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End of Sequence Profiling (ESP): The Structural Analysis of Tumor Genomes

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Tumor genomes can be highly rearranged. Recurrent genome rearrangements involve genes that mediate a wide range of mission-critical tumor functions that are increasingly targeted by anti-tumor therapeutics. Current technologies for studying tumor genome structure are not capable of elucidating the structural organization of tumor genomes at high resolution or relating it to the underlying sequence. Consequently, the role of translocations and inversions in solid tumors is poorly understood. ESP is a sequence-based method for simultaneously determining the structural organization of tumor genomes and cloning ***all*** types of structural rearrangements. We applied it to the analysis of a breast cancer cell line and primary tumors of the brain, breast, ovary and prostate, accurately mapping copy number, and revealing direct evidence for the packaging of amplified DNA from multiple loci, extensive rearrangements of packaged DNA, and for independent mechanisms of amplification operating within a single tumor genome. Robust mathematical methods for tumor genome assembly and for integrating disparate whole-genome data sets are being developed, enabling the formal testing of hypotheses on tumor genome evolution and function. ESP can also be carried out on tumor cDNAs for large-scale identification of tumor-specific transcripts. We have demonstrated the utility of this by analyzing full-length enriched and normalized cDNA libraries from MCF7 and a primary brain tumor. Multiple tumor-specific transcripts were identified and analyzed.

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Protein Knockout, A Novel Reverse Genetic Tool for Dissecting the Function of Oncoproteins During Normal Development and in Antagonizing Tumorgenicity

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The ubiquitin-proteasome pathway is a major piece of proteolytic machinery that selectively targets cellular proteins for degradation. We have developed a new technology, designated "protein knockout," to harness the SCF (Skp1, CUL-1, and F-box-containing substrate receptor) ubiquitination machinery, to direct the degradation of otherwise stable cellular proteins. The objective is to develop an efficient reverse genetic tool for generating loss-of-function mutations in somatic cells, and in mice, and to antagonize tumorigenicity of oncoproteins in animal models. The engineered ubiquitin-protein ligase consists of the F-box-containing b TrCP fused in-frame to a binding peptide (BP) for the intended target.

We have previously shown that such engineered b TrCP-BP ubiquitin-protein ligases efficiently target cellular proteins for destruction in culture mammalian cells. To demonstrate the *in vivo* application of the protein knockout system, we used the *in utero* gene transfer method to deliver a recombinant adenovirus carrying the engineered b TrCP-Max to the lung and intestinal epithelium of mid-gestational mouse embryos, and showed that targeted eradication of the Myc family transcription factors significantly impaired late gestation of these organs. Using the nude mice tumor model, we showed that induced expression of a chimeric b TrCP-Ecad ubiquitin-protein ligase efficiently degraded otherwise stable b-catenin in colon carcinoma cells, and significantly inhibited their tumorigenic potential. Future studies are aimed at evaluating the therapeutic value of the protein knockout system in a mouse model for leukemia.

Kyle Furge, Ph.D.
Van Andel Research Institute

Identification of Frequent Chromosomal Aberrations in Hepatocellular Carcinoma from Gene Expression Microarray Data

Karl J. Dykema and Kyle A. Furge, Ph.D.   Bioinformatics Program   Van Andel Research Institute

Hepatocellular carcinoma (HCC) is a leading cause of death worldwide, and cytogenetic profiling studies of HCC have consistently identified a subset of cytogenetic abnormalities that frequently occur within this cancer type. While molecular genetic techniques such as comparative genomic hybridization (CGH) have traditionally been used to identify cytogenetic aberrations, it may also be possible to identify cytogenetic abnormalities indirectly from gene expression studies. A technique we have termed comparative genomic microarray analysis (CGMA) predicts regions of cytogenetic change by searching for regional gene expression biases. CGMA was applied to HCC gene expression profiles to identify regions of frequent cytogenetic change and to identify genes whose expression is misregulated within these regions. 104 HCC gene expression microarray profiles were analyzed using CGMA. CGMA identified 13 regions

of frequent cytogenetic change in the HCC samples. Ten of these regions have been detected in previous CGH studies. The results of this study suggest that CGMA analysis of gene expression microarray data sets is a practical alternative to CGH profiling. In addition, CGMA may be useful in identifying candidate genes within cytogenetically abnormal regions.

Brian Davis, Ph.D.
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Functional-Based Identification of New Target Genes for Cancer Treatment

Igor Roninson, Ph.D. , Brian Davis, Ph.D., Michael Shtutman, Anil Maliyekkel, Elena Levina, Mirza Baig, and Liana Rodriguez Cancer Center, Ordway Research Institute

We have developed a general method for identifying genes essential for cell growth, based on the selection of growth-inhibitory Genetic Suppressor Elements (GSEs), short cDNA fragments that inhibit the function of their cognate genes. We have previously used a library of normalized (reduced-redundance) cDNA fragments from breast carcinoma cells in an inducible retroviral vector, to select GSEs inhibiting breast cancer cell growth. 57 genes, involved in different cellular functions, gave rise to verified growth-inhibitory GSEs. These genes are investigated as potential targets for new chemotherapeutic drugs (Primiano et al., Cancer Cell 4:41-53, 2003). In the present project, we are expanding this approach to different types of tumor and normal cells. RNA from 17 cell lines representing different cancers and leukemias is used to generate normalized cDNA comprising essentially all the human genes, known and unknown. This cDNA will be used to produce a library of random fragments for GSE selection as well as a short hairpin RNA (shRNA) library of RNAi inhibitors. We are also developing inducible lentiviral vectors for high-efficiency transduction of GSEs or shRNAs into different cell types. The resulting libraries will be used for function-based selection to generate comprehensive lists of human genes, inhibition of which stops the growth of different tumor cell types, or telomerase-immortalized normal cells. Comparison of the data generated in tumor and normal cells should pinpoint the most promising target genes with a tumor-specific role in cell proliferation.

Niroshan Ramachandran, Ph.D.
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Development of a Self Assembling Protein Array

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One of the most compelling steps in the post-genomic era will be learning the functional roles for all proteins. The Harvard Institute of Proteomics (HIP) has initiated a project to create a sequence-verified collection of full-length cDNAs representing all human coding regions in a recombinational vector system that allows the immediate in-frame

transfer of all coding regions into virtually any protein expression vector. These transfers allow the addition of peptide tags to either or both end of the proteins. This repository, called the FLEXGene Repository (for **F** ull- **L** ength **Ex** pression-ready), will enable the high-throughput (HT) screening of protein function for the entire set (or any customized subset) of human genes using any method of *in vitro* or *in vivo* expression.

One such approach is the use of target protein microarrays, where multifunctional assays, such as protein-protein interactions, nucleic acid-protein interactions, and small molecule or enzyme-substrate screens can be performed on hundreds of different proteins. However, they have not found wide acceptance, in part, because of the challenges in producing proteins to spot on the arrays. We developed a novel approach to generate protein microarrays by printing cDNAs onto glass slides, and then translating target proteins with mammalian reticulocyte lysate. This robust method obviates the need to purify proteins, avoids protein stability problems during storage and captures sufficient protein for functional studies. The versatility of this technology, **N**ucleic **A**cid **P**rogrammable **P**rotein **A**rray (**NAPPA**), was demonstrated by mapping pairwise interactions among 29 human DNA replication initiation proteins , recapitulating the regulation of Cdt1 binding to select replication proteins, and mapping its geminin binding domain.

The most exciting part of this project has been the ease with which the clones from the repository can be rapidly incorporated into this type of HT biological experimentation. NAPPA could be readily adapted to assess the binding selectivity of small molecules to a family of related proteins (e.g., kinases), or to a mutant series of a single protein, to screen for immune responses to a large panel of antigens, or to screen for substrates for an active enzyme. The increasing availability of large repositories of protein-expression ready cDNA clones in recombinational vectors, namely, FLEXGene collection, will provide a rich content source that will amplify the power of this technique to study protein function.

Arun Majumdar, Ph.D.
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Optomechanical Chips for Molecular Profiling of Cancer

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It is becoming increasingly evident that high-throughput identification and quantitation of a large number of serum proteins is important for generating a molecular profile that is critical in diagnosis, monitoring, and prognostic evaluation of complex diseases such as cancer. Recent work has shown that when specific biomolecular binding occurs on one surface of a microcantilever beam, changes in intermolecular forces bend the cantilever, which can be optically detected. In contrast to labeled approaches, the cantilever-based

technique is label free and uses a single reaction step for analysis. Using microcantilevers of different geometries, we have been able to detect two forms of prostate specific antigen (PSA) over a wide range of clinically relevant concentrations and conditions found in sera. To enable multiplexed serum tumor marker analysis, we have developed a technology that involves: (i) mechanical, fluidic, and optical design of a cantilever microarray containing 1000 cantilevers; (ii) fabrication of the chip using standard semiconductor processing techniques; (iii) laser-based optical readout technique that can simultaneously measure the motion of 500 cantilevers with 1 nm resolution; and (iv) software for data acquisition and processing. Furthermore, we have spent considerable effort on developing the adequate surface chemistry to maximize binding-induced deflections and to prevent non-specific binding. Currently, this 5th generation chip is being successfully used to detect PSA, b -hCG, and CEA in purified form and in sera to develop a tumor marker profile.

Steve Sommer, M.D., Ph.D.
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PAP: A Platform Technology

Steve Sommer, M.D., Ph.D. and Q. Liu Department of Molecular Genetics,City of Hope National Medical Center/Beckman Research Institute

Pyrophosphorolysis activated polymerization (PAP) is a novel approach for retrieving multiple types of information from nucleic acids in complex genomes. The exceptional specificity of PAP derives from an inactive 3' blocked oligonucleotide (P*). P* is activated by pyrophosphorolysis of the 3' terminal nucleotide, followed by extension of the activated oligonucleotide by DNA polymerase. We demonstrate that PAP is a platform technology. 1. Allele-specific detection of a single nucleotide change in the presence of 10 6 -10 9 normal alleles (PAP-A) to direct detection of spontaneous somatic mutations in the mouse and human genome. 2. Microarray-based resequencing (PAP-R). PAP exhibits a high selectivity to mismatches along the length of the blocked oligonucleotide in solution and on microarray surface. This property is applied to resequencing on microarray where P*s are anchored to a surface and processed in a high throughput manner. 3 . Analysis of *in vivo* chromatin structure (LM-PAP). LM-PAP greatly increased the signal to noise ratio in four genomic regions. The applications include: a) ultra sensitive detection of minimal residual disease or measurement of mutation load (PAP-A); b) High throughput molecular diagnosis of sequence variants that predispose to complex disease (PAP-R); and c) analysis of chromatin structure as a function of imprinting, X-inactivation and gene expression or quantitation of the level of methylation (LM-PAP).

Annelise Barron, Ph.D.
Northwestern University

Rapid and Sensitive Genetic Mutation Detection by Tandem SSCP/Heteroduplex Analysis in a Microchip Electrophoresis System

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Genetics promise to revolutionize cancer treatment, by enabling correlation of prognosis with specific sequence alterations. Single-strand conformational polymorphism (SSCP) and heteroduplex analysis (HA) are two excellent and complementary electrophoretic methods for genetic mutation detection, because of their simplicity, breadth of application, and low cost. However, it can be difficult to obtain high sensitivity of mutation detection by SSCP or HA alone, unless multiple experimental conditions (i.e., different temperatures) are used. Also, slab gel-based SSCP and HA methods are slow and labor-intensive. Sample preparation methods for SSCP and HA are similar, and we have optimized conditions to allow simultaneous production of both SSCP and HA conformers. We have shown that tandem SSCP/HA, when implemented on a rapid, high-throughput capillary electrophoresis (CE) system, offers much higher sensitivity of mutation detection (~100%) than SSCP or HA alone. In order to implement SSCP/HA by CE, careful optimization of the polymeric separation matrix and wall coating was critical. A highly entangled polymer mesh can separate similarly sized DNA chains, due to differences in molecular conformation deriving from minor sequence alterations, even single-base changes. To develop a faster (min. vs. hr.), clinically relevant mutation detection method, we are now developing microchip electrophoresis-based SSCP/HA. We show that single-base mutations in the p53 gene can be detected with separation times under 2 minutes. The research is being extended to breast cancer tumor samples, to create a more clinically applicable system for mutation detection in cancer-related genes.

Clive Taylor, M.D., Ph.D.
University of Southern California

Recovery of DNA, RNA, and Protein from Archival Formalin-Fixed, Paraffin-Embedded Tissue Sections Based on the Antigen Retrieval Principle

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Following upon the success of heat-induced retrieval for proteins, further studies have focused on identification of optimal chemical reagents, for simpler and more effective heat-induced retrieval of DNA and RNA.

For DNA retrieval, heating formalin-fixed, paraffin-embedded (FFPE) tissue sections under the influence of pH was demonstrated to be an efficient approach for the recovery of DNA that has sufficient structural integrity to be useful in a variety of molecular analyses. Heating tissue sections at higher pH values of the retrieval solution achieved the highest efficiency of retrieval of DNA. A total of 15 chemical solutions were tested, in conjunction with a simple alkaline solution. Two 10 m m sections each from four FFPE human lymphoid tissues were subjected to DNA extraction, using the heating retrieval

method to compare different concentration of alkaline solution versus Britton and Robinson buffer solution (BRB) at pH 12, and various inorganic chemical solutions (SDS, Tween-20, or GITC at various concentration). DNA products were evaluated by spectrophotometry, PCR amplification, and kinetic thermocycling (KTC)-PCR. For DNA, boiling tissue sections in 0.1 M NaOH or KOH produced higher yields and better quality of DNA compared to BRB, or chemical solutions alone, or mixed with alkaline solution. **For RNA** retrieval from the FFPE tissue sections, a cell model system, using the MDA-MB-486 human breast cancer cell line that was fixed in formalin for various periods ranging from 6 to 30 days, was used to establish and validate an optimal protocol of heat-induced retrieval of RNA. In a study involving careful comparison between fresh cell line and FFPE cell line preparations fixed in formalin for 1 to 30 days, RNA extraction was successfully performed using heat-induced retrieval method with BRB. RT-PCR was used to evaluate the efficiency of retrieval method, using pairs of primers for 10 human genes. Comparable results could be obtained for FFPE cells as those obtained by fresh cells.

Conclusion. Boiling FFPE tissue sections in 0.1 M alkaline solution is a simple and effective heat-induced retrieval protocol for DNA extraction. Boiling tissue sections in the BRB of pH 7 - 7.5 is the most efficient retrieval protocol for RNA extraction from the archival tissues.

Tim Huang, Ph.D.
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Use of Oligonucleotide-Based Microarray to Interrogate Promoter Hypermethylation

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Silencing of tumor suppressor genes via promoter CpG island hypermethylation offers tumor cells growth advantage. This epigenetic event is closely associated with transcriptionally repressive heterochromatin in human cancers. We hypothesize that by lining up hypermethylated sites along a stretch of promoter CpG island, it is possible to uncover the path of how this heterochromatin environment is established for gene silencing. Bisulfite DNA sequencing is a standard technique for detailed mapping of methylated CpG sites within a gene promoter. However, this technique is limited in throughput for analyzing a large number of DNA samples. We have developed an oligonucleotide-based microarray technique capable of simultaneously surveying the methylation status of multiple CpG sites along a stretch of DNA. Genomic DNA samples are bisulfite-treated and PCR-amplified to distinguish CpG dinucleotides that are methylated from those that are not. Fluorescently labeled PCR products are hybridized to oligonucleotides arrayed on microscopic slides that can discriminate between methylated and unmethylated alleles at multiple interrogating sites. Using oligonucleotide pairs that span the promoter and the first exon region of *RASSF1A* , we have observed that DNA methylation is present in the exon of all the samples (primary

breast tumors, adjacent normal tissues, and breast cancer cell lines). The promoter region is largely unmethylated in normal controls and in a subset (32%) of the primary tumors analyzed. However, varying degrees (42-65%) of hypermethylation are seen in the remaining primary tumors and breast cancer cell lines. This result suggests that the *RASSF1A* promoter is differentially methylated and that this methylation event progressively spreads from the first exon into the promoter area in some breast tumors. Chromatin profiling also confirms a repressive state of the methylated *RASSF1A* promoter in breast cancer cells. Taken together, detailed mapping of promoter hypermethylation, coupled with chromatin profiling, may reveal information important to the mechanism(s) of epigenetically mediated gene silencing.

Nancy Allbritton, M.D., Ph.D.
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Profiling Ras-Activated Signal Transduction Pathways

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Mutations in *ras* family genes are the most common mutations found in human cancers and clearly contribute to the cancerous state of the cells. Our work focuses on applying the new technology CACE/LMS to study Ras signaling pathways. The CACE/LMS measures the activation state of many kinases simultaneously within the same single cell. This work requires a collaborative effort in the fields of analytical chemistry, synthetic organic chemistry, and cell biology to design, test, and validate novel reporters of the intracellular activity of kinases downstream from Ras. The initial reporter developed and validated was for protein kinase B (PKB) (Li 2004). Our studies demonstrated the measurement of PKB activity in cell lines and tumor cells from patients, as well as quantitative measurements in single-cells after growth factor stimulation. A challenge in reporter development for many of the kinases in Ras-regulated pathways, particularly the MAP kinase cascades, is the design of reporters that specifically measure the activity of the kinase of interest. This need is being addressed by the addition of docking domains derived from MAP kinase-binding proteins to the substrate portion of the reporter molecule. Conjugated molecules with improved *in vitro* kinetics for Erk1/2 have been developed and tested in living cells. This method is now being applied to the MAP kinases p38 and JNK. In addition, tri-domain reporters composed of substrate, docking, and protein transduction domains are also under development to create membrane-permeant reporters.

Cheng Lee, Ph.D.
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Gel Protein Extraction Platform for Ultrasensitive Gel Protein Identification

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We have successfully combined the gel protein capillary extraction platform with a miniaturized trypsin membrane reactor for achieving ultrafast and ultrasensitive identification of gel resolved proteins, using MALDI-MS (Cooper, J. W., Lee, C. S., "Integrated and Ultrasensitive Gel Protein Identification," *Anal Chem* 2004 in press). This integrated gel protein identification technology is demonstrated for the effective (~90% recovery), rapid (less than 5 min), and sensitive identification (as low as 1 ng gel protein loading) of gel resolved proteins using model proteins with molecular mass ranging from 14 kDa (cytochrome C) to 116 kDa (ß-galactosidase). We have further examined the capabilities of this integrated gel protein identification technology for handling complex proteome mixtures, by revisiting the yeast 2-D PAGE in similar isoelectric point, and molecular mass ranges as studied by Gygi and co-workers [Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., Aebersold, R., *Proc. Natl. Acad. Sci.* USA 2000, 97, 9390 -9395]. We are, additionally, able to identify a large number of low abundance proteins with codon adaptation index (CAI) values of <0.2 and increase the proteome coverage to nearly 50%. The CAI value distribution for identified yeast proteins now more closely approximates that predicted for the entire yeast proteome. Besides the simplicity in the procedures and instrument requirements, the translation of single-capillary setup to a microfluidic format will provide automated, comprehensive, high throughput, and robust analysis of gel resolved proteins for proteome applications in key markets, including clinical diagnosis, drug discovery and screening, and life science research.

Victor Levenson, M.D., Ph.D.
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Gene-specific DNA Methylation for Cancer Diagnosis: A Study of 53 Promoters in 10 Breast Cancer Patients

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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.

Ruth Hogue Angeletti, Ph.D.
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Proteomics of Hepatic Neoplasia in a Synchronous Rat Model

Ruth Hogue Angeletti, Ph.D. , Qilie Luo, Ph.D., Linda Siconolfi-Baez, M.S., Pallavi Annamaneni, Ph.D., Mark Bielawski, Ph.D., and Phyllis Novikoff, Ph.D. Albert Einstein College of Medicine

Hepatocellular carcinoma is the one of most frequent causes of death by cancer in the world. There is no reliable diagnosis prior to late stages of disease and no hope for cure, except surgery. It is important to understand molecular changes in the early stages of the disease, not only to identify biomarkers, but also to determine potential targets for early intervention. The overall goal of the project is to discover and identify distinctive alterations in protein expression in early precancerous lesions isolated from their physiological microenvironment and in sera from these same biological stages. The data obtained will provide early molecular markers with diagnostic and therapeutic potential for human liver cancer. The well-characterized resistance hepatocyte (RH) animal model of liver carcinogenesis exhibits well-defined, synchronous stages of initiation and progression of liver cancer that are strikingly similar to those in liver cancer in humans. Three types of analysis were tested: laser capture microdissection (LCM) of stage 4 preneoplastic nodules for proteomic analysis, identification of serum protein differences by cICAT labeling followed by mass spectrometry, and identification of changes in the proteins participating in the cytoskeletal signaling scaffold.

Steve Kron, M.D., Ph.D.
University of Chicago

Bcr-Abl Kinase Assays for Gleevec Resistance in Chronic Myeloid Leukemia

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The primary goal of CA103235, "Bcr-Abl kinase assays for STI571 sensitivity or response," is to develop quantitative assays to measure Bcr-Abl tyrosine kinase activity and inhibitor sensitivity in extracts of circulating leukemic cells from CML patients. This effort is stimulated by the recent development of IM, an ATP analog that blocks the activity of the oncogenic Bcr-Abl kinase expressed in hematopoietic cells with the reciprocal translocation t(9;22) characteristic of CML. Typically, IM induces remission, but patients may develop resistance, leading to recurrence of disease. We hope to develop a clinical assay for IM response to guide CML therapy. Our approach has been to incubate immobilized protein and peptide Bcr-Abl substrates with CML cell extracts and ATP, with or without IM, and quantitate specific tyrosine phosphorylation by incorporation of radionuclides, phosphotyrosine immunoreactivity and/or MALDI-TOF mass spectrometry. The exploratory phase has led to several simple and practical prototype Bcr-Abl assays in distinct formats that each measure specific kinase activity and inhibition in cell extracts with >10:1 specificity and signal to noise. We are poised to develop these tools for use in clinical assays, high-throughput screens, and discovery of novel kinase substrates.

Paul Lizardi, Ph.D.
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Analysis of Genetic Alterations in DNA Archives Generated from Small Lesions and Tumors of the Head and Neck

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The assessment of cancer risk is a valuable strategy for utilization of limited resources in cancer prevention; however, tissue biopsy samples are often not large enough to allow for the archiving of sufficient material for comprehensive genetic analysis. Methods are now available which enable amplification of the entire human genome, starting from samples of a few hundred cells. A recently described method developed in our laboratory has been shown to preserve relatively unbiased DNA sequence representation. We are using head and neck cancer as a model to establish and validate procedures for DNA archiving of neoplastic and pre-neoplastic lesions. Samples of head and neck malignancies and premalignancies are being collected prospectively. Archival DNA is stored prospectively, after whole genome amplification. Most of the archival DNA samples will be assayed for human papillomavirus (HPV) infection. The samples are additionally being processed for comprehensive analysis of

gene loses and gains, using microarray-based comparative genomic hybridization (BAC-array-CGH), which be performed at UCSF. We will use a bioinformatics framework for analysis of the entire data set of genetic alterations generated from these studies. Computational and statistical tools will be used to construct classification schemes based on distance-based trees, as well as different clustering algorithms, utilizing the complete data set of array-CGH and HPV infection status observations. The collection of data can be used to derive a conditional risk model for those patients that develop additional head and neck cancers in the future; that is, at each time point with respect to each molecular marker, say a genetic event detected by array-CGH, we can estimate the conditional probability of final malignant cancer, where the conditioning is with respect to the marker. Thus, given a large enough data set derived from the prognostic follow-up sampling, we will make an effort to generate a multi-factorial model for risk prediction.