Genomic Biomarkers of Lung Cancer: Predictors of Risk, Prognosis, and Therapeutic Response

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Polymorphisms in DNA repair genes have been postulated to increase the risk of certain cancers in the presence of environmental exposure to carcinogens such as cigarette smoke. Cell cycle control is also central to the repair of DNA damage before cell division. As part of the University of Pittsburgh Lung Cancer SPORE Project 4, we have proposed to extend these observations to include a pathway haplotype association analysis, focusing on all of the 25 genes involved in the NER pathway and 5 key cell cycle control genes in order to capture all of the common genetic variation within the selected genes in the two pathways of interest and evaluate how this variation contributes to individual lung cancer risk. To test the prognostic significance of the NER and cell cycle control pathway gene haplotypes, we will also perform a prospective study by genotyping the PLuSS high-risk subcohort. In addition, the NER gene haplotypes may predict not only lung cancer risk but also drug resistance and survival. It is well known that resistance to platinum-based drugs, a chemotherapeutic regimen often used in the treatment of lung cancer, is associated with upregulation of NER proteins. In order to further evaluate this haplotype/phenotype relationship, we have proposed to study the relationship of the NER/cell cycle control haplotype with response to platinum-based drug treatment among the lung cancer cases. This haplotype-based approach will provide a great amount of information about genes and pathways and will help to evaluate how genomic variation relates to lung cancer risk. The ability to rapidly screen individuals for risk and prognosis, using noninvasive procedures, has tremendous potential for future clinical application.

Here, we summarize our single nucleotide polymorphism (SNP) selection strategy for the selected NER and cell cycle control genes which incorporates haplotype tagSNPs defined by predicted linkage disequilibrium scores, functional SNPs characterized by amino acid substitutions, evolutionary conservation across species, published epidemiological data, and a minor allele frequency (MAF) of 10%. An algorithm to score SNPs on the basis of these inclusion criteria has been developed. The high-throughput Illumina BeadStation GX has been used to generate a 384 SNP GoldenGateTM genotyping assay that we have now validated and are currently applying to a Pittsburgh case control series (n = 2,200 subjects).

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Keywords: DNA repair; haplotype; pharmacogenomics

Mouse Model of Type II Endometrial Carcinogenesis

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Endometrial cancer is generally recognized to be composed of two broad categories of tumors, known as Type I and Type II, with distinct epidemiologic/clinical features and genetic basis. Mouse models of endometrial cancer have enormous potential to serve as preclinical models and lead to an improved understanding of the genetic mechanisms underlying the initiation and progression of endometrial cancer. Mouse models of endometrial cancer described to date exhibit endometrioid differentiation and thus represent Type I models. However, mouse models of Type II neoplasia are also desirable because of their distinct underlying biology and because Type II tumors have an especially high risk of disease progression and metastasis. We hypothesized that telomere shortening is an important mechanism that promotes genomic instability in Type II tumors and that the absence of Type II histology in mouse models to date is related to the fact that mice have much longer telomeres than those of humans. To explore these hypotheses, we generated and analyzed cohorts of mice with critically short telomeres and analyzed endometria of aged mice. We document in such mice a distinctive endometrial lesion that closely resembles endometrial intraepithelial carcinoma, the accepted in situ precursor of Type II serous carcinomas. Although additional work will be required to build upon these initial efforts, this study points to the feasibility of generating mouse models of Type II endometrial carcinogenesis.

Keywords: endometrial cancer; mouse model; telomeres

Targeting EGFR in Lung Cancer With EGFR Guanidinium Antisense Peptide Nucleic Acids

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Dysregulation of growth factor receptors including the epidermal growth factor receptor (EGFR) in lung cancer results in a promotion of growth, increased survival, and reduced apoptosis. Several EGFR inhibitors currently in clinical trials that inhibit receptor activation and function have demonstrated limited antitumor efficacy. An alternate approach is to downmodulate EGFR protein levels using antisense gene therapy. Although intratumoral administration of EGFR antisense gene therapy has been demonstrated to have antitumor efficacy, it is not a viable route of administration for tumors that are metastatic or difficult to access including lung cancer. Traditional antisense DNA- or RNA-based molecules cannot be administered systemically because of rapid degradation by serum nucleases. To circumvent this problem, we have developed antisense agents with a peptide backbone called guanidinium-peptide nucleic acids (GPNA). This novel class of molecules binds the target DNA or RNA in a highly sequence-specific manner, is resistant to nucleases and proteases, has a strong affinity for complementary DNA and RNA sequences, and has very good uptake into cells. We have designed a GPNA antisense oligomer complementary to 16 bases on the EGFR mRNA (EGFRAS GPNA). We have tested uptake of the EGFRAS GPNA in lung cancer cells and found the efficiency to be almost 100%. EGFRAS GPNA treatment results in growth inhibition in lung cancer cells 201T and 273T in vitro. Unlike conventional oligonucleotides, EGFRAS GPNA is resistant to nuclease degradation and hence can be delivered systemically in animal models. We hypothesize that EGFRAS GPNA will effectively downmodulate EGFR in lung xenograft tumors, resulting in growth inhibition. Further, since EGFRAS GPNA are resistant to nuclease degradation, systemic delivery may be a feasible option for treatment of lung tumors in vivo. Strategies that downregulate EGFR expression when systemically administered have immense potential to improve current therapeutic approaches for lung cancer.

Keywords: antisense; epidermal growth factor receptor (EGFR); peptide nucleic acid

Activation of Akt via Dysregulation of IGF-I Signaling in the Endometrium of Neonatal DES Exposed Rodents

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Neonatal exposure of the rodent to the xenoestrogen diethylstilbestrol (DES) imparts a permanent hormonal imprint on the developing reproductive tract, causing an increase in expression of estrogen-responsive genes and an increased incidence of endometrial hyperplasia (in 58% of rats) and uterine adenocarcinoma. We previously reported that endometrial complex hyperplasia with atypia in postmenopausal women is linked to overexpression of the IGF-I receptor, loss of the negative regulator of the IGF-I pathway (PTEN), and subsequent activation of Akt (see reference). We therefore compared the level of expression of components of the IGF-I signaling pathway (IGF-I, IGF-II, IGFBP3, IGFBP5, IGF-IR, IGF-2R, and IRS-1) as candidate genes involved in endometrial hyperplasia from rodents exposed neonatally to DES compared to vehicle-treated controls in the proestrus/estrus phase. Neonatal DES exposure results in compromised ovarian function; however, developmental exposure to this xenoestrogen resulted in persistent expression of estrogen-responsive genes in adult animals despite their hypoestrogenic milieu. The expression of estrogen-responsive genes IGF-I, and IGFBP3, was consistently high in the endometrium of rats exposed neonatally to DES as compared to proestrus/estrus endometrium of vehicle controls, indicating that these genes have been reprogrammed to be hyper-responsive to estrogen. Additionally, the estrogen responsive gene IRS-1, a major substrate and mediator of insulin and IGF-I receptor signaling, was overexpressed in the endometrium of rats exposed neonatally to DES as compared to the proestrus/estrus endometrium of vehicle controls. IGF-IR and IRS-1 were activated as judged by tyrosine phosphorylation in the majority of DES-treated endometria. We also evaluated the expression and activation of the downstream signaling component of the IGF-I pathway, Akt. Akt expression and phosphorylation were significantly increased in endometrium of rats exposed neonatally to DES as compared to the endometrium of vehicle controls. Consistent with activation of IGF signaling, there was a significant increase in KI-67 positive cells in endometrium of rats exposed neonatally to DES compared to vehicle-treated controls, indicating that this pathway was promoting cell proliferation. The phosphorylation of serine 639 of IRS-1 by S6 kinase negatively regulates the function of IRS-1. Phosphorylation of serine 639 of IRS-1 correlates with phosphorylation of S6 ribosomal protein in the vehicle-treated endometrium. Interestingly, although phosphorylation of S6 ribosomal protein was present in all DES-treated endometria, the phosphorylation of serine 639 of IRS-1 was lost in the majority of neonatal DES-treated endometria. These results suggest that neonatal DES exposure results in developmental reprogramming of components of the IGF signaling pathway in the rodent endometrium. However, activation of this signaling pathway was present in both "normal" endometrium of rats exposed neonatally to DES and the hyperplasias that occurred in these animals, suggesting that additional molecular alterations are required to progress to endometrial hyperplasia in this model system. A loss of negative feedback inhibition of IRS-1 in endometrial hyperplasia may lead to unrestrained activation of proliferation and survival signaling in the neonatal DES exposed rat endometrium. Our studies indicate that IGF signaling and activation of Akt play a central role in both human endometrial complex hyperplasia with atypia and the rodent model of endometrial hyperplasia induced by neonatal DES exposure.

Reference

McCampbell AS et al. *Clin Cancer Res,* 2006.

Keywords: Akt; endometrium; hyperplasia

Immunotherapy for Prostate Cancer: Combining PD-1 Checkpoint Blockade With Attenuated Listeria-PSCA-Based Vaccination

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Immunotherapy represents a promising approach to prostate cancer treatment. Recent data from our laboratory (as well as others) shows that the immune checkpoint mediated by interactions between the T cell surface molecule known as PD-1 and the molecule B7-H1 on cancer cells can inhibit an antitumor immune response. As PD-1 is markedly upregulated on prostate-infiltrating CD8 T cells, blockade of this interaction using monoclonal antibodies directed against PD-1 may play a role in treatment. Our group has also shown that anticancer vaccines based on attenuated *Listeria monocytogenes* (LM) show a striking synergy with blockade of the PD-1/B7-H1 checkpoint. On a mechanistic level, this synergy occurs through an enhancement of both the number and cell-per-cell function of antiprostate T cells. On the basis of these extensive preclinical data, we have designed a Phase I trial combining these two agents for men with prostate cancer. As disease burden plays a major role in the outcome of immunotherapy, we have chosen to target men with minimal disease, i.e., men who have undergone radiation therapy for high-risk disease. The trial includes several critical immune correlates to test the central hypothesis that the combination of PD-1 blockade and PSCA-specific LM-based vaccination will "break tolerance" and result in the accumulation of activated CD8 T cells in the prostate gland. If successful and well tolerated, a larger Phase II trial with relevant clinical endpoints will be initiated.

Keywords: immunotherapy; PD-1; vaccine

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A Phase I/II Trial of Sorafenib in Combination With Anastrozole in Patients With Metastatic Hormone Receptor-Positive (ER/PR+) Breast Cancer Resistant to Aromatase Inhibitors

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Background: Most patients with ER/PR+ MBC develop resistance to endocrine therapy. Activation of growth signaling pathways such as the ras-raf-MAPK pathway has been proposed as a mechanism of resistance to aromatase inhibitors (AIs). Hence, we are evaluating the use of sorafenib, a multi-kinase inhibitor targeting primarily raf-kinase, to attempt to overcome AI resistance.

Methods: This is an ongoing multi-institution Phase I/II study of sorafenib in combination with anastrozole in MBC patients utilizing an optimal two-stage design. Eligible patients include postmenopausal women with ER/PR+ MBC with disease recurrence following AI therapy or progression while on an AI. Participants may have received ≤2 prior chemotherapies for MBC. Primary objectives are to determine the recommended dose and clinical benefit rate (CBR) of sorafenib with anastrozole. Secondary objectives are to determine toxicity, enumerate circulating endothelial cells (CECs) as an angiogenesis biomarker, and analyze the effect of treatment on CYP3A4-metabolized steroids.

Results: 0/3 patients in cohort 1 (200 mg BID) and 0/3 patients in cohort 2 (400 mg BID) had dose-limiting toxicities in cycle 1. Our recommended Phase II dose was therefore 400 mg BID. Interim analysis after recruiting 12 patients showed >30% CBR, allowing continued accrual to 35 patients (current n = 17). Median patient age is 55.8. All patients had ECOG PS of 0-1. The most common grade 1/2 adverse effects (AEs) were diarrhea (64%) and fatigue (43%). The most common grade 3/4 AEs were hand-foot syndrome (36%) and hypertension (14%). Among 13 assessable patients, 2 had stable disease for >24 weeks, 2 had durable PR >6 months, 6 had progressive disease, and 3 were not evaluable. CBR was 30.8%. Preliminary analysis suggests that fall in CECs from baseline to 1 week after treatment predicts for response. Additionally, fall in estradiol, estrone, and 16-OH estrone was noted following initiation of therapy. Accrual is ongoing as are the correlative science analyses.

Conclusions: The addition of sorafenib to anastrozole in patients with ER/PR+ AI-resistant MBC appears to be beneficial, and fall in CECs appears to predict response. Given the negligible activity of single-agent sorafenib in MBC, we believe that the benefit may be attributable to the restoration of sensitivity to AIs through inhibition of the ras-raf-MAPK pathway.

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310 High-Level Expression of Inhibitory B7 Molecules Is Associated With High Risk of Clinical Failure Following Prostatectomy

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Members of the extended CD28/B7 family play critical roles in regulating T cell responses. We have previously shown that blockade of interactions between B7-1,2 and the inhibitory CTLA-4 receptor can greatly potentiate antitumor responses in mice. Clinical studies with CTLA-4 blockade as treatment for several forms of human cancer, including prostate, are currently under way. We recently identified a new member of the B7 family, B7x (also known as B7-H4). B7x is expressed on normal tissues and binds to an unknown receptor expressed by activated T cells, thereby partially inhibiting T cell-mediated killing and limiting damage to normal tissues by aberrantly activated autoreactive T cells. These observations raised the possibility that expression of B7x, or its close homolog B7-H3, by tumor cells might play a role in tumor cell evasion of immune responses. We therefore sought to determine whether expression of either of these molecules might have an impact in prostate cancer.

We used tissue microarrays to conduct a retrospective study of B7-H3 and B7x in prostate cancer. Triplicate cores for 948 patients were stained and scored for frequency and intensity of expression. These results were evaluated in the context of clinical grade and outcome following radical prostatectomy. We found that high-level expression of these molecules was associated with a greater risk of clinical failure. Hazard ratios were 2.79 for B7-H3 ($p<.0005$), 2.22 for B7x ($p<.0005$), and 2.50 for both ($p<.0005$). These results suggest that expression of these inhibitory B7 molecules on tumor cells may have an impact on disease progression, possibly by interfering with immune surveillance.

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156 Ex Vivo Analysis of Prostate Cancer Tumor-Infiltrating Lymphocytes Demonstrates Immunosuppressive CD4+CD25+FOXP3+ T Cells

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Regulatory T cells are important to maintaining immune homeostasis; however, in the setting of neoplasia, regulatory T cells may play a critical role in inhibiting effective immune responses against cancer cells. To investigate the status of regulatory T cells in prostate cancer, we enrolled patients onto an IRB-approved protocol and performed ex vivo FACS analyses to assess prostatectomy samples for the presence of regulatory T cells within the tumor microenvironment. Tumor infiltrating lymphocyte data were compared to information obtained from normal prostate tissues. Circulating lymphocyte data were also obtained from blood samples of prostate cancer patients and healthy donors. The tumor microenvironment of prostate cancer samples demonstrated increased numbers of CD4+CD25+FOXP3+ T cells as compared to normal prostate tissues. Ongoing studies are investigating cytokine production, antigen specificity, and functional status of these T cells.

Keywords: immunobiology; regulatory T cells; tumor microenvironment

311 The Antiangiogenic Effect of Histone Deacetylase Inhibitors Interferes With DNA Methyl Transferase Inhibitor Activity In Vivo in Prostate Cancer Models

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The combination of histone deacetylase inhibitors (HDACI) and DNA methyl transferase inhibitors (DNMTI) has been shown to act synergistically in reexpressing silenced genes in many cancer cell lines. We tested the efficacy of valproic acid (VA), an HDACI, and 5-Aza-2'-deoxycytidine (DAC), a DNMTI, in inhibiting the growth of prostate cancer (PCa) cell lines PC-3 and DU-145 and found that synergism in growth inhibition and gene reexpression was seen in vitro but not in vivo.

Methods and Results: In vitro: The decrease in number of colony-forming units in PC-3 cells after 48 hours of pretreatment with single-agent DAC (0.25 μM) was 53.2%, VA (1mM) was 19.1%, and the simultaneous and sequential combinations were 84% and 86.8%, respectively. The decrease in colony-forming units of DU-145 cells after 48 hours of pretreatment with single-agent DAC (0.25 μM) was 32.5%, VA (1mM) was 28.4%, and the simultaneous and sequential combinations were 76.2 % and 81.8%, respectively. Thus, irrespective of the differing sensitivities of the two cell lines to the single agents, the combination of VA and DAC displayed a significant growth-inhibitory effect on DU-145, and PC-3 PCa cell lines in vitro. In vivo: While growth of DU-145 and PC-3 PCa cell lines in vivo was inhibited by the two drugs used singly, the combination did not have any additive effect in mediating this growth inhibition. Our hypothesis is that one of the molecular events that inhibit the combination of drugs from acting effectively, at least in PC-3 cells, is the antiangiogenic effect of VA. The antiangiogenic effect was assayed by staining for the endothelial cell marker, CD31, a determinant of blood vessel density and also, in lieu of direct measurements of intratumoral DAC, a modification of the Mile's assay (a permeability assay using the Evan's blue dye). PC-3 but not DU-145 xenografts had a significantly lower CD31 count in the VA-treated group compared to untreated controls (average CD31 count/40X field in untreated PC-3 xenografts = 32.97 ± 4.57, and in 0.4% VA treated xenografts = 17.13 ± 3.27, a 48% decrease in vessel density). However, xenografts of both cell lines demonstrated at least some decrease in permeabilty of Evan's blue dye, after prolonged treatment with VA.

Conclusions: VA, an HDACI, acting as an antiangiogenic agent, may inhibit the permeability of the second agent, DAC, into the xenograft. As a result, the synergism seen in combining DAC with VA in vitro may not be translated in vivo.