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14. ABSTRACT : The goal of this project is to develop biopsy based assays to assess the probability that patients with a negative biopsy or with a prostate cancer (CaP) Gleason score 6 (GS6) biopsy actually have "significant" CaP of Gleason score 7 or higher which was missed during the biopsy evaluations due to insufficient sampling. Based on the concepts of the CaP "field effect", this goal will be achieved by gene expression and epigenetic analysis of preneoplastic lesions and non-cancerous areas of prostate tissues from patients with CaP and benign prostate tissues from patients free of CaP. According to the DOD regulations, all research activities began after the approval of the research protocol by the HRPO in March 20 2012. Since then, we have identified the appropriate cases for the study and completed collection by laser captured microdissection (LCM) of all but two of the samples needed for genomic profiling. The collection of samples by LCM was an important part of the experiments in the first year of this project. Procedures for the collection of benign prostate tissues from patients free of CaP have been established. Internally funded collaborations have been established which will enhance the chances of success in this project by expanding the scope of genomic profiling. Two 2012 publications describe our findings related to the CaP field effect. Encouraged by these findings, we are actively pursuing the objectives of this project.					
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Introduction:

Each year in the U.S., more than a million men with an elevated serum PSA or abnormal digital rectal exam undergo a prostate biopsy, and nearly 200,000 are found to have prostate cancer (CaP). Decisions to treat CaP are heavily influenced by the Gleason score (GS) of the tumor in the needle biopsy specimen. Gleason score is a measure of tumor differentiation based on the two most prevalent patterns of tumor growth. Patients whose entire tumor is composed of GS6 rarely progress, and recently, more men diagnosed with GS6 tumors on needle biopsy are selecting active surveillance rather than surgery or radiation therapy. In contrast, men with more poorly differentiated tumors (GS7 and higher) have a significantly increased risk of progression, and require treatment. Choosing the best treatment options for patients with biopsy GS6 is complicated by the fact that a biopsy procedure only samples a very small part of the prostate, and in about 30% of men, it underestimates the GS (Griffin, Yu et al. 2007). In those cases, men with GS7 and higher (GS7⁺) prostate cancer are assumed to have GS6 tumors potentially leading to inappropriate treatment. In addition, because of the limited sampling and 30% false negative rate for detecting cancer (Stewart, Leibovich et al. 2001; Patel, Jones et al. 2004), many men with a negative biopsy result may have clinically significant prostate cancer. Because of that, many of the 800,000 patients with a negative biopsy undergo repeat biopsies which can be frustrating for both patients and urologists. When a pathologist examines a prostate needle biopsy specimen, the focus is on the identification of prostate cancer, and appropriate Gleason scoring, and little attention is paid to the “normal” areas which often comprise the majority of biopsy samples. This is despite a considerable body of evidence suggesting that molecular alterations associated with tumor in adjacent non-neoplastic cells, the so called “tumor field effect”, can provide valuable clues regarding the status of the tumor. Remarkably, the field effect alterations have also been associated with aggressive prostate cancer (Malins, Gilman et al. 2004).

Body / Results:

The objective is to develop clinically relevant molecular models to predict significant prostate cancer with GS7⁺ based on the prostate cancer field effect markers. This proposal will focus only on identification of significant tumors with GS7⁺ because Gleason score is the single strongest predictor of outcome in men with prostate cancer, and has the greatest influence on the clinical management of men with prostate cancer. This proposal will concentrate on the “omics” areas where prostate cancer field effect has been best demonstrated, namely transcriptomic and epigenomics. There are two Aims. Aim I will identify and validate prostate cancer field effect markers associated with GS7⁺ tumors. Aim II will develop and test molecular models for predicting upgrading in GS6 biopsies and for predicting GS7⁺ cancer in a repeat biopsy.

Aim I will analyze 4 types of samples (Table I). These include non-cancerous tissues from CaP patients with (i) indolent GS6 CaP (N₁₆ = 5), (ii) GS3+4 CaP (N₃₊₄ = 5), and (iii) GS 8 and higher CaP (N₈₊ = 5). We also analyze benign prostate tissues from patients free of CaP (BP = 5) as controls. BP samples are resected prostate tissues from patients who were not diagnosed with CaP but had their prostates resected in cystoprostatectomy operations because of bladder cancers. During the first phase of the project, gene expression and epigenetic alterations are to be analyzed by next generation sequencing. Laser captured microdissection (LCM) is used to collect high grade

prostatic intraepithelial neoplasia (HGPIN) lesions in 15 samples and is an important part of the experiments in year 1. The remaining samples are collected using bulk macro-dissection (Table I).

Table I: Bulk and LCM samples proposed in the application for the biomarker discovery step by the next generation sequencing

Sample	Bulk	LCM (HGPIN)
BP	5	
N _{i6}	5	5
N ₃₊₄	5	5
N ₈₊	5	5
Total	20	15

Research Accomplishments:

1. Established procedures for collecting cystoprostatectomy specimens: In coordination with the Tissue Request Acquisition Group (TRAG) at the Mayo Clinic, a process has been implemented for the collection of resected prostates from cystoprostatectomy patients. Mark Manemann, the research coordinator on this project has been in charge of consenting patients according to our IRB protocol (IRB# 11-004215). Collected tissues are sent to the Histology Lab and the Biospecimen Processing (BAP) facilities for further processing. So far we have been collecting about 1-2 samples per month.
2. Cases for discovery step have been selected: Under the supervision of Dr. Cheville and other pathologists in the team we identified in our frozen prostate tissue registry cases that had adequately large areas of HGPIN for LCM collections and benign areas that did not include any preneoplastic or tumor regions by macrodissection in each of the N_{i6}, N₃₊₄, and N₈₊ categories. We also selected some of the cases to be used in the subsequent validation studies. All these samples are from consented patients according to the IRB protocol.
3. Collection of samples by LCM and bulk macro-dissection: Under the direct supervision of Dr. Cheville, all but two of the LCM samples have been collected with adequate cells to produce quantities of RNA and DNA needed for sequencing (10-100 ng). This has been accomplished by a meticulous and carefully implemented process to minimize degradation of nucleic acids, especially the RNA. Bulk areas for macro-dissection from the same LCM cases were identified and are being processed by the BAP core facilities.
4. Testing sequencing protocols for epigenetics profiling: We worked closely with the Mayo Genomics Facility to test reduced representation bisulfite sequencing (RRBS) at concentrations in low nanogram regions. We are also testing a recently published protocol that putatively enhances RRBS (Akalin, Garrett-Bakelman et al. 2012). This modified protocol will allow identification of relevant DNA methylation changes beyond CpG islands and thus we believe will significantly enhance our chances for discovery of the epigenetics field effect markers that can be tested in biopsy based assays.
5. Tested procedures for extracting nucleic acids from FFPE samples collected by LCM: In the validation step of this project we will be collecting HGPIN samples from FFPE blocks by LCM. In preparation for that step, we examine staining procedures and isolation kits for collecting nucleic acids. The best procedure we

found uses Paradise stain (Arcturus) for the LCM slides and PicoPure (Arcturus) kit for isolation as it outperformed the Recoverall (Ambion) in our hand.

Additional related research activities: In addition to the steps described above, we have engaged in other research activities which will enhance our abilities to accomplish PC100553 goals. These include:

- New collaborations and funding: We secured additional funding through the Mayo Clinic Center of Individualized Medicine (CIM) to expand the size and cell types collected by LCM. We have started a new collaboration with Dr. Jeffrey Karnes who is interested in identifying indolent cancers based on the genomic rearrangements (such as chromosomal translocations or deletions, gene fusions, etc.) in tumor cells. Through these collaborations and supplemented funding, in addition to the HGPIN samples for the PC100553 project, we have been collecting the non-neoplastic epithelial cells adjacent to tumors and also tumor cells from indolent GS6 and from GS7 and higher tumors by LCM. The plan is collect samples from up to 100 cases. We will examine the gene expression and epigenomic profile as well as chromosomal abnormalities in a subset of these samples in the coming year. These data will complement our findings from the current DOD project and will ultimately allow more robust statistical models for testing biopsies at the end of this project.
- Publications and presentations: We published our previous results related to the CaP field effect in an article in the American Journal of Pathology (Kosari, Cheville et al. 2012). We presented these results in the AACR annual meeting in April 2012 with the acknowledgement of the DOD sponsorship. Additionally, our investigations of genomic rearrangements and chromosomal abnormalities identified strong evidence for prostate cancer field effects. Some of these data were published earlier this year (Murphy, Cheville et al. 2012).
- Androgen receptor (AR) splicing variations as a marker of CaP field effect: In collaborations with Dr. Lucas Nacusi at the Mayo Clinic, we tested if various splicing events in the AR can be a field effect biomarker. AR variant 4 predicted by Dr. Nacusi to be a tumor suppressor was down regulated in prostate cancer in a pilot study. Also, the expression of this variant tended to be lower in GS 8⁺ tumors compared with GS6 tumors. Since the androgen receptor has been implicated in the cancer field effect (Li and Cannizzaro 1999; Cheng, Gu et al. 2002), we are currently testing this variant in BP, N_{i6}, N₃₊₄, and N₈₊ samples for prediction of significant cancers.

Reportable Outcomes

We are close to submitting samples to the Mayo Clinic genomic core facilities for gene expression and epigenetics profiling by modern sequencing. Through internally funded research, we have collected by LCM non-neoplastic prostate epithelial cells from CaP patients and tumor cells of various grades. These samples will be analyzed in addition to the HGPIN and bulk non-cancerous tissues that are part of this DOD grant. Genomic analysis will include large genomic rearrangements such as chromosomal translocation and deletions in addition to gene expression and epigenetics profiling. We believe these additions will greatly enhance our chances of

success in this project. A recent publication describes some of these genomic events in the CaP adjacent non-neoplastic cells (Murphy, Cheville et al. 2012). Another publication published earlier this year (Kosari, Cheville et al. 2012) describes our previous work related to the CaP field effect. With the acknowledged support from this grant, this work was presented in the AACR meeting in April 2012.

Conclusions:

We have been able to put in place many of the plans and procedures needed for this project to move forward. We expect the pace of this research to accelerate in the coming months. Through additional collaborations we have found new evidence of the genomic abnormalities due to the cancer field effect. We are very encouraged by these data and are actively pursuing experiments towards the goals of this proposal.

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