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Abstracts

Human papillomaviruses: From bench to countryside

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Human papillomaviruses are ubiquitous infectious agents of the cutaneous skin or the mucosal epithelia. The 8 kB circular double-stranded DNA genome replicates in the nucleus of infected cells as low to moderate copy number autonomous plasmids. More than 100 viral genotypes are known, each with restricted tropism for different anatomical sites. Infections with some viral types are almost always subclinical, while others cause hyperproliferative warts, papillomas or condylomas. Infections may be transient, lasting about one year, or they may persist, with or without clinical symptoms. Laryngeal papillomas are caused by HPV-6 and HPV-11, the same viruses responsible for most genital warts. These lesions, while rare, can require aggressive surgical removal but are likely to recur. Obstruction of the airway can be life-threatening. Certain higher risk HPV types closely related to HPV-16 and HPV-18 have oncogenic potential. Indeed, these HPVs are responsible for essentially all cancers of the cervix, and they are associated with a substantial fraction of penile and anal carcinomas and with some pharyngeal and tonsilar cancers. Approximately 600,000 HPV cancers are newly diagnosed each year worldwide, primarily in medically underserved populations. Viral activation is often linked to transient or long-term immunoinsufficiency. Relatively effective clinical management is achieved through Pap smear cytology combined with surgical ablation of dysplasias. Molecular probing for viral DNA directly or after PCR amplification can enable identification of genotypes, determination of possible multiple infections and assessment of overall viral activity. Such testing is becoming a valuable component of preventive screening and treatment follow-up. Prophylactic vaccines are in worldwide clinical trials, while potential therapeutic vaccines are in earlier stages of development. Antiviral pharmaceuticals such as interferons and IFN-inducers, the nucleoside analog cidofovir, and cytotoxic podophylins are available, but their efficacy is not assured for individual patients.

The control of HPV mRNA production, the properties of the encoded proteins, the regulation of DNA replication, and the nature of virus-host interactions are all known in considerable detail. Papillomavirus gene expression and vegetative DNA amplification are largely differentiation-dependent in the mid-strata of squamous epithelia. In particular, the viral E7 protein binds to the host retinoblastoma susceptibility protein pRB and induces all the host proteins needed for S-phase reentry, restoration of dNTP pools, and DNA synthesis. The E6 protein binds to and inactivates the p53 tumor suppressor protein, decreasing anti-viral responses including apoptosis to enable viral reproduction. New targets for drug discovery are emerging from enzymologic characterizations of the viral replication origin binding protein E2 and the di-hexameric DNA helicase E1, along with the determination of the roles of recruited host proteins needed for synthetic activity of the replication complexes.

Ultimately, nascent virions are shed during desquamation of the superficial keratinocytes. While relatively rare, aberrant expression of E6 and E7 can occur in the less-differentiated, cycling cells. Their effects on p53 and pRB can result in excessive cell cycling, induction of host gene mutations, and successively more severe stages of dysplasia characterized by cytologic and nuclear abnormalities. Initiation of cancer is generally accompanied by integration of the viral plasmid replicons, often as tandem arrays, the induction of host chromosome instability, and the translocation of the integrated viral DNA to multiple loci, together serving as important biomarkers of progression. Domestic and international education concerning HPV infections and diseases has become a pressing priority as more effective forms of prevention, detection and treatment are becoming possible on a universal scale.

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Molecular Cantilevers for PSA

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Early detection of cancer can reduce disease-related mortality as well as treatment-related morbidity and costs. With recent advances in development and quantitative analysis of biomarkers, the goal of screening for cancer using serum tests can be achieved using multiple markers. Availability of an inexpensive platform for detecting these markers in a sensitive, specific and high-throughput manner will therefore be highly desirable. Over the past 4 years we have developed a successful collaboration between the University of Southern California, University of California, Berkeley, and Oakridge National Laboratory to devise a microcantilever-based biosensing platform that is label-free, single-step and cost effective. We have shown that this biosensing approach allows detection of both nucleic acids as well as proteins, providing a universal biosensor platform, and has a potential to analyze, in a high-throughput manner, a large number of clinical serum samples. In contrast to existing biochip technologies, the microcantilever-based biochemo-optomechanical sensors (BioCOM chips) developed do not require any fluorescent labels. We have demonstrated the utility of these BioCOM chips in detecting clinically relevant protein biomarkers, such as PSA for prostate cancer, over a wide range of concentrations (0.2 ng/ml to 60 μg/ml). With rapid identification of numerous biomarkers under development and validation for other malignancies, the BioCOM chip will provide an inexpensive assay platform for detecting these markers in a sensitive, and specific high-throughput manner. Our future plan includes expansion of the scope of our research by collaborations with University of Pittsburgh Cancer Institute, MD Anderson Cancer Center, Duke University Medical Center and Northwestern University Cancer Center as a part of the NCI Early Detection Research Network, where we will use serum resources to perform serum marker profiling studies in malignancies including ovarian cancer, breast cancer and lung cancer. These studies may provide the basis for providing low cost, serum-based screening tests for early cancer detection.

Translational Research on Breast, Endometrial, and Ovarian Cancer: An Epidemiologist's View

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The basic scientist's view of translational cancer research is to use knowledge of biology to develop and test the feasibility of prevention, early detection, or treatment strategies in humans. The epidemiologist's vision of translational research focuses on determining the biological basis for observations made in people with cancer or in populations at risk for cancer that may again yield clues about prevention or early detection. Epidemiologic studies of breast, endometrial, and ovarian cancer reveal many intriguing similarities. Early menarche and late menopause are risk factors for breast, endometrial, and less so for ovarian cancer, while early age at first birth, high parity, and breastfeeding decrease risk for all three cancers. Oral contraceptives decrease risk for endometrial and ovarian cancer. Number of ovulatory cycles is a composite of these variables, which directly correlates with breast, endometrial, and ovarian cancer risk. Hormonal replacement therapy may increase risk for all three cancers, although the association may vary by whether progesterone was included in the regimen. Other risk factors apparent for one of more of these cancers include tubal ligation, intrauterine device use, body mass index, and, of course, family history. Many biologic models have been suggested to explain these observations. Estrogen excess provides a biologic underpinning for all three cancers; with progesterone reversing the risk for endometrial cancer but amplifying risk for breast cancer. Incessant ovulation is a model proposed for ovarian cancer mediated through damage and repair of the ovarian epithelium. Since "incessant ovulation" also appears to increase risk for breast and endometrial cancer, the explanation must be more complex, perhaps involving enhanced steroid secretion from the ovary that has gone through repeated ovulations and has a greater number of residual stromal cells capable of steroid production. It has also been suggested that inverse associations with high parity or late age at last birth might point to exfoliation of pre-cancerous endometrial or ovarian cancer cells. Other theories suggested for ovarian cancer include gonadotropin excess and inflammation. Finally, to explain the protective effect of even a single pregnancy that lasts a lifetime, the largely neglected theory that pregnancy produces immunity to antigens later expressed by cancer cells should also be considered. Human mucin family mem-

ber, MUC1, is a potential mediator of this hypothetical immunity-inducing effect of pregnancy since MUC1 circulates during pregnancy (and breastfeeding) and is overexpressed by breast, endometrial, and ovarian cancer cells. The study of MUC1 auto-antibodies as potential markers of risk, prognosis, and early detection would be a useful target for EDRN collaborative studies.

HLA-DQB1 Allele Distribution and Other Risk Factors For Cervical Cancer nn 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 cooccurrence 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 crosstabulated against cytological and HPV infection status no significant differences were found.

Coexistence at high frequency of two known susceptible and resistant HLA-DQB1 alleles in this Vietnamese population, strongly suggests other interacting genetic determinants besides HLA-DQB1 which deserve further research.

Distinct Genetic Pathways Leading to Melanoma Depending on Anatomic Site and UV Exposure

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UV light is regarded as a major pathogenic factor for melanoma. However, some melanomas arise in anatomic sites that are relatively or completely sun protected. We have analyzed 126 primary melanomas from anatomic sites in different sun exposure (skin with ($n = 30$) or without (= 40) chronic sun damage (CSD and no CSD), glabrous (non-hair bearing acral skin, $n = 36$), and mucosa ($n = 20$)) for DNA copy number aberrations using array CGH and for the mutational status in BRAF and RAS genes. We found significant differences in the type of genomic instability, regional DNA copy number changes and mutation frequencies in BRAF between the four groups suggesting varying genetic pathways between subtypes.We were able to accurately predict melanoma subtypes based on their genomic profiles only. This may be clinically relevant in patients who present with metastatic disease and unknown primary.

Previously, it has been shown that BRAF is mutated in about 70% of melanoma cell lines and that cell lines that are wild type frequently have RAS mutations. In our collection of primary tumors, mutations in BRAF were found to be significantly more frequent in cases with no CSD. Mutation in RAS genes occurred exclusively in samples without BRAF mutation and most commonly affected the NRAS gene. However, a significant proportion of melanomas (57%) showed mutations in neither BRAF nor RAS genes. BRAF mutants had significantly higher frequency of PTEN locus deletions and lower frequency of CCND1 or CDK4 amplifications, suggesting cooperation between these genetic events. Interestingly, among no CSD melanomas, cases with mutations in BRAF or RAS genes frequently showed losses of the CDKN2A region on 9p, whereas cases without mutations did not. If confirmed, this suggests that the oncogene active in melanomas that are BRAF and RAS wild-type does not require loss of p16 and thus may operate outside of the MAP kinase pathway.

In summary, the divergent pattern of genetic alterations in melanomas of different anatomic sites and sun exposure patterns suggest distinct genetic pathways likely to require distinct targeted interventions in the future. More detailed comparisons are necessary to identify potential candidate genes in cases with no detectable mutations in the MAP kinase pathway.

Bench to Bedside Direct: EGFR Mutations and the Pathogenesis of Lung Cancers

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Recent reports of mutations in the tyrosine kinase (TK) domain of *EGFR* indicate that they predict for response to TK inhibitors. These observations led to extensive clinical trials of gefitinib (Iressa) a small molecule inhibitor of EGFR that targets its TK domain. Responses have varied from about 10% (USA) to 30% (Pacific Rim countries) and they target specific subpopulations, especially oriental women never smokers with adenocarcinoma. *EGFR* mutations target the same subpopulations. Mutation incidences range from 4% (unselected US and Australian cases) to 25% in unselected Pacific Rim cases. However subpopulations have rates as high as 74%. While other rare mutations have been described, in our experience they fall into two categories: in frame deletions (usually 15 bp in length, range 9–18 bp) that center around a common 8 bp deleted region in the °C helix loop (exon 19), while the other is an exon 21 missense mutation (codon L858R). Both of these mutational types are predicted to narrow the ATP binding cleft (which gefitinib targets). Multiple cell lines have now been identified with these or related mutations and they confer greatly enhanced sensitivity (>100 fold) to gefitinib. While the mutations appear to relate to patient responses, evidence is accumulating that other mechanisms of sensitivity to targeted therapy may occur. In particular the role of increased gene dosage (via amplification or polysomy) in tumors with and without mutations needs to be explored. Another important question is how lack of tobacco exposure predisposes to mutations. EGFR is over expressed in many non-small cell lung cancers (NSCLC) and this abnormality can be detected in dysplastic bronchial epithelium, suggesting that it is an early event in pathogenesis. Immortalized bronchial epithelial cell lines are new in vitro models for lung carcinogenesis. These cells are dependent on EGFR activation for anchorage dependent and independent growth. These findings suggest that TK inhibitors could play a role in chemoprevention.

The Human Mitochondrial Proteome, Aging and Degenerative Diseases

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Mitochondria are the powerhouses of cells, and also play an integral role in ion homeostasis, fatty acid oxidation, intra-cellular signaling and in the regulation of oxidative stress and cell death processes. Furthermore, recent evidence suggests that mitochondria are closely linked to the aging process and to many degenerative disorders such as Alzheimer's disease, Parkinson's disease, cancer and diabetes mellitus. The emerging role of mitochondrial dysfunction in disease has led to a surge of interest in studying mitochondrial proteomes to identify potential diagnostic and therapeutic targets.

In recent years, we have sought to define the protein composition of the human mitochondria, i.e., the 'mitochondrial proteome', using a variety of mass spectrometry based strategies [1–4]. To date, we have identified close to 700 unique proteins. More recently, we have developed new chemical methodologies to help identify a variety of posttranslational modifications that we believe are essential to understand the changes in the mitochondrial proteome that occur in these degenerative diseases [5–7]. In this presentation, we will discuss the current status of the human mitochondrial proteome and our strategies to define posttranslational changes that may contribute to mitochondrial dysfunction, particularly phosphorylation and oxidative modifications.

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Proteomic Global Profiling for Cancer Biomarker Discovery

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There is substantial interest in applying proteomics to cancer biomarker discovery. Proteomics is particularly suited for profiling biological fluids and uncovering circulating markers and is currently being applied to the identification of novel biomarkers for a variety of cancers. Novel proteomic approaches include direct profiling of serum using mass spectrometry, application of a variety of strategies to harness immunity for cancer diagnosis, protein tagging to capture subproteomes rich in diagnostic markers, the use of protein microarrays, and the use of multi-dimensional liquid protein separation technologies for comprehensive profiling of serum and other biological fluids. It is likely that the application of a wide range of proteomics tools to cancer will yield a panel of markers that have utility for early cancer diagnosis.

The Icelandic Cancer Project: Locating Cancer Susceptibility Genes by LD Mapping

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Cancer initiation and progression is characterized by the complex interactions of genetic, environmental and clinical factors. The *Icelandic Cancer Project* (ICP) was initiated to build a population-based clinical genomics database and biobank that can be used to study the evolution of cancer – from genetic predisposition to clinical outcome. The ICP is a collaborative effort between Iceland Genomics, the Icelandic Can-

cer Registry, the major medical centers in Iceland and a group of cancer clinicians. Participants include all cancer patients in Iceland, their relatives and controls. To date over 19,000 individuals have volunteered, including over 5000 cancer patients. Participants donate blood and permit access to tissue samples and relevant clinical, epidemiological and genealogical data.

A major aim of the ICP is to identify common, low-penetrance cancer alleles through genetic association studies based on LD mapping. We therefore undertook an investigation of candidate regions including the regions of known susceptibility genes, *BRCA1* and *BRCA2*, with the aim of both characterizing patterns of LD in Iceland and testing the feasibility of LD mapping. Furthermore, we performed a simulation study aimed at determining the power of LD mapping for the low-penetrance scenario.

We typed 195 microsatellite markers in thirteen 15 cM regions of the genome (among these the regions of *BRCA1* and *BRCA2*) in 2400 cases and 1400 control individuals from the Icelandic population (approx. 740,000 genotypes). More than half of the pairs of markers within 2 cM of each other and more than 15% of the marker pairs 2–5 cM apart, show significant LD in the control population, however there is no detectable LD between the surveyed regions. This proves that LD extends over megabases in the population and that population stratification is very limited. Thus, association studies can be performed in Iceland using fewer markers than in a typical European population. LD mapping was performed using model based, multipoint analysis in a Bayesian setting. The positions of the *BRCA1* and *BRCA2* genes were determined with accuracy greater than the average marker spacing. Examples for other regions where no known susceptibility genes are present will be given.

Artificial data sets of microsatellites and SNP markers and low penetrance mutations were generated using coalescent simulations. A single or multiple (heterogeneity) disease mutations were also simulated but their physical locations were not included in the analysis except for the effect they have on causing disease. The results show that the methods can accurately predict location of the disease mutation even in the presence of allelic heterogeneity and low penetrance, and that family information increases accuracy. Spacing of markers 1 cM apart appears sufficient for the initial screening.

In conclusion, the Icelandic population is well suited for using LD mapping to identify cancer susceptibility genes with low penetrance mutations.

Colon Cancer Families

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Familial colorectal cancer family syndromes: Familial forms of colorectal cancer (CRC) are uncommon but important. Their importance lies in their impact on years of life given that they affect young subjects and diagnosis is often delayed because cancer is not generally associated with the young. Additionally, familial CRC has provided opportunities for new cancer gene discovery and may be used as a model for sporadic CRC. The two most important forms of autosomal dominant familial CRC are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC).

FAP is caused by a germline mutation in the tumour suppressor gene *APC* and all affected subjects develop myriads of colorectal adenomas by the mid-teenage years and CRC at a mean age of 40 years. Particular APC mutations may result in an attenuated phenotype. HNPCC is caused by a germline mutation in a DNA mismatch repair gene, the two most frequently implicated genes being *hMLH1* and *hMSH2*. Most CRCs show the microsatellite instability-high (MSI-H) phenotype and there is loss of expression of the implicated DNA mismatch repair protein as shown by immunohistochemistry. Penetrance is less than 100% and affected subjects are at increased risk of developing extracolonic malignancy including small intestinal, endometrial and gastric cancer. Adenomas are few in number but are 'aggressive' with a high likelihood of rapid evolution to CRC.

An autosomal recessive form of familial polyposis has been shown to devolop in subjects carrying pathogenic *MYH* variants (homozygous or compound heterozygote) [1]. *MYH* is a DNA repair gene that predisposes to somatic G to T mutation in both APC and K-ras. Adenomas are multiple but not as numerous as in classic FAP. It has been suggested that *MYH* could result in an autosomal dominant syndrome following 'passenger loss' of the wild-type copy of *MYH* on chromosome 1p [2]. CRCs in subjects carrying a single *MYH* variant are more likely to have G to T mutations in APC or K-ras, more likely to show 1pLOH and less likely to be MSI-H.

Other rare hereditary CRC syndromes include juvenile polyposis, Peutz-Jeghers syndrome, Cowden's syndrome and the Bannayan-Riley-Ruvalcaba syn-

drome [3]. Familial risk of colorectal adenoma and carcinoma has been linked to unknown loci on 15q [4] and 9p [5]. Germline E-cadherin mutation can result in CRC as well as gastric cancer (mainly signet ring cell type) [6].

Approximately 40% of CRC are characterized by extensive DNA methylation. It has been noted that subjects with CRCs showing DNA methylation are at an increased risk of having a positive family history of CRC and extra-colonic malignancies [7]. DNA methylation is also associated with early onset CRC and with the condition hyperplastic polyposis [8]. It is therefore likely that genetic factors predispose some individuals to DNA methylation and to neoplastic pathways that implicate genes that may be silenced by promoter region methylation. The Queensland Familial Colorectal Cancer Registry includes 25 'HNPCC-like' families in which CRCs show variable MSI status (MSI-H, MSI-L and MSS) across families. Both MSI-H and MSI-L CRCs are more frequent than would be expected by chance and hyperplastic polyps are over-represented also. Cancers and polyps commonly show DNA methylation and also *BRAF* mutation (see below) regardless of MSI status. These 'Serrated Pathway Syndrome' families could be explained by a genetic predisposition to DNA methylation.

Sporadic and familial CRC: is one the late onset counterpart of the other? FAP is often viewed as the familial counterpart of sporadic CRC showing 'chromosomal instability' (CIN) while HNPCC is often viewed as the familial counterpart of sporadic MSI-H CRC. It has been assumed that the 'vast majority' of CRCs begin as adenomas that are initiated though bi-allelic inactivation of *APC*. Mutation of K-*ras* and *TP53* are conceived as key steps in the growth of an adenoma and its transformation into carcinoma. Genetic instability is viewed as a mechanism for accelerating the stepwise development of tumorigenic mutations.

The preceding views on the evolution of CRC are widely held but are not well substantiated. Only 6.7% of CRCs have mutation of all three 'key' CRC genes (*APC*, K-*ras* and *TP53*) and there is a negative correlation between mutation of K-*ras* and *TP53* [9]. FAP adenomas are a poor model for sporadic adenomas because it takes decades for one out of many thousands to become malignant. Even sporadic adenomas rarely grow when observed over time. Interestingly, adenomas have been shown to progress more rapidly in subjects with a family history of colorectal neoplasia (excluding those with HNPCC) [10]. The low rate of conversion of adenoma to carcinoma in FAP and in sporadic neoplasia probably reflects the fact that the evolutionary process is unlikely to culminate in malignancy without the establishment of genetic instability. Mechanisms for generating genetic instability include the loss of direct DNA repair, loss of base excision repair and loss of DNA mismatch repair. Genes implicated in these forms of DNA repair in sporadic CRC include *MGMT*, *MYH* and *hMLH1* respectively. Of these, *hMLH1* and *MYH* may be inherited as pathogenic variants while *MGMT* and *hMLH1* are silenced somatically by DNA methylation [11,12].

Mechanisms in alternate pathways to CRC: The conventional views on the evolution of CRC take no account of alternate genetic mechanisms for initiating neoplasia, such as mutation of K-ras or BRAF and mutation. They take little account of precancerous lesions other than conventional adenomas. Lastly, conventional models do not place much importance on DNA methylation even though multiple genes can be silenced through this chemical modification of DNA. These missing elements converge within the 'serrated' or 'methylator' model of colorectal tumorigenesis [13]. The earliest lesions are believed to arise through inhibition of apoptosis. Oncogenic K-*ras* leads to activation of pro-survival Akt/PKB and subsequent inactivation of caspase-9 and BAD. Oncogenic *BRAF* acts further downstream by blocking cytochome c dependent caspase activation [14]. Serrated polyps (hyperplastic polyps and serrated adenomas) are frequently characterized by either K-*ras* or *BRAF* mutation (not both) and extensive DNA methylation [14,15]. Hyperplastic polyps with *BRAF* mutation are often large, multiple, and located in the proximal colon. They differ subtly from conventional hyperplastic polyps and have been named fisessile serrated adenomas' [16,17].

Most sporadic MSI-H CRCs are characterized by both *BRAF* mutation and extensive DNA methylation and it is likely that the majority arise within serrated polyps (following methylation and silencing of *hMLH1*) and not within conventional adenomas. By contrast, MSI-H CRCs in HNPCC do not have *BRAF* mutation or DNA methylation and most arise in conventional adenomas [14]. It has also been suggested that serrated polyps may serve as the precursors of remaining non-MSI-H CRCs with extensive DNA methylation [18]. These cancers will have mutation of either *BRAF* or K-*ras* and frequently show low-level MSI (MSI-L). MSI-L may arise through silencing of *MGMT* and the subsequent generation of numerous methylG:T mismatches that would saturate the DNA mismatch repair system [13]. Therefore, the signature genetic al-

terations in CRCs developing via the 'Serrated Pathway Syndrome' will include *BRAF* or K-*ras* mutation (not both), extensive DNA methylation, and patterns of genetic instability as determined by the silencing of *hMLH1* or *MGMT*.

The true lesson of familial CRC: The actual lessons of familial CRC syndrome relate to three conceptual principles: (1) the importance of germline and somatic mutation occurring within steps (exemplified by FAP), (2) the role of DNA repair deficiency and genetic instability (exemplified by HNPCC), and (3) the silencing of genes through promoter methylation (exemplified by the 'Serrated Pathway Syndrome'). Most sporadic CRCs arise through different combinations of these principles and do not represent the direct counterpart of a familial form of CRC. CRC is a heterogeneous disease arising through several discrete evolutionary pathways. Molecular classifications of CRC that utilize MSI status, methylation status or combinations of the two provide reasonably discrete clusters of CRC with particular clinical, pathological and molecular features [19,20]. Progress in early detection and the prevention of CRC as well as the development of new treatment modalities depends on the achievement of logical groupings of CRC with differing pathogenic pathways.

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Molecular and Cellular Events in the Multi-Step Development of Human Cancer

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Neoplasia is a genetic disease, but in a dynamic rather than static way. Its severity and location change with time, and its genetic basis evolves over a period of years. Systems under change require that we understand snapshots of the progression as well as the forces of movement that propel the changes. This basic understanding of neoplastic progression goes back one hundred years.

Cancer results from the interplay of selective pressures with the underlying genetic changes. Like urban

dwellers becoming suburbanites, cells can acquire combinations of changes that allow them to out-compete their neighboring cells in the nutritionally poor tumor environment and in the ability to move far beyond their initial environment. Human cells have an innate resistance to this process of neoplasia, but the multistep progression of neoplasia and the acquisition of genetic instability, in a minority of people, allow the development of malignancy. Many of the genetic changes of cancer are highly patterned. These patterns provide unmistakableclues to enable us to do useful things, such as to individualize the assessment of cancer risk, develop diagnostic tests, and design rational therapies that can be embraced enthusiastically by not only the health care system, but by patients.

p110 sEGFR as a Serum Biomarker in Breast and Gynecologic Cancers

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In recent years, substantial progress has been made in our understanding of the biology of breast and ovarian cancer, yet these diseases remain major public health problems in the US. In 2004, it is estimated that breast cancer will be the leading cancer diagnosed, and breast and ovarian cancer will be the second and fourth leading causes of cancer deaths, respectively, in women in the US. Activation or altered expression of the growth promoting oncogenes encoded by the EGFR/ErbB family have been shown to play a critical role in the development and progression of these malignancies. Clinically, altered EGF/ErbB receptor expression in tumors has been shown to correlate with disease recurrence and patient prognosis, as well as with responsiveness to therapy. In addition to the full-length EGF/ErbB transmembrane isoforms of these receptors, alternate EGFR/ERBB transcripts which encode "soluble" EGF/ErbB receptor isoforms (sEGFRs/sErbBs) have been identified. These sEGFRs/sErbBs appear to be expressed in a tissue- and cell-specific manner in normal human tissues as well as in malignant tissues the expression patterns of these receptor isoforms are being studied intensively as potential tumor/serum biomarkers in breast and ovarian cancer patients. In particular, we have identified a 3.0 kb alternate transcript that encodes a 110 kDa soluble EGFR (sEGFR) isoform, which can be detected in normal as well as cancer patient sera. Serum sEGFR concentrations are altered by normal physiological processes, such as aging and menopause, and by pathological conditions such as cancer. This seminar will summarize recent discoveries in the emerging field of soluble EGF receptor biology, as well as review the potential clinical utility of sEGFR as both a prognostic and/or diagnostic serum biomarker in breast and ovarian cancer patients. Supported by the NIH/NCI grants (Ro1 CA 57531 and Uo1 CA85133), NIH Office of Women's Health Research, Friends You Can Count On, and the Prospect Creek Foundations.

Statistical Issues in the Analysis of High-Throughput Biologic Data

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This talk will provide an overview of statistical challenges and common pitfalls in the analysis of high-throughput biologic data. The talk will begin with an outline of the main types of scientific questions that investigators aim to answer from high-throughput experiments, accompanied by recommendations of basic analysis strategies appropriate for each of the various aims. Examples of clustering methods, permutation tests, multiple comparisons procedures, and class prediction methods applied to gene expression microarray data will be briefly presented. Considerable time will be devoted to discussion of proper interpretation of analysis results, avoidance of circular reasoning, and proper validation of class predictors.

Epigenetic Events as Biomarkers In Barrett's Esophagus Neoplastic Progression"

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Introduction: Patients with Barrett's esophagus (BE) are at increased risk of developing esophageal adenocarcinoma (EAC). For many years, dysplasia grade has been the sole means of risk stratification for patients

with BE. Significant problems have emerged in studies of dysplasia, including poor reproducibility of dysplasia interpretation and sampling error, that illustrate the need for improved early detection and stratification markers.

Materials and methods: To identify frequently hypermethylated genes, we first screened 77 EAC, 69 BE and 64 normal esophagus specimens (NE) in a cross-sectional analysis by performing methylation profiling of 13 genes using real-time quantitative methylation-specific PCR (qMSP). From these 13 genes, we further investigated eight genes that were frequently methylated and had a high specifity for BE/EAC in a series of BE patients with known outcome after long-term follow-up. We included esophageal biopsies from 13 BE patients who later developed dysplasia and/or EAC (Group P) (median follow-up 62 months) and compared them to an age- and sex-matched cohort of 26 BE patients who did not develop dysplasia or EAC during a median follow-up of 82 months (Group NP).

Results: Normal squamous esophageal epithelium, from non-EAC or non-BE patients or from patients with BE or EAC, rarely underwent hypermethylation at the loci studied. By contrast, frank EAC were frequently methylated at these same sites, often at five or more loci. Five of the markers studied were novel methylation targets of EAC and BE (HPP1, CRBP1, RIZ1, RUNX3, and OST-2). Regarding 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, four genes were significantly more often methylated in BE tissues derived from P compared to NP (HPP1, TIMP3, p16, RUNX3). A panel of these four genes was able to detect progressors with a sensitivity of 69% and specificity of 88%.

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 EAC (P) vs. patients who did not progress during long-term endoscopic follow-up (NP). These findings have important implications regarding early detection, risk stratification, and surveillance strategies.

3D Cell Tomography for High-Throughput Orientation Independent Feature Analysis

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There is a persistent interest in developing new cell analysis tools that achieve adequate sensitivity and specificity for a given analytical and classification task while providing ever increasing throughput. Most cell and tissue morphological analyses are slide-based; however, the measurement of slide-based cell features may be compromised due to the potential orientation dependence of such features, and this problem may ultimately constrain the accuracy of algorithmic classifiers. In an effort to improve feature quality by overcoming their orientation dependence, a flow-based 3D tomographic cell analysis instrument is under development.

The VisionGate Flow Optical Tomography (FOT) instrument generates 2D optical projections (shadowgrams) from hundreds of different perspectives (rotational views), each projection being an absorption image having an extended depth-of-field beyond the thickness of a cell. From these 2D projections, the FOT computes the 3D cell image using modified image reconstruction techniques similar to those used in radiological CT. The resulting 3D cell image can be analyzed with both 2D and 3D feature extractors, and automated classifiers can be constructed to leverage the enhanced feature input. The orientation independence of 3D features, including nuclear texture, provides for improved classifier accuracy, while the flow capability provides for high-throughput.

We will discuss the method of generating 2D shadowgrams and their characteristics such as resolution and point spread function, and similarly we will discuss the 3D tomographic image reconstruction method and its impact on image quality. Current results from the FOT instrument will be presented utilizing phantoms and cells embodying features in 2D that are orientation dependent, particularly with regard to nuclear texture, but which become orientation independent in 3D.

Somatic Genetic and Epigenetic Alterations in Gastrointestinal Cancer

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The bimodal distribution of microsatellite mutations in microsatellite instability (MSI) positive cancer defines the microsatellite mutator phenotype (MMP), a

critical determinant of tumor cell fate driving tumorigenesis through a mutator pathway (1). Silencing of the DNA mismatch repair gene *hMLH1* in MSI positive tumors illustrates the importance of epigenetics in cancer. A CpG island DNA methylator phenotype (CIMP) has been postulated to explain *hMLH1* silencing in cancer of the MMP (2). The CIMP hypothesis, as begetting the MMP, adds a more fundamental earlier step in carcinogenesis. To test this model, we analyzed methylation in CpG islands from six genes, and thirty random genomic sites, in a panel of over 250 colorectal cancers (3). Tumor-specific somatic hypermethylation was a widespread age-dependent process that followed a gradual distribution. Similar results were obtained after analyzing over 120 CpG loci in a panel of nearly 100 gastric carcinomas. These results undermine the CIMP hypothesis because it is not possible to distinguish which tumors have a methylation rate that is higher than normal. We also show that the mutator phenotype dominates over the gradual accumulation of DNA hypermethylation in determining the genotypic features that govern the phenotypic peculiarities of colon cancer of the mutator pathway. The epigenetic origin of mutator phenotype and cancer does not seem to be initiated by a pathogenic methylator phenotype, but rather by a gradual age-dependent disintegration of the epigenetic code. A picture is emerging in which epigenetic alterations may precede and determine the occurrence of genetic alterations in gastrointestinal tumorigenesis. However, in the MSI mutator pathway for cancer, the occurrence of the critical inactivation of the *MLH1* mutator gene, either by a genetic alteration or by epigenetic silencing, determines the fate of the tumor because it will accumulate a defined spectrum of altered genes determined by the mutator phenotype. We thus conclude that genetics supersedes epigenetics in the manifestation of the cancer phenotype.

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Validation: Definitions, Pitfalls and Approaches

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Extraordinary claims have been made about the use of molecular markers to screen for and to predict prognosis of cancer. For example, to screen for ovarian cancer, a serum proteomics pattern-recognition assay is $\sim$100% sensitive and specific; to predict prognosis of breast cancer, an RNA expression array pattern-recognition assay is better than any available clinicopathological methods. Historically, however, the field of cancer marker research is characterized by strong initial claims that are not, in subsequent research, reproducible.

A major problem in assessing the "validity," or strength, of a claim about molecular markers is that the word "validation," while referring to general concepts of reproducibility and generalizeability, is so broad as to actually be a source of confusion. The methodologic "rules of evidence" to evaluate research about diagnosis and prognosis are not nearly as well developed as for research about therapy.

The purposes of this talk are:

- To identify concepts of "validation," developed over the last 2 decades in the field of clinical epidemiology, that may be useful to plan and interpret research about molecular markers;
- To describe specific rules of evidence that may be applied to current molecular marker research.

Even if concepts and rules of evidence are not well-developed, research in this field could be substantially advanced by requiring *explicit attention*, in Methods (i.e., design, by investigators) and Discussion (i.e., interpretation/review, by investigators, reviewers, editors) of 3 fundamental questions relating to "validation":

1. *Chance*. Are results (discrimination) due to chance (e.g., overfitting of a multivariable model, without checking for reproducibility)?
2. *Bias*. Are results due to bias (e.g., non-biological features "associated" with cancer, that can become hard-wired in a study and cannot be separated out by multivariable mathematical analysis)?
3. *Generalizeability*. Are results generalizeable to appropriate clinical populations?

The problems incurred by (1) and (2) are so fundamental that they *must be addressed in every research study, even if the study is preliminary, exploratory, or "proof of principle."* Unless problems of (1) and (2) are satisfactorily addressed, then conducting research about (3) is not relevant and may be wasteful. Problem (1) will be easy to deal with, through proper attention to design. Problem (2) requires sometimes-subtle application of already-existing concepts. Problem (3) requires assembly of specimens in appropriate biorepositories.

Proper attention to problems 1–3 will be necessary to understand the potential of molecular markers for use in screening and in predicting prognosis of cancer.

Methylation of Key Genes in Urological Cancers

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Genetic and Epigentic changes drive the progression of human tumors. Genetic changes much as micro satellite analysis of urine DNA have shed light on the development of biomarkers for cancer detection. More recently, promoter hypermethylation of key genes gave been integrated into progression models for kidney, bladder, and prostate cancer. Promoter hypermethylation has been detected in the urine and serum DNA of all these human cancers. We will explore the use of these markers alone and in combination for the detection of cancer. With the use of new quantitative assays, methylation can be used to monitor these tumors. Moreover, quantitative analysis of primary tumor tissues holds great promise in augmenting the current histologic diagnosis of prostate cancer after PSA screening. We will discuss the status of these markers and the necessary next steps for their continued development.

Modeling Ductal Pancreatic Cancer in Mice

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Pancreatic ductal adenocarcinoma (PDA) is the fourth-leading cause of death due to cancer, with few patients living longer than one year. The lethality of PDA has been ascribed to a lack of effective therapies and the relatively advanced stage of disease at the time of detection. Pursuits into the etiology of PDA have demonstrated multiple genetic alterations across the histological spectrum of preneoplastic and frankly invasive carcinoma. Oncogenic *KRAS* mutations are identified in the earliest preinvasive pancreatic ductal neoplasms, suggesting that oncogenic *KRAS* may play a role in the initiation of pancreatic cancer. Conversely, mutations in the tumor suppressor genes *Ink4a, p53* and *DPC4/SMAD4* are noted in advanced preneoplastic and invasive cancer, suggesting a role for these genes in tumor progression. Although mutant mice predisposed

to the development of exocrine pancreatic cancer have been previously described, none develop the spectrum of histological progression resembling human preinvasive and invasive ductal pancreatic cancer. To construct a murine model of ductal pancreatic cancer, we have engineered a mutant mouse strain that harbors an endogenous, conditionally-expressed, oncogenic $Kras^{GabD}$ allele. Compound mutant mice were produced by interbreeding the conditional $Kras^{GabD}$ mutant mouse strain with those that express Cre recombinase in pancreatic progenitor cells (Pdx1-cre and p48-cre), and these mice but neither parental strain developed pancreatic intraepithelial neoplasms (PanIN-1 through 3) that strikingly resemble their human counterpart. PanINs develop with complete penetrance and are detectable at weanling age, with histological progression to PanIN-3 observed in older mice. Additionally, a subset of aging mice develop invasive and metastatic PDA, supporting the premise that PanIN lesions are precursors for PDA. Analysis of murine PanIN demonstrated the aberrant activation of multiple pathways that could serve as therapeutic targets because they have been previously noted in human PanIN, including the Notch, Cox-2, erbB2, and hedgehog pathways. Furthermore, serum proteomic analysis has revealed a diagnostic pattern for PanIN in the low molecular weight range, suggesting that such strategies may be applicable to the identification of patients with PanIN. This murine model of PanIN offers the opportunity to identify and develop methods to detect, prevent and eradicate preinvasive ductal pancreatic cancer; additionally, it should serve as a genetic platform suitable for the construction of advanced models of PDA.

The Genetics of Inherited and Sporadic Breast Cancer: How Understanding Genes Can Reduce Risk

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Every breast cancer, whether inherited or sporadic, is the result of multiple genetic alterations in critical regulatory genes. Breast cancer is the most common malignancy among women with a lifetime risk of over 10%. Germ-line mutations in BRCA1 and BRCA2 are responsible for the vast majority of inherited predisposition to breast and ovarian cancer. Other, rare forms of inherited breast cancer are due to mutation in p53, PTEN, CHK2, and ATM. How genes that are responsible for inherited forms of the disease contribute to the far more common forms that are completely somatic in origin remains to be determined. Recently, we have confirmed that breast and ovarian cancer risk among BRCA1 and BRCA2 mutation carriers is high and thus clinically important. We have also shown that non-genetic factors can significantly influence risk in mutation carriers. The focus of our current research is to identify new breast cancer genes. At the most basic level, the very large number of genetic alterations in breast tumors, and their genetic heterogeneity, strongly suggest that some mutator mechanism must be involved in breast tumorigenesis. Thus, we are attempting to identify novel mutator genes as well as genes that are targets of mutators in breast epithelium by performing a novel, yeast-based screen. In addition, we are attempting to identify new breast cancer genes in high risk families using genomic approaches. Finally, we are evaluating whether circulating tumor DNA can be used as a biomarker of disease.