GI diseases combined) samples, the 3370 Da, 3441 Da, 3510 Da and 10055 Da markers ranked best.

The exact identification of the proteins or peptides corresponding to m/z 3370 Da, 3441 Da and 10055 Da is currently under investigation.

Conclusion: In this discovery-phase pilot study we have identified several biomarker candidates for pancreatic cancer by SELDI analysis of duodenal juice. Potential applications of these markers include early diagnosis of pancreatic cancer by a screening of high-risk populations and the differential diagnosis of pancreatic mass lesions.

Future studies will be needed to validate these biomarker candidates and to determine their exact identity by tandem mass spectrometry.

Microfluidics-Based Comprehensive Proteome Profiling for Biomarker Discovery

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The majority of current protein analysis methods are still largely dependent upon traditional two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), due to its ability separate thousands of proteins from complex biological samples. Even though 2-D PAGE is capable of providing detailed views of complex mixtures through its high resolving capabilities, it is a relatively time consuming, labor intensive, and cumbersome technology that is not readily amenable to automation and suffers from poor reproducibility. A microfluidics-based multidimensional protein separation system is presented as next-generation 2-D PAGE for high throughput and ultrasensitive analysis of complex proteomes. Complex protein mixtures are initially separated in a single first-dimension microchannel by non-native isoelectric focusing providing a pI-based separation and concentration of constituent proteins. The focused proteins are then simultaneously transferred to an array of orthogonal microchannels wherein parallel, size-based SDS-PAGE further resolves the proteins in a second dimension. Simultaneous monitoring of each microchannel in the second-dimension array is performed by multi-channel laser-induced fluorescence detection, enabling highly sensitive and differential display capabilities for comparative proteome studies. Complex protein patterns obtained from normal and cancerous samples can be analyzed using image processing methods for the discovery of cancer markers.

Detection of Prostate Cancer Using Serum Proteomic Pattern in a Histologically Confirmed Population

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Purpose: To retrospectively identify a panel of serum proteins that can discriminate men with prostate cancer (clinically organ confined) from men with benign prostate disease.

Materials and methods: A contemporary set of 345 men who had an archival serum sample available were included in this study. The cancer group consisted of 246 men who underwent radical prostatectomy at the Johns Hopkins Hospital between March 1999 and April 2001. The non-cancer group included 99 men with no histological evidence of prostate cancer on biopsy between April 1997 and April 2001 at the same institution. Serum proteomics mass spectra of these patients were generated using ProteinChip® arrays and a ProteinChip Biomarker System II laser desorption/ionization (SELDI) time-of-flight mass spectrometer (Ciphergen Biosystems, Inc., Fremont, CA). The cases and controls were randomly split into a training and a testing group by a stratified sampling procedure. A combination of bioinformatics tools including ProPeak (3Z Informatics, Charleston, SC) was used to reveal the most optimal panel of biomarkers for maximum separation of the prostate cancer and the benign prostate disease cohorts.

Results: A panel of 3 proteins (PC-1, PC-2, and PC-3) was selected using the training data. Performance of each of the protein markers and a linear regression derived composite index (PC-com3) were evaluated on the testing data. The area under the curve for PSA, PC-1, PC-2, PC-3 and PC-com3 was 0.542, 0.585, 0.600, 0.636 and 0.643, respectively. Improvement of PC-com3 over PSA is observed at specificity range 30% to 80%. At a selected specificity of 45%, sensitivity of PC-com3 is 76%, significantly better than PSA, which has a sensitivity of 57% ($p < 0.0001$).

Conclusions: Serum proteomics patterns may potentially aid in early detection of prostate cancer.

Gene-Specific DNA Methylation in Breast Cancer Patients: A Study of 53 Promoters

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A new technique of DNA methylation analysis in heterogeneous samples – methylation-sensitive restriction enzyme (digest) – PCR (MSRE-PCR) – has been applied to DNA extracted from formalin-fixed paraffin-embedded tissues. This high-throughput analysis allows fast detection of methylated fragments and deletions in clinical samples without prior purification of tumor-containing regions. Complete assay can be performed using less than three genomic equivalents; for clinical samples one standard section from a paraffin block is sufficient for a comprehensive assay.

Using MSRE-PCR we analyzed methylation in promoters of 53 cancer-related genes using DNA extracted either from tumor or from tumor-free margins of the same surgical specimen. MSRE-PCR assay detected changes in methylation status and tentative deletions in over 20 genes, including X-chromosome-linked genes, putative oncogenes, hormone receptors and growth regulators. Tentative deletions in locus 9p21, which contains tumor suppressors p15Ink4A and p16Ink4B, were by far the most frequent events in both tumor-containing samples and their tumor-free counterparts. Over half of the genes analyzed were similarly methylated in both tumors and tumor-free samples. The number of tentative deletions was directly proportional to the age of the patients.

MSRE-PCR allows fast and comprehensive screen for both methylation and deletion of selected promoters in heterogeneous samples. The method can be applied to analysis of imprinting, normal and disease-dependent silencing, and development-induced changes. In its first application MSRE-PCR has revealed both genetic and epigenetic features of breast cancer. Accumulation of MSRE-PCR data for clinical applications will allow more precise diagnosis and stratification of different malignancies, ultimately facilitating more individualized therapy as we identify in greater detail the molecular perturbations of human tumors.

Technical Variation, Peak Alignment, and Biomarker Discovery in MALDI-TOF Proteomics Profiling

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Keywords: variation, amplitude, phase, MALDI-TOF, peak alignment

The use of MALDI-TOF mass spectrometry as a means of displaying the proteome has been evaluated extensively in recent years. One of the limitations of this technique that has impeded the development of robust data analysis algorithms is the variability in the location of protein ion signals along the x-axis. We systematically studied technical variations of MALDI-TOF measurements in the context of proteomics profiling. By acquiring a benchmark data set with five replicates, we estimated 76% to 85% of the total variance is due to phase variation. We devised a lobster plot, named because of the resemblance to a lobster claw, to detect these phase variations. To remove phase variation in replicates, we investigated a peak alignment algorithm. This operation is analogous to the normalization step in microarray data analysis. Only after this critical step can features of biological interest be clearly revealed. By principal component analys! is, we demonstrated that after peak alignment, the differences among replicates are reduced. Computer source codes in this paper are incorporated into a larger open-source analytical solution for proteomics profiling called Rproteomics.

Carcinoma Cell-Specific Mig-7: A New Potential Early Marker for Circulating and Migrating Cancer Cells

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Identification of genes that are expressed in a cancer cell-specific manner can provide markers for detection, diagnosis, and disease progression. We have previously reported that receptor tyrosine kinase ligands in concert with ligation of ávâ5 integrin induce expression of Mig-7 restricted to carcinoma cells. Because of this highly specific expression, we hypothesized that Mig-7 could be used as a marker of occult tumor cells. The objective of this study was to begin to test this hypothesis by generating Mig-7 specific antisera and RT-PCR methods for detection of Mig-7 expression in tissues and blood from cancer patients as compared to those from normal subjects. By immunohistochemistry and by RT-PCR, we detected Mig-7 mRNA in lymph nodes from 7 out of 9 (77.8%) endometrial carcinoma xenograft mice but not from any of the five negative control animals. Mig-7 expression was more specific than Met expression, the RTK that binds Scatter factor and is used as a marker of poor progression, in endometrial carcinoma as compared to normal endometrial tissue samples. In 87.3% of tumors from various tissues including breast, lung, colon and ovary, we detected Mig-7 expression. Blood samples from untreated metastatic cancer patients also displayed Mig-7 mRNA in contrast to a lack of expression in chemotherapy treated or normal individuals. In conclusion, we report the first immunohistochemical and RT-PCR assays for Mig-7 as a marker and discuss this highly specific localization to cancer cells in contrast to an absence in normal cells. Our preliminary data indicate that Mig-7 may be a potential early marker of migrating and circulating carcinoma cells.

MarkPap(TM) Technology: >From Idea to Commercial Products

A biomarker-based, *in vitro* diagnostic medical device with potentials for cervical cancer screening

Nenad Markovic*, Olivera Markovic and the BioSciCon's Cervical Cancer Study Group

Sponsor: BioSciCon, Inc., Rockville, MD, USA, http://www.markpap.com

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Objectives and background: Based upon discovery that cervical acid phosphatase can be used as a biomarker specific of cervical dysplasia, we have initiated a translational research to transform this idea into commercially available products for cervical cancer screening, thus, to benefit all women. The core of this research is an accurate, inexpensive and easy-to-perform test for detection of the biomarker. Our new technology (trademark MarkPap^{TM}) includes three products: (1) MarkPap Test (MPT, assay, method, service), MarkPap Research Kit (an assembly of reagents, controls and instructions), and the MarkPap Solution (for specimen collecting and transport). Together, as an in vitro diagnostic medical device, this technology enhances the visibility of abnormal cells on Pap smears and/or monolayers made of cervical specimens collected in liquid (LBP). The three products are available for research in US and for sale globally.

We present results of two clinical laboratory trials supported in part by NIH via SBIR Phase-1 and Phase-2 grants (2-R44CA086767-02)

Study design: Multicenter, random assignment, assessors blinded, concurrent control, split-specimen, clinical trial to compare the new technology with standard Pap smear and the ThinPrep Pat test in a general and a high-risk population. Duration: Two-year observation following an initial exam and specimen acquisition. Performance measures: *Primary efficacy* (a) portion of abnormal specimens, (b) false negative rate. *Secondary efficacy:* accuracy as determined by

sensitivity and specificity of the test and the control groups compared versus (1) adjudicated cytology verified with colposcopy/biopsy/histology, and (2) clinical outcomes. *Tertiary efficacy:* test versus historical control. Safety: frequency of adverse events described according to severity and relevance to testing.

Results:

1. General population (Report of April 2003, accepted for publication in Disease Markers).
   MPT: 1,088, PAP: 1,286. Test positive/abnormal: MPT = 181 (16.6%), PAP = 106 (8.2%). Negative/normal: MPT = 882 (81.1%), PAP (1167 (90.7%. RR: MPT = 0.205, PAP = 0.091. 95%CI of difference: 0.060 – 0.114. OR = 2.26. Chi-square (S[O − E]^{b}/E = 40.69 (P = 0.000000002).
2. High-risk population (Presented at ASCCP 2004 Biennial Meeting, February 2004).
   MPT: 428, ThP: 466. Test positive/abnormal: MPT = 79 (0.184), ThP = 59 (0.132). 95%CI of difference: 0.33 – 10.13.
   SE: MPT = 0.937, ThP = 0.833. SP: MPT = 0.90, ThP = 0.99. Hypothesis testing: Pe = Ps + d (0.3Ps).
3. In both populations false negative rates were reduced from 0.09 – 0.11 to 0.02 – 0.05.
4. No adverse event related to the testing occurred for two years in 2,000 subjects (1,220 still in follow-up).

Conclusion and prospective: Due to the biomarker's visibility, the cytoscreeners have increased the rate of detection of verified positive/abnormal specimens for 50%. The rate of false negatives has been reduced for more than 50%. The new test has been more accurate than either Pap smear or ThinPrep due to better sensitivity with equivalent specificity. No adverse events were recorded. The results warrant another study to meet FDA requirements for Pre-Marketing approval (PMA). A proposal for SBIR Continuation Phase-II (Phase III) study is submitted (April 1, 2004 deadline) and is pending review.

An Isothermal, PCR-Free Method for Detection of RNA, DNA, Protein and CpG Methylation Sites

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We have developed a rapid, gel-free, isothermal method for quantifying levels of specific proteins and nucleic acids based on the production of RNA signal molecules. The core signal-generation method, called Abscription™, relies on the reiterative synthesis of short RNA oligonucleotides (abscripts) by abortive transcription from artificial abortive promoter cassettes (APCs). Abscripts of defined size and sequence can be made with the inclusion of dinucleotide initiators and incomplete mixtures of NTPs. Site-specific APCs can be made by annealing non-template DNA oligonucleotides to target DNA strands thereby converting them into structures that resemble open promoter complexes. Inclusion of target strands in the APC allows the characterization of SNPs through the production of 1 of 4 possible abscripts. Alternatively, modular preformed APCs can be attached to targets via probes. Oligonucleotide extensions on the APCs (target site probes) are used for detection of DNA or RNA targets while antibody-APC conjugates are used for protein detection. Abscription products can be detected directly by mass spectrometry, or through reporter tagged nucleotide analogs (RiboLogs®) that are incorporated into abscripts by RNA polymerase.

A series of applications are under development for the detection of nucleic acids and proteins. The RiboMET assay has been used to detect CpG methylation levels using a modified SNP assay. The assay is based on the discrimination of sequence differences resulting from deamination of unmethylated C to U by sodium bisulfite treatment. The sequence difference at CpG verses the deaminated UpG sites is reflected in a sequence difference in the corresponding abscripts. The RiboMET assay has been used to accurately detect methylation levels as low as 6% at a single defined site. The feasibility of RNA and protein detection abscription assays has been tested with the development of target-specific modular APCs.

Discovery of Biomarkers that Identify Her2 Positive Breast Cancer Patients versus Controls and Revert to Normal Levels in Plasma Post-Surgery

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Background: Breast cancer is the most common non-skin malignancy affecting women in the United States. Early detection increases survival. As yet, there are no simple, biomarker based diagnostic tests that can be used for population screening and early detection. PSA is an example of such a biomarker test that is being used for Prostate Cancer. We set out to discover a set of easily accessible protein markers in plasma that would be useful for disease detection and revert to normal levels following surgical treatment.

Methods: Plasma samples from a total of 75 breast cancer patients with Her2 positive disease and 61 normal controls were fractionated and analyzed by SELDI proteinchip-mass spectrometry. Peaks were detected using a stringent criterion of signal to noise ratio greater than 10 to 1. A data quality control protocol was applied to the spectra including both single spectrum assessments and reproducibility tests based on multiple runs. A recursive-support vector machine (RSVM) algorithm was used to select the biomarkers that distinguished cancer from normal plasma, build the classifiers, and estimate error rates by cross-validation. The selected biomarkers were further validated in pre- and post- surgery samples from a subset of patients in order to assess changes in response to decreased tumor burden.

Results: We obtained a leave-one-out cross validation error rate of 17.6% using eight biomarkers to segregate cancer versus control specimens. Using two separate runs of the cancer and normal samples, our biomarker set produced an inter-run error rate of 25%. The eight markers were further tested in a pre- and post-surgery study. One of the markers reverted back to normal expression levels after surgery in a statistically significant manner. This marker was present at higher levels in control specimens as compared to cancer and increased to normal levels post-surgery in a majority of patients.

Conclusion: Our data suggest that differentially expressed protein signatures exist in the plasma of patients with Her2 positive breast cancer as compared to controls. Markers discovered and identified may be become useful breast cancer diagnostics as well as indicators of response to therapy.

A New Approach for Proteomic Profiling of Serum Samples and Identification of Serum Peptide Using Functionalized Magnetic Beads, Anchorchiptm Technology, and MALDI-TOF, and MALDI-TOF/TOF Mass Spectrometry

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Most cases of ovarian cancers are detected at late stages and are rarely curable; thus there is an urgent need to develop early detection assays with the hope of improving survival. Multiple strategies have been applied to identify differentially expressed proteins in serum samples of ovarian cancer patients and have already provided promising results. However, there are still only a limited number of proteomic platforms that can be used to generate reliable protein profiles from serum samples to help us identify early diagnostic markers for the disease. We have developed a new integrated method for proteomic profiling using: 1) magnetic beads and AnchorChip^{TM} technology for sample fractionation and preparation before mass spectrometry (MS) analysis, 2) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS

and MALDI-TOF/TOF MS to generate proteomic profiles in both linear and reflector modes, 3) MALDI-TOF/TOF MS to identify peptides from the same sample spot, and 4) bioinformatics (*ClinProTools*$^{\mathrm{TM}}$ software) for data mining, visualization and generation of proteomic patterns. Our results showed that this new approach is reproducible and the magnetic beads are able to fractionate human serum. In addition, the AnchorChip technology provided high sensitivity in MS detection by concentrating the matrix/fractionated serum samples to a 600um spot. We also found that both linear and reflector modes in MALDI-TOF analysis offer different advantages in proteomic profiling in terms of number of peaks detected, their resolution and mass accuracy. We have demonstrated that this new approach can generate differential proteomic profiles from serum samples obtained from stage I ovarian cancer patients and age-matched healthy women.

Discrimination of Malignant and Normal Serum Samples Using the Broad-Spectrum Cancer Marker RECAF. Part I: Ovarian Cancer

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Aims: The purpose of this work was to determine if the concentration of soluble RECAF (receptor for AFP) is elevated in the serum of ovarian cancer patients.

Methods: The study included 353 normal individuals, 48 patients with confirmed ovarian cancer and 114 women with unconfirmed diagnosis but CA-125 levels $>$35 U/ml. The test consisted of a RIA in which the solid phase was coated with a rabbit anti-RECAF and the sample competed with pure abeI-RECAF. The standard curve was calibrated in arbitrary Units using an MCF-7 cell extract. CA-125 values were from accredited clinical laboratories.

Results: Serum RECAF was significantly elevated in cancer patient samples or individuals with CA-125 higher than 35 U/ml as compared to normal individuals (independent t-test $p <= 2.41 \times 10^{-aa8}$). Using a cut-off of 4,612 RECAF Units to discriminate between malignant and non-malignant, the sensitivity of the test was 97.5% with 97.7% specificity. There was a certain degree of correlation between CA-125 and RECAF values ($r^b = 0.81$).

Conclusions: The data strongly suggest that RECAF could prove useful as a marker for ovarian cancer detection. Furthermore, since ovarian cancer is often silent, a general cancer-screening test based on RECAF would detect a significant number of sub-clinical ovarian cancers.

Discrimination Of Malignant And Normal Serum Samples Using the Broad-Spectrum Cancer Marker RECAF. Part II: Breast Cancer

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Aims: RECAF (receptor for AFP) is a ubiquitous oncofetal antigen. Our group has developed a RIA to measure RECAF in serum as described in Part I. Previous histological work indicates that RECAF is present in breast cancer cells but not in normal breast tissue or benign lesions. The objective of this work was to determine if the serum RECAF test could prove useful for detecting breast cancer.

Methods: Sera from 64 histologically confirmed breast cancer patients and 353 normal individuals were tested. The RIA format is described in Part I.

Results: Patients with breast cancer exhibited a significant increase of circulating RECAF as compared to normal individuals (t-test $p <= 10^{-8a}$). The area under a ROC curve was 0.997 and selecting a cutoff value of 4,612 RECAF Units resulted in 96.9% sensitivity with 97.7% specificity.

Conclusions: The RIA used in this study differentiated breast cancer samples from normal donors with high specificity and high sensitivity. RECAF is negative in adenomas by immunohistology hence circulating RECAF levels should not be higher than normal. There is no current breast cancer marker attaining these levels of sensitivity and specificity and therefore RECAF could find clinical use in monitoring and screening for this disease.

Discrimination of Malignant and Normal Serum Samples Using the Broad-Spectrum Cancer Marker RECAF. Part III: Lung Cancer

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Aims: Many types of fetal and cancer cells express RECAF (a receptor for AFP). In previous sections we showed elevated RECAF levels in the serum of patients with breast and ovarian cancer. Herein we describe the presence of RECAF in sera and tissue sections from lung cancer patients.

Methods: Part I describes the RIA assay used to test 32 lung cancer samples and 353 normal individuals. Immuno-histology was carried out on 8 squamous carcinomas, 8 adenocarcinomas and 8 small cell carcinomas.

Results:

Serology: RECAF was significantly higher in the serum of lung cancer patients as compared to normal individuals (t-test $p <= 3.233 \times 10^{-gc}$). The sensitivity of the assay was 96.9% and the specificity was 97.7%.

Histology: The results are shown in the Table below:

	Very positive	Positive	Negative
Squamous carcinoma	7/8	None	1/8
Adenocarcinomas	4/8	4/8	None
Small cell carcinomas	5/8	2/8	1/8

Conclusions: Both serological and histological RECAF tests detected most lung cancers. The latter might also detect cancer cells on sputum samples.

Discrimination of Malignant and Normal Serum Samples Using the Broad-Spectrum Cancer Marker RECAF. Part IV: Stomach Cancer

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Aims: We have developed a RIA to measure circulating RECAF (a receptor for AFP) in serum. An independent immunohistochemistry study presented elsewhere in this Congress shows positive RECAF staining in a high percentage of stomach cancers. In this study, we investigated the serum levels of RECAF in patients with stomach cancer as compared to normal individuals.

Methods: Sera from 31 stomach cancer patients (histologically confirmed) and 353 normal individuals were tested. The RIA design was described in Part I.

Results: Circulating RECAF values are significantly higher in stomach cancer patients than in normal individuals (t-test $p <= 2.743 \times 10^{-e9}$). The area under the ROC curve is 0.988. Using 4,612 RECAF Units as the cutoff value between malignant and non-malignant samples resulted in a sensitivity of 93.5% with 97.7% specificity.

Conclusions: The serum RECAF RIA used discriminated stomach cancer and normal donors with high specificity and sensitivity. Thus, RECAF might find clinical use in monitoring and screening for this type of malignancy.

The broad-spectrum of cancers in which we were able to detect elevated serum RECAF throughout this series of studies strongly suggests that this cancer marker could be successfully used for cancer screening in general.

Diagnostic Markers That Distinguish Colon and Ovarian Adenocarcinomas: Identification by Genomic, Proteomic, and Tissue Array Profiling

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Colon and ovarian cancers can be difficult to distinguish in the abdomen, and the distinction is important because it determines which drugs will be used for therapy. To identify molecular markers for that differential diagnosis, we developed a multi-step protocol starting with colon and ovarian cancer cell lines from the 60 human cancer cell lines used by the National Cancer Institute to screen for new anticancer agents. The steps included: (i) identification of candidate markers using cDNA microarrays; (ii) verification of clone identities by re-sequencing; (iii) corroboration of transcript levels using Affymetrix oligonucleotide chips; (iv) quantitation of protein expression by fireverse phase' protein lysate microarray; (v) prospective validation of candidate markers on clinical tumor sections in tissue microarrays. The two best candidates identified were Villin for colon cancer cells and Moesin for ovarian cancer cells. Among 133 colon and ovarian tumor specimens on TARP (Tissue Array Research Program) tissue microarrays, Villin appears at least as useful as the currently used colon cancer marker CK20, and Moesin also appears to have utility. Combination of the conventional markers (CK7 and 20) and the new markers (Villin and Moesin) increased the accuracy of the differential diagnosis. Because Moesin stained stromal elements in both types of cancer, it would probably not have been identified as a marker if we had started with mRNA or protein profiling of bulk tumors. The multi-step process introduced here has the potential to produce additional markers for cancer diagnosis, prognosis, and therapy. To facilitate the molecular marker discovery step, we have developed a high-throughput proteomic screening system that includes higher density 'reverse-phase' protein lysate microarrays and a relational database of high quality antibodies (Nishizuka et al., PNAS 2003).

Proteomic Profiling Of Hematopoietic Malignancies

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Proteomic profiling has been used recently to define protein characteristics of serum from adult patients with a variety of tumors, with promise for use in early detection. However, the application of these technologies to staging and prognostics is not as well studied and in spite of the identification of prognostic factors, which predict for patients falling into predictably good outcome groups or into groups requiring more intensive therapy, a sizable minority of patients will still have recurring disease. There is a great demand to develop new methodologies to stratify hematopoietic malignancies based on molecular phenotype which would allow for a more intelligent therapy that is tailored to the specific stage involved. In order to identify protein patterns, which predict outcomes more accurately, we have begun a pilot project to determine which proteomic markers associate with malignancy using Surface-Enhanced Laser Desorption/Ionization time of flight mass spectrometry (SELDI). The model system described here is pediatric Hodgkin's disease (HD).

The objective of this study was to determine if protein profiling using SELDI could accurately distinguish HD patients in different stages and to generate diagnostic protein "fingerprints" unique to each stage. These results are a part of our efforts to develop expression profiles with potential for direct clinical utility and as guides toward protein identification efforts. The combined information, when expanded to a larger more statistically significant study, will be used to stratify tumors into different prognostic groups, enabling more intelligent choices for therapy. We retrospectively acquired 13 serum samples from stage II and 9 serum samples from stage III/IV Hodgkin's disease (HD) patients. Many protein peaks were determined to be differentially expressed significantly in different HD stages (2, 3 and 4). Using SELDI/TOF-MS, we achieved 83.3% to 100% accurate classification for HD stage 2, 3 and 4 rapidly and reproducibly.

With WCX ProteinChips®, a peak at 5845 Da was found to be under-expressed in stage 3 compared to

stage 4, and a peak at 11529 Da was under-expressed in stage 4 compared to stage 2. Another peak at 17256 Da was over-expressed in stage 2 compared to stage 3&4. With IMAC ProteinChips®, a peak at 4647 was under-expressed and a peak at 5744 Da was over-expressed in stage 4 compared to stage 3. Work is ongoing to characterize these proteins and determine their biological behavior. These protein peaks are the priority nodes in classification trees created with this set of sera, and are one example of how SELDI technology may aid in the discovery of new biological markers for different HD stages, as well as provide analysis of differences in protein expression patterns.

Global Amplification of Sense RNA: A Novel Method to Archive and Replicate RNA for Cancer Biomarker Discovery and Validation

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The finite amount of mRNA available from each sample and the lability of RNA during long-term storage significantly limit the number of cancer biomarker discovery and validation studies that can be supported by these priceless collections. Several clever amplification approaches have been developed that allow gene expression profiling to be performed on even the few cells available from microdissection. However, these methods do not address the problem of the lability of RNA during long-term storage, nor the compatibility of the RNA for the variety of platforms and approaches to gene expression profiling.

We developed a novel procedure to amplify mRNA into sense RNA (sRNA). The cDNA intermediate forms a stable biorepository capable of regenerating the complex mRNA profile in the original sample. The procedure exploits the template switching activity of reverse transcriptase to incorporate RNA polymerase binding site upstream of single stranded cDNA. Limited PCR was used for double stranded DNA synthesis. sRNA was synthesized from PCR products by *in vitro* transcription. sRNA was evaluated by real-time RT-PCR and microarrays.

sRNA synthesized from RNA extracted from human cell lines, tissues, blood and fixed exfoliated cervical cells yielded successful results with microarrays and real-time RT-PCR. The size of sRNA ranged from 3.0–0.1 kb. sRNA synthesis preserved the relative differences in mRNAs spiked at abundance ranging 5 orders of magnitude (0.00001–0.1%). This reflects the high-fidelity of sRNA synthesis for mRNA as low as 0.3 copies/cell. One round of sRNA amplification resulted in approximately a million-fold amplification of mRNA, and highly reproducible micrarray results (Pearson correlation coefficient 0.97 using MWG 10K arrays and Resonance Light Scattering technology for signal detection) from 40 nanograms of input RNA.

sRNA is amplified synthetic mRNA in $5' \rightarrow 3'$ direction; the appropriate template for any gene expression analysis. sRNA amplification should find applications in RNA archiving throughout large scale cancer biomarker discovery and validation projects.

The Utility of Karyometric Analysis for Detection of Colorectal Cancer and Prediction of Recurrent Adenomas

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Purpose: To evaluate the karyometric features of rectal biopsies as potential biomarkers of risk for colorectal neoplasia.

Methods: Karyometry analyzes the spatial distribution patterns of chromatin in nuclei from formalin-fixed, hematoxylin and eosin (H&E) stained tissue sections. Automated image analysis provides 93 features that can be used to derive average nuclear abnormality (ANA, a robust measure based on all 93 features) and discriminant function scores (DF, a sensitive measure based on the most highly discriminating features) that summarize deviation of nuclei from normal.

Results: Karyometric analysis of 100 nuclei per subject from normal-appearing rectal mucosa at baseline in 36 subjects with negative colonoscopies showed statistically significant differences at the nuclear level between subjects with no family history or personal history of adenomas vs. subjects with a family history but no personal history of adenomas vs. subjects with

a personal history of adenomas. The 24 subjects with no family or personal history of adenomas were then used as a reference group for karyometric analysis of 100 nuclei per sub ject from normal-appearing rectal mucosae from 138 participants who had participated in an adenoma recurrence trial. Karyometry demonstrated statistically significant nuclear differences at baseline on the basis of size of largest adenoma, adenoma location, and adenoma histology. There were also significant nuclear differences at baseline as a function of recurrence status and location of recurrent adenoma as seen in a 3–5 year follow-up period. A discriminant function was developed that showed statistically significant differences at baseline between subjects on the basis of recurrence status. Further analyses were conducted on individuals with extensive longstanding ulcerative colitis (UC), who were at increased risk for colorectal cancer (CRC). Karyometric DF scores were derived for biopsies of histologically normal-appearing rectal mucosae from patients in four categories: no dysplasia ($n = 8$), low-grade dysplasia ($n = 16$), high-grade dysplasia ($n = 9$), and coexisting adenocarcinoma elsewhere in the colorectum ($n = 10$). Some cases in all categories showed DF scores that did not differ substantially from normal, but a proportion of subjects in each group with dysplasia or synchronous CRC showed progressively worsening DF scores.

Conclusion: These results suggest that karyometry could substantially increase the prognostic information to be derived from rectal biopsies. If confirmed in larger studies, this fully automated technique, performed on routine H&E stained sections, might have important clinical applications.

A New Human Prostate Cell Model for the Study of African American Prostate Cancer

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African American men have the highest incidence of prostate cancer in the world. Generation of suitable *in vitro* models is critical for understanding processes associated with development and progression of prostate cancer in high-risk African-American men. The actively proliferating early RC-165N cells derived from benign tissue from a radical prostatectomy specimen were transduced through infection with a retrovirus vector expressing the human telomerase catalytic subunit (hTERT). Both infected and uninfected cells were passaged for serial subcultivation and were characterized. A high level of telomerase activity was detected in RC-165N/hTERT cells. RC-165N/hTERT cells are currently growing well at passage 50, while RC-165N cells senesced within passage 3. These immortalized cells showed no cell growth in soft agar, poor growth above the agar layer, and no tumor formation in SCID mice. AR, PSCA, NKX3.1, cytokeratin 8 except PSA were all expressed in this immortalized cell line. Growth is stimulated by dihydrotestosterone (DHT), and lysates are immunoreactive with AR antibody by Western blot analysis. The telomerase expression in human prostate epithelial cells can itself induce immortalization and does not induce changes associated with a transformed phenotype, and that these immortalized cells express prostate-specific markers. The establishment of this cell line provides an opportunity for further development of an *in vitro* model of carcinogenesis for African American prostate cancer.

A Comparative Study of Fluorescence In Situ Hybridization (FISH) and Routine Cytology in the Diagnosis of Pancreatic Carcinoma in Cytological Samples from Billiary Tract Strictures

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Introduction/purpose: The purpose of this prospective study was to assess the sensitivity and specificity of FISH and routine cytology in the detection of pancreatic carcinoma from strictures of the biliary tract.

Material and methods: Bile duct brushing and bile aspirate specimens were collected from 100 patients at the time of ERCP. A ThinPrep? preparation slide was made for cytologic diagnosis. In the FISH lab, cells from the biliary stricture were harvested, fixed,

and placed on a slide. Fluorescent DNA probes to the centromeres of chromosomes 3, 7, and 17 and the 9p21 locus (site of the P16 gene) were hybridized to the cells on the slide. A case was considered positive for malignancy if 5 or more cells exhibited polysomy.

Results: Cytological specimens from 100 patients with biliary tract strictures were diagnosed both by FISH and exfoliative cytology. Of these patients, 17 were clinically and/or histologically diagnosed as having pancreatic carcinoma. The sensitivity of FISH and cytology were 41.2% (7/17) and 17.6% (3/17), respectively. The specificity of both tests was 100%.

Conclusion: Our results suggest that FISH has a higher sensitivity but similar specificity to routine cytology for the detection of pancreatic carcinoma in cytological samples from biliary strictures. FISH may be a valuable adjunct test to routine cytology in the diagnosis of pancreatic carcinoma in biliary tract strictures.

Auto-Antibodies In Early Breast Cancer

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Background: Immune responses to a number of tumour associated antigens have been reported, although their suitability, individually, as diagnostic indicators has not been demonstrated. Rather than consider individual immune responses, this study has taken a panel approach to demonstrate the diagnostic potential afforded by the detection of multiple autoantibodies to known tumour associated proteins.

Methods: Auto-antibodies to MUC1, p53, c-myc and c-erbB2 were measured, by ELISA, in five groups – normal controls ($n = 100$), primary breast cancer patients (PBC) ($n = 200$), patients with benign breast disease ($n = 50$), PBC patients deemed to be 'at risk' of breast cancer from whom serum samples had been taken at least 6 months prior to clinical diagnosis ($n = 9$) and patients with auto-immune disorders ($n = 25$).

Results: At a specificity for cancer of 100%, elevated levels of auto-antibodies were seen in 82% of PBC patients. No significant differences were seen in overall detection when these patients were sub-divided either by detection methodology (screen-detected vs symptomatic presentation), lymph node status at diagnosis or menopausal status. Of those individuals with pre-diagnosis samples, 55% had elevated auto-antibodies at the 100% confidence level. In this group, the lead-time for cancer detection with auto-antibodies, over clinical detection, ranged from 6 to 27 months, with a median of 15 months.

Conclusions: This study raises the possibility of using a combination of assays to detect auto-antibodies to cancer-associated antigens for screening and early diagnosis of breast cancer.

From Gene Expression Patterns to IHC Diagnostics: Classification of Carcinoma and Translation into Antibodies for Patient Stratification

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Our research group at Applied Genomics Inc. set out with the goal almost fours years ago of producing a set of immunohistochemistry (IHC) reagents that would distinguish the biologic diversity of carcinomas and could be used to search for biomarkers that would predict response to existing therapeutics and novel therapeutics in development. In collaboration with scientists at Stanford University, we have used gene expression data to target the production of over 700 novel antisera and screened them using tissue arrays with greater than 300 breast or lung carcinoma cases for their patterns of staining of breast and lung carcinoma. We have settled upon "Panels of Diversity" comprised of approximately 45 novel IHC reagents that we intend to use in both retrospective and prospective studies with the

goal of identifying biomarkers useful for determining optimal therapy for breast and lung carcinoma patients. In breast cancer, we have used our breast "Panel of Diversity" to perform a retrospective study on 550 patients diagnosed at the Comprehensive Cancer Institute of Huntsville (CCIH) between 1989 and 2000 (average follow-up of 65 months). In a preliminary analysis of our data, we have used a classification and regression tree (CART) approach to identify a subset of nine biomarkers that predict recurrence in lymph node negative, estrogen receptor expressing breast cancer patients ($HR = 7.1$ $p < 0.001$). Work is ongoing to narrow the panel to the most robust reagents with the best predictive value. We believe that the expanded panel, which distinguishes broader biologic diversity in breast cancer, should prove useful for retrospective and prospective studies using paraffin embedded diagnostic tumor tissue to identify and validate biomarkers that distinguish responder populations in adjuvant clinical trials.

Development of Riboflavin Carrier Protein (RCP) as a Biomarker for Reproductive Cancers

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Riboflavin carrier protein (RCP) is an estrogen-induced protein which we have shown to be over-expressed in breast and hepatocellular carcinoma tissues with elevated serum levels. In this study, we investigated its potential to serve as a biomarker of reproductive cancers. Using a monospecific, polyclonal antibody to chicken RCP (cRCP), the expression pattern of RCP was studied in ovarian, prostate and endometrial adenocarcinomas and cell lines. It was found to be highly over-expressed in the cytoplasm of these adenocarcinoma tissues as well as in LNCaP, PC3 (prostate adenocarcinoma) and SKOV3 (ovarian adenocarcinoma) cell lines. In normal endometrium and stage 1 endometrial adenocarcinoma, RCP was mainly localized in the apical region of the columnar epithelial cells; this polarity was lost in later stages of this cancer, while retaining cytoplasmic expression. Addition of antiRCP antibody to PC3 and SKOV3 cells in culture resulted in cell-rounding, loss of attachment to substratum and gradual cell death over 24 hours. AntiRCP treatment also effected a 98% reduction of ^{c}H-riboflavin uptake and a 50% reduction in ^{c}H-thymidine uptake over 24 hours in PC3 cell cultures, indicating reduced DNA synthesis. Absorption of the antibody with cRCP abrogated the observed effects, demonstrating the antibody's specificity of action. To characterize human RCP, we immunoprecipitated the protein from cell lysates of prostate and ovarian cancer cell lines using an antiserum to chicken RCP. SDS-PAGE/Western blotting using several monoclonal antiRCP antibody probes consistently revealed three protein bands from the human cancer cells lines that were of approximately 31–37 kDa. Further characterization of human RCP protein from cancer cell lines and the RCP gene is currently in progress. These investigations indicate that RCP may play an important role during development of these cancers and may have potential for use as a biomarker for early detection/diagnosis of these cancers. Supported by grant CA91185-2 from NCI.

Methylation Profiling for the Prediction of Barrett's Esophagus Progression

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Introduction: Patients with Barrett's esophagus (BE) have an increased risk of developing esophageal adenocarcinoma (EAC). For years, dysplasia grade has been the sole means of risk stratification for patients with BE. Significant problems have emerged in studies of dysplasia that make it imperative for the BE field to incorporate additional detection and stratification markers including poor reproducibility of dysplasia interpretation and sampling error.

Material and methods: We performed quantitative methylation profiling of 13 genes using real-time quantitative MSP of of tumor tissues from 88 patients with frank EAC, 72 patients with BE and matched normal esophageal tissue, as well as from 10 patients without esophageal disease. After identifying frequenlty methylated genes in this cross-sectional analysis, we then investigated longitudinally derived multiple esophageal biopsies from 11 BE patients who progressed to dysplasia and/or EAC (Group P) (median follow-up 50.8 months) and compared them to an age- and sex-matched cohort of 24 BE patients who did not progress to dysplasia or EAC during a median follow-up of 64 months (Group NP).

Results: Normal squamous esophageal epithelium, whether from noncancer non-BE patients or from patients with BE or EAC, rarely undergoes hypermethylation at the loci studied. By contrast, frank EAC and BE are frequently methylated at these same sites, often at five or more loci. Five of the markers studied are novel methylation targets of EAC and BE (HPP1, CRBP1, RIZ1, RUNX3, and OST-2). In the longitudinal outcome study of BE, the methylation pattern was significantly different in BE tissues derived from P vs. NP. The number of methylated genes was significantly higher in the P group. Moreover, three genes were significantly more frequently methylated in BE tissues derived from P compared to NP (HPP1, TIMP3, p16). A panel of these markers was able to define low, intermediate and high risk groups of progression to dysplasia and/or cancer.

Conclusions: We identified five novel methylation targets in BE and EAC. In addition, methylation panel profiling of BE tissues showed significantly different methylation patterns in patients who later progressed to dysplasia and/or cancer (P) vs. patients who did not progress during long-term endoscopic follow-up (NP). These findings may have important impact regarding early detection and risk stratification and may influence current surveillance strategies if confirmed in large prospective trials.

Gene Expression Signatures of Benign and Malignant Epithelial Cells Differentiate Prostate Cancers with Moderate Risk or High Risk of Progression

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Our laboratory has been focusing on the discovery, biology and translational utility of prostate cancer (CaP) specific gene expression biomarkers defining CaP onset and/or progression. This study focuses on CaP-associated gene expression signatures in specific cell types of the human prostate gland with the objective to carefully define the pathobiology of epithelial component in prostate tumorigenesis.

Gene expression analysis was performed in paired laser capture microdissected (LCM) benign and cancer epithelial cells from two patient groups (10 patients each) with "high risk" or "moderate risk" of CaP progression after radical prostactomy based on PSA recurrence, Gleason score, pathological stage, and tumor cell differentiation. Total RNA isolated from the LCM cells was linearly amplified and analyzed by U133a Affymetrix GeneChips. Gene expression clusters predictive of "high risk" or "moderate risk" form of the disease were evaluated based on a multidimensional scaling (MDS) method. Validation of the differential expression was done by real time RT-PCR (TaqMan).

Comparative gene expression analysis of LCM derived benign and malignant epithelium from 20 patients (40 gene chips) revealed common tumor cell specific over- or under-expression of genes such as AMACR, NPY, PAP, PSP94. MDS plots were generated between genes expressed in epithelial cells of benign and tumor glands of "high risk" and "moderate risk" CaP patients. Two hundred genes were selected as a basis for separation of the groups on each MDS plot. A subset of the 20 genes that strongly separate the "high risk" and "moderate risk" CaP patients represent genes associated with oncogenic activity, apoptosis- and cell growth regulation, and antioxidant activity.

Gene expression clusters that define "high risk" or "moderate risk" CaP have potential in identifying epithelial cell type specific prognostic bio-markers. Gene expression signatures characteristics of "high risk" CaP also have potential in defining novel therapeutic targets. Further studies along these lines are in progress.

SELDI Analysis of Serum from Pre-operative Ovarian Cancer Cases versus Normal Controls Identifies Combination of Peaks in Training Set with High Sensitivity at 98% Specificity in Independent Validation Data Set

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Background: Ovarian cancer is often detected in late stage disease (75%) with poor prognosis, while if detected in early stages 5-year survival rates can exceed 90%. The stark contrast in survival rates between early and late stages, and the relatively stable mortality rate over the past 30 years despite some advances in therapeutics, makes early detection an attractive approach to investigate for possible reduction in disease specific mortality. Identifying serum biomarkers accurately distinguishing ovarian cancer cases from women without ovarian cancer is a first step towards development of an ovarian cancer early detection strategy. Mass spectrometry techniques applied to serum, such as surface enhanced laser desorption and ionization (SELDI) may identify peaks and combinations of peaks that separate a high proportion of cases (sensitivity) from most controls (specificity). Setting specificity at a high fixed level (98%) and evaluating sensitivities at this specificity provided direct comparisons between different combinations of peaks.

Methods: Pre-operative serum from 100 ovarian cancer cases and 172 healthy age-matched control subjects were analyzed with SELDI using IMAC3 chips. Randomized allocation split the subjects into two equally sized samples, a training data set and a validation data set. Over 100 peaks were identified as consistently detected in more than 20% of the spectra. Stepwise backwards logistic regression on the training data reduced this initial set to 20 potential peaks. Optimal subsets of sizes one to ten were determined through maximizing the likelihood statistic over all possible subsets of a fixed size from the 20 peaks. Bias results when applying predictions to the same data set on which are parameter estimated. Bias was reduced through two methods, namely cross-validation and evaluation on an independent validation data set.

Results: Sensitivity at 98% specificity is listed for optimal sets with 3 and 8 peaks, achieving validation sensitivities of 54% and 77% respectively. The peaks in the optimal subset of size 3 were at 5.37, 11.62, and 22.38 kDa, while the peaks in the optimal subset of size 8 were at 3.57, 4.14, 5.37, 5.91, 7.81, 8.95, 11.62, 22.38 kDa. Subsequent analysis of 11.62 kDa peak identified the corresponding protein as α-haptoglobin.

Conclusions: While the sensitivity in the validation data set did not reach other reported sensitivities using SELDI for ovarian cancer, it does confirm the presence of a signal in SELDI spectra that warrants further investigation. Our group plans to develop immunoassays to proteins/peptides identified via SELDI that are likely to contribute to the separation of cases and controls through measurement in serum.

Sensitivity at 98% specificity			
Number of Peaks	Training set	Cross-validation	Validation set
3	0.53	0.50	0.54
8	0.69	0.53	0.77

Comparison of Concentration and Activity of Matrix Metalloproteinase 2 & 9 in Plasma of Patients with Breast Cancer, Breast Disease and at Risk of Developing Breast Disease

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Matrix metalloproteinases (MMPs) are involved in extracellular matrix modification and specifically, MMP2 and MMP9 are implicated in both early and late processes of tumor development. In biological samples, MMPs occur as inactive precursors, active enzymes or enzyme inhibitor complexes. However, the specific role of each of these enzyme forms in the physiological/pathological processes associated with tumor development or the risk for developing breast cancer has not been clearly elucidated. The aim of this study was to determine if patients with breast cancer, benign disease and at risk for developing breast cancer display characteristic levels of active and/or total MMP2 and MMP9 in plasma. Concentration and activity of MMP2 and MMP9 were determined quantitatively in the plasma of patients diagnosed with breast cancer ($n = 31$), benign disease ($n = 38$), or determined by the Gail Model to be at high risk ($n = 31$) or low risk (controls, $n = 24$) of developing breast cancer. Data obtained was statistically analyzed to reveal differences/patterns characteristic of each category and build a predictive/descriptive model using canonical discriminant analysis. Control individuals displayed significantly lower concentrations of total MMP2 than benign and high risk ($p < 0.001$, respectively) and also the breast cancer patients ($p = 0.002$). Active MMP9 was also significantly lower in controls than benign, high risk ($p < 0.001$ respectively) and cancer ($p = 0.015$). However, in the control individuals, concentrations of active MMP2 was significantly higher than in cancer ($p = 0.038$), benign ($p = 0.022$) and high risk ($p = 0.001$) and so was the concentration of total MMP9 (control/cancer, $p = 0.013$, control/benign $p < 0.001$ and control/high risk $p < 0.001$). Based on activity measurments, controls had significantly lower activity of total MMP2 than cancer, benign and high risk ($p < 0.001$ respectively) and significantly lower activity of MMP9 active than cancer, benign ($p < 0.001$ respectively) and high risk ($p = 0.008$). The high risk group exhibited significantly lower activity of MMP2 active than the control, cancer ($p < 0.001$, respectively) and benign groups ($p = 0.013$). Discriminant analysis of all eight features (concentrations and activity levels of active/total MMP2 and MMP9) also enabled the distinction of the control group from the high risk, benign and cancer. Plasma concentration and activity of active/total MMP2 and MMP9 may have clinical significance in determining breast disease status.

Quantitative Mrna Transcript and Promoter Methylation Profiling of Carcinogen and Oxidant Metabolizing Genes in Exfoliated Buccal Mucosal Cells

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Exfoliated mouth (buccal) epithelial cells are sufficiently accessible to permit cytologic collection of viable cells, and assay of gene expression. Buccal cell collection may allow identification of relevant gene-environment interaction signatures. We offer a first report of evaluation of human gene expression in exfoliated buccal cells collected by cytologic brush, extracted by total RNA techniques, and amplified by a recently-developed RNA-specific strategy, employing real-time quantitative RT-PCR. In a pilot study, metabolic activity of exfoliated cells was verified by MTT assay; timed exposure to mainstream tobacco smoke resulted in clear induction of CYP1B1, but not GSTP1 and other genes, in serially collected buccal cells from the one subject. Subsequently, quantitative assays for 11 target (Ahr, CYP1A1, CYP1B1, GSTM1, GSTM3, GSTP1, GSTT1, NQO1, CAT, GPX, SOD1) and 3 reference housekeeper gene transcripts (GAPDH, β-actin, 36B4) were performed on buccal specimens from 19 current and 23 former or never-smokers recruited from an ongoing lung cancer case-control study. Wide inter-individual variability (range >1000-fold) was demonstrated for most genes. In multivariate analyses, age, gender, tobacco exposure and other factors were associated with the level of expression of each transcript, on a gene-specific basis, but most inter-individual variability remained unexplained by these non-genetic factors. Gene expression signature was not clearly predictive of lung cancer case or control status, within study power. Additional experiments demonstrated ex-vivo exposure of primary cultures of these cells in high throughput fashion is feasible. Finally, a recently-developed tag-modified bisulfite genomic sequencing approach to assay detailed, continuous promoter methylation spectra has also been applied to exfoliated buccal cells. Overall, the buccal cytologic brush method is noninvasive,

quantitative, and amenable to high throughput applications in humans.

Proteome Comparison Of Human Papillomavirus (HPV) Oncogene-Transduced Keratinocytes With HPV-Induced Cervical Lesions In Order To Identify Cellular Markers Signaling Malignant Progression Of Virus-Induced Lesions

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Mucosatropic HPVs infect anogenital epithelia causing neoplasia during their productive live cycle. HPV infection with a viral type belonging to the so called "high risk" HPV subgroup is a prerequisite for the development of cervical carcinomas. Detection and analysis of HPV-induced lesions rely on cytological and histological grading systems to evaluate morphological changes. Exfoliated cervical cells (Papanicolaou (Pap) smears) are classified as normal, atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSILS), or high-grade squamous intra-epithelial lesions (HSILS). Tissue biopsies are graded into normal, cervical intraepithelial neoplasia (CIN) grade I, II, and III, referring to the increasing proportion of non-differentiated cells in the squamous epithelium. Currently it is still necessary to surgically remove all lesions which possess an increased potential for carcinogenic progression (CIN II and III) even though it is known that a subset of these lesions will regress spontaneously. Moreover, reliable cellular molecular markers have not yet been identified which allow discrimination between ASCUS, LSILS, HSLIS and CIN I to III.

We are using an *in vitro* HPV oncogene expression-dependent epithelial cell immor-talization model and a cervical biopsy bank in an approach to i) identify cellular markers associated with the morphological changes described by the cytological and histological grading and to ii) identify hallmarks of a benign to malignant progression of HPV-induced neoplasia. Currently we are analyzing the proteome of human keratinocytes transduced with none or either one (E6 or E7) or both HPV oncogenes (E6 plus E7) using 2-Dimensional Differential In-Gel Electrophoresis (2 D-DIGE) and MALDI-TOF (matrix-assisted laser desorption ionization – time-of-flight) technology in order to reveal differences in the protein expression pattern. Preliminary results indicate that the cellular protein disulfide-isomerase ER 60, a serine proteinase inhibitor, peroxiredoxin 2, and the keratin expression profile of epithelial cells are differentially influenced by a HPV E6 or E7 oncogene expression. Furthermore, we are in the process of comparing the proteome of CIN I to III lesions matched to same patient normal cervical tissue. Neoplastic tissue is collected from the investigated biopsies by laser capture microscopy (LCM) before proteome analysis is carried out through the combined use of 2 D-DIGE/MALDI-TOF technology. A comparison between the findings obtained through the analysis of the *in vitro* HPV onocogene-transduced human keratinocytes and the results of the analyzed biopsies will be presented.

The Use of Surface-Enhanced Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry to Identify Biomarkers for the Detection of Breast Cancer in Saliva

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In the last 10 years, the use of saliva as a diagnostic fluid has become a translational research success story. Technologies are now available enabling saliva to be used to diagnose disease and predict disease progression. This pilot study describes the use of Surface-Enhanced Laser Desorption/Ionization Time-of-Flight (SELDI) to detect putative breast cancer markers in saliva. In this pilot study, three protein chip arrays were used for salivary protein profiling. Eight salivary specimens were analyzed: two pooled saliva specimens (cancer & control), three healthy women and three women diagnosed with carcinoma of the breast. The specimens were analyzed on the SELDI using the

H4, SAX, and the WCX protein chip arrays and washed at pH of 3.5 and pH 7.5 with each array having unique biochemical properties to separate proteins. The results of the pilot study demonstrated that the WCX protein chip array when prepared and washed at pH3.5 yielded the most promising results. The analyses revealed 4 major protein peaks that were higher in concentration among the cancer subjects as compared to the controls. These protein peaks were 117 kDa, 170 kDa, 228 kDa, and 287 kDa in molecular weight. The investigators feel that that there are distinct proteins in saliva that may have diagnostic potential. Additionally, these proteins appear to be elevated among cancer patients. Considering the logistical advantages of salivary diagnostic testing, it would be extremely useful to continue to explore the possibility of using saliva as a diagnostic medium.

Human Urine Contains Small, 150–250 Nucleotide Sized, Soluble DNA Derived From the Circulation and May Be Useful in the Detection of Colorectal Cancer

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Human urine has been shown to possess submicrogram per milliliter amounts of DNA. We show here that DNA isolated from human urine resolves into two size categories: the large species, greater than 1 kilobase, being predominantly cell associated and heterogeneous in size, and the smaller, between 150–250 base pairs, being mostly non-cell associated. We showed that the low molecular weight class of urine DNA is derived from the circulation, by comparing the mutated K-ras sequences present in DNA isolated from tumor, blood and urine derived from an individual with a colorectal carcinoma (CRC) containing a mutation in codon 12 of the K-ras proto-oncogene. In the urine, mutated K-ras sequences were abundant in the low molecular weight species, but far less abundant in the large molecular weight derived DNA. Finally, the possibility that detection of mutant K-ras sequences in DNA derived from the urine correlates with the occurrence of a diagnosis of CRC and polyps that contain mutant K-ras was explored in a blinded study. There was an 83% concurrence of mutated DNA detected in urine and its corresponding disease tissue from the same individuals, when paired urine and tissue sections from 20 subjects with either CRC or adenomatous polyps were analyzed for K-ras mutation. The possibility that the source of the trans renal DNA is apoptotic cells, and the potential use of this finding for cancer detection and monitoring is discussed.

Comprehensive Profiling of Human Serum for Biomarker Discovery Using Protein Array-Pixelation, A 4-D Strategy Combining Top-Down and Bottom-Up Separation Methods

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Identification of low abundance proteins from human plasma or serum by either top-down methods such as 2-D gels or bottom-up LC/LC-MS/MS methods is complicated by the very wide range in protein concentrations. Reduction of the proteome complexity and addition of more separation modes are required to substantially extend protein profiling capacity so that potential biomarkers of human diseases can be identified. Hence, we developed a multi-dimensional protein profile analysis strategy that combines many of the advantages of top-down protein separations with the power of LC-MS/MS for analyzing tryptic peptides from complex mixtures of proteins. Our separation of human serum starts with a first dimension depletion of six abundant plasma proteins using an Agilent MARS antibody column. The sample is then separated into well-resolved fractions using MicroSol-IEF fractionation on a ZOOM IEF device, and aliquots of each fraction are separated on 1-D gels. Each lane is cut into slices which are digested with trypsin. The solution IEF and SDS PAGE produce a two-dimensional grid or array where each point (pixel) contains a mixture of proteins with defined pI and molecular weight ranges. Trypsin digests are analyzed using nanospray LC-MS/MS with a linear ion trap mass spectrometer (Thermo Electron LTQ), and proteins are identified using SEQUEST. Da-

ta are filtered using a combination of scoring systems, and results are analyzed and displayed using custom software. When fully optimized, this method should be capable of detecting at least 2,000 to 3,000 proteins in serum or plasma, including low abundance biomarkers of disease, which are often present at the low ng/ml level.

Methylation Profiling of Lung Cancer Using Restriction Landmark Genomic Scanning (RLGS) Analysis

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DNA hypermethylation of CpG islands in promoter regions of several genes has been reported in lung cancer. To develop a potential panel of markers useful for early detection and screening, we compared genome-wide methylation profiling of lung cancer using restriction landmark genomic scanning (RLGS). DNA samples from 14 pairs of lung tumor and normal tissues resected from same individuals were assayed for differential methylation at over 2,000 loci. Sites of aberrant methylation were identified by comparing cleavage profiles obtained with *NotI*, *EcoRV*, and *HinfI* in paired tumor and normal DNA samples by visual inspection of two-dimensional gels. Hypermethylation events were recognized as failure of samples to cleave with NotI and end-label, resulting in reduction of spot intensity in tumor as compared to normal samples. Among 162 sites of differential methylation, identified from at least one tumor/normal pair, 21 hypermethylated genes were identified and were not reported previously as hypermethylated in lung tumor tissue. An additional 17 sites have been cloned and located within the genome. We are currently identifying the remaining candidate genes associated with the hypermethylated fragments. Hypermethylation was observed at loci on chromosomes 3p25.3, 9q33.3, and 22q13.31 sites where loss of heterozygosity has been frequently reported in lung cancer. These data suggest that differential promoter hypermethylation is a frequent event in lung cancer and that detection of hypermethylation by RLGS may have utility in detecting novel methylation sites, identifying previously unrecognized tumor suppressor genes, and clarifying pathways of lung carcinogenesis leading to novel screening approaches.

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PBOV1 (UROC28): A Novel Biomarker Overexpressed in Epithelium and Serum of Prostate Cancer patients Treated by Radical Prostatectomy

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PBOV1 (UROC28) is a novel gene which was identified by Differential display RNA fingerprinting and found to be up-regulated in prostate, breast and bladder cancers [Cancer research 60: 7014-20, 2000]. This gene maps to human chromosome 6q23-24 and was shown to be over-expressed in prostate cancer with increasing Gleason grade at both mRNA and protein levels. The *PBOV1* protein has also been found in increased levels the blood of prostate cancer (PCa) patients. Rabbit monoclonal antibodies against two synthetic peptides were produced in rabbits and mice and their reactivity was assessed *in vitro* using human PCa and control cell lines. Expression rates and the *in vitro* membrane-specific localization of the *PBOV1* protein were evaluated using Flow Cytometry and Con-focal fluorescence microscopy with LNCaP and derivative cell lines and controls. We also utilized quantitative IHC analysis to evaluate both the peripheral zone cancer and adjacent normal areas for expression of the *PBOV1* protein in a $n = 186$ case tissue microarray (TMA) with long term follow-up. The *PBOV1* antigen IHC expression was evaluated using quantitative image analysis with the AutoCyte Pathology Workstation (TriPath Inc, Burlington, NC). The TMA consisted of 0.6 mm spots of each case ($n = 186$) in quadruplicate. The mean follow-up time for the biochemical recurrence only group ($n = 90$) and the biochemical recurrence with distant metastasis and/or death groups ($n = 96$) were 12.5 years (median = 13 years, range = 4–19 years) and 10.6 years (median = 6 years, range = 2–20 years), respectively. The *PBOV1* specific antibodies demonstrated significant membrane-specific up-regulated activity of the *PBOV1* protein *in vitro* versus

several LNCaP cell lines when assessed by Flow cytometry and Con-focal fluorescence microscopy. In addition, sodium phenylbutyrate at concentrations between 5–25 mM further increased the production of *PBOV1* protein *in vitro*. A preliminary assessment demonstrated increased levels of *PBOV1* protein in the serum of PCa patients. Additional work is planned to more fully characterize the *PBOV1* antibody reagents' utility in breast, bladder and prostate cancers.

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Cancer Risk in EDRN High Risk Registrants

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Background: In 2001, The EDRN Clinical and Epidemiologic Center at Creighton University began recruiting individuals to a High Risk Registry. All persons recruited were judged to be at high risk for specific types of cancer because they were known carriers of cancer-associated mutation, most commonly in the BRCA1, BRCA2, MLH1, or MSH2 gene.

Methods: Registrants complete a baseline questionnaire and yearly follow-up questionnaires. Kaplan-Meier estimation was used to calculate the cumulative risk of neoplasia.

Results: Presently, the Registry includes 319 persons, and 176 of these have completed the baseline questionnaire and at least one follow-up questionnaire. Among these 176 persons, 13 in-situ or invasive cancers have been diagnosed during follow-up. Nine additional persons have been diagnosed with a benign tumor which could be classified as pre-cancer. At one year the cumulative risk of cancer was 4%; at two years it was 10%. At one year the cumulative risk of any lesion was 7%; at two years it as 17%. The specific diagnoses are given in the table.

Number (Gene)	Site	Description
7 (various)	Colon	Adenoma(s)
1 (APC)	Ampulla	Adenoma
1 (BRCA2)	Endometrium	Hyperplasia with atypia
1 (MLH1)	Urinary tract	Carcinoma in situ
3 (MLH1,MSH2)	Urinary tract	Transitional cell carcinoma
2 (BRCA1)	Ovary	Carcinoma
2 (MLH1,MSH2)	Endometrium	Adenocarcinoma
2 (MLH1)	Colon	Adenocarcinoma
1 (BRCA2)	Breast	Ductal carcinoma
1 (BRCA1)	Pancreas	Carcinoma
1 (MLH1)	Prostate	Carcinoma

Conclusion: The High Risk Registry members are at very high risk for the development of neoplasia. Further growth of the registry will make it an increasingly valuable resource for EDRN validation studies. Routine collection of serum at baseline and follow-up for use in future EDRN projects is warranted.