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TITLE: Prospective Evaluation of Intraprostatic Inflammation and Focal Atrophy as a Predictor of Risk of High-Grade Prostate Cancer and Recurrence after Prostatectomy

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14. ABSTRACT
We are evaluating, in two nested case-control studies, intraprostatic inflammation and focal atrophy, a prostate lesion that is often inflamed, as tissue markers for risk of future diagnosis of high-grade prostate cancer, and for prognosis at the time of surgery for clinically localized prostate cancer. For prostate cancer incidence, in Year 2, we completed the pathology review of inflammation and focal atrophy the H&E stained biopsy core images for the linked PCPT-SELECT data (incident prostate cancer). We worked with the Fred Hutchinson Cancer Research Center (subcontractor) to identify and generate needed covariates for the statistical analysis. We performed a preliminary analysis of the overall prevalence of inflammation and atrophy. To make the work more efficient, we have optimized a higher-throughput method for image analysis of the immunohistochemical stained slides than we had originally proposed. We documented the comparability of the counts from the image analysis method and manual (visual) counting for one of the markers. Our work is progressing slower than as proposed in the Statement of Work, but results are being produced, and with state-of-the art technology.

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INTRODUCTION: With respect to healthy men, at this time, we do not know how to prevent the development of prostate cancer that has the potential to be aggressive, nor do we have a tool to identify men who would most benefit from preventive interventions for aggressive disease. With respect to men with early prostate cancer, at this point, we still cannot predict with certainty which men are more likely to suffer and die of their prostate cancer after prostatectomy. In this population-based research project, we are directly addressing these major problems. We are evaluating, in two nested case-control studies, intraprostatic inflammation and focal atrophy, a prostate lesion that is often inflamed, as tissue markers for risk of future diagnosis of high-grade prostate cancer, and for prognosis at the time of surgery for clinically localized prostate cancer. Our overall hypotheses are: 1) Chronic intraprostatic inflammation is a cause of prostate cancer that is more likely to be aggressive and recur. 2) Focal atrophy, a prostate lesion that is often inflamed, is a risk and prognostic indicator.

BODY: This work is being performed collaboratively by three institutions: Johns Hopkins Bloomberg School of Public Health, Fred Hutchinson Cancer Research Center, and the University of Colorado, Denver School of Medicine. We obtained all required IRB approvals for both the PCPT-SELECT linked study on prostate cancer incidence and the Brady prostate cancer recurrence study, including from the DOD IRB **(Task 1 completed in Year 1)**. A Materials Use Agreement and Data Use Agreement were executed between SWOG and Johns Hopkins University for the PCPT-SELECT linked study. Drs. Platz and De Marzo previously created the prostate cancer recurrence case-control study (in part with prior DOD funding) and associated TMAs. This TMA set is now part of the Prostate Cancer Biorepository Network. For equitable use and tracking purposes, we applied for access to these TMAs and received approval from the PCBN.

Prostate cancer incidence: We developed the source population, prostate cancer case, and control definitions for this work. Based on those definitions, we identified eligible men who participated in both the PCPT and SELECT. From the source population, we identified all prostate cancer cases and selected two controls per case who were frequency matched on age, race, and treatment arms for the two trials. The expected sample size for this work was 100 cases and 200 controls. Tissue was sufficient for 291 of the men **(Task 2 completed in Year 1)**. We pulled prostate biopsy tissue samples from the PCPT biorepository for the 291 men. We cut and mounted 5 sections from one paraffin-embedded prostate tissue biopsy core per man onto slides. These slides were shipped in batches to Johns Hopkins (total of 31 boxes). We received and accessioned the slides. We imaged the 6th H&E stained section of the paraffin-embedded prostate tissue biopsy core per man and uploaded it into a program for remote review **(Task 3, items a-d completed in Year 1)**.

We recruited and trained the pathology fellow (Dr. Ibrahim Kulac) on the methods of scoring inflammation and focal atrophy. We completed the review of the images of the H&E stained sections to determine the prevalence and extent of inflammation and atrophy **(Task 4, items a and b completed in Year 2)**. We reviewed 588 slides for 291 men. 50.5% of men had 2 slides and the mean number of biopsy cores mounted per slide was 2.3. Most men had biopsy cores from the apex and mid regions. For focal atrophy and by slide, we scored: the presence of focal atrophy, the type of focal atrophy, the number of cores with focal atrophy, the percentage of the area of each core with focal atrophy as well as the total core area with atrophy, of the focal atrophy the percent that was simple, post-atrophic hyperplasia, simple with cyst formation, and partial. For inflammation and by slide, we scored: the presence of inflammation, type of inflammation (acute, chronic, acute and chronic), number of cores with inflammation, percent of core area with inflammation, chronic extent (total), chronic grade I stromal extent, chronic grade II stromal extent, chronic grade III stromal extent, chronic grade I intraepithelial extent, chronic grade II intraepithelial, extent chronic grade III intraepithelial extent, chronic grade I luminal extent, chronic grade II luminal extent, chronic grade III luminal extent, acute extent (total), acute grade I stromal extent, acute grade II stromal extent, acute grade III stromal extent, acute grade I intraepithelial extent, acute grade II intraepithelial extent, acute grade III intraepithelial extent, acute grade I luminal extent, acute grade II luminal extent, and acute grade III luminal extent.

We electronically sent the inflammation and focal atrophy data to the Fred Hutchinson Cancer Research Center for merging with prostate cancer case-control status and covariate data, including: 1) *Demographic, anthropometric, dietary and lifestyle information:* age at prostate biopsy (PCPT), attained education (SELECT), race/ethnicity (SELECT), family history of prostate cancer (SELECT), body mass index (SELECT), waist circumference (PCPT [not collected in SELECT), physical activity (PCPT [not collected in SELECT), cigarette smoking status at randomization (SELECT), use of aspirin/NSAIDs at randomization (SELECT), use of statin

drugs at randomization (SELECT), history at randomization (SELECT) of cancer (at during SELECT), cardiac disease, diabetes, chronic lung disease, BPH, prostatitis, intake at randomization (SELECT) of energy, protein, carbohydrate, fat, alcohol, red meat (as a main dish), total polyunsaturated fatty acids, total fruit, total vegetables; 2) *Trial-related information:* date of end of study biopsy in PCPT, date of entry into SELECT, arm of the PCPT trial, arm of the SELECT trial; 3) *Clinical and pathology information:* serum PSA closest in time to end of study biopsy in PCPT, PSA level leading to the diagnosis of prostate cancer in SELECT (cases), clinical and pathologic stage and Gleason sum (cases), history of prior negative biopsy in PCPT, HGPIN in end of study biopsy in PCPT. The source of data is shown above in parentheses: our decisions about whether to obtain the variables from PCPT or SELECT depended on whether the data were collected in both trials, the quality of the data collected, and the timing of the data collection relative to the PCPT end of study biopsies. The variables have been finalized, the datasets merged, and received electronically by Hopkins **(Task 4, item f completed in Year 2)**. The biostatistician we had identified to work on this project has left the institution (she is now a PhD student in biostatistics at Harvard); she continues to work for us on an hourly basis during less busy times during the school year. However, we felt it was prudent to hire a new full-time biostatistician, including for this project. John Barber, who is master's trained in biostatistics, will begin on August 11, 2014. On his arrival, he will perform the detailed statistical analysis of these data. We have prepared this progress report **(Task 4, item g completed in Year 2)**.

The next step is to IHC stain, image, and perform analysis the PCPT-SELECT biopsy cores for the immune markers, Ki67, and GSTP1.

Prostate cancer recurrence: The Brady recurrence nested case-control study already exists; no additional work was needed to define the study population, cases, or controls. We pulled the recurrence TMAs (N=16 TMAs, which includes 524 cases and 524 controls) and cut and mounted 6 TMA sections. H&E stained one TMA section **(Task 5, items a-c completed in Year 1)**. In Year 1, we had optimized the IHC stains.

In Year 2, we implemented a more efficient method of image analysis for IHC-stained TMA sections **(Relevant to Task 3 item f, and 5 item e)**. Digital image analysis is a computational technique used to extract meaningful information from digital microscopy images. Whole slide images (WSI) are a convenient and increasingly popular means for creating digital images for image analysis. However, there are limited tools enabling image analysis on WSI using regions of interest (such as tumor versus benign areas) annotated with virtual microscopic tools. To address this need for this study, we worked with Drs. Toby Cornish and Nathan Cuka in the development of their PIP (PIP is for Image Processing) program, a Java-based software framework designed to apply arbitrary image analysis pipelines to WSI using a configurable thread execution model.

As an initial test and to optimize the image-analysis method, we have evaluated the PIP software using a test TMA designed to represent the cases on the recurrence TMAs. This test TMA contains tissue cores from 10 of the same cases contained in the recurrence TMAs with tumor (4 cores) and normal (4 cores) along with various control tissues, across 1 block. The test TMA was IHC stained for mast cell tryptase (Vector Red Alkaline Phosphatase, red stain), cytokeratin 8 (CK8, DAB brown stain for quantification of epithelial area), and hematoxylin (nuclei). The TMA was scanned as a WSI using an Aperio Scanscope and 64 individual cores were manually segmented as annotation layers in Aperio Imagescope. We implemented a mast cell-counting algorithm (Fig. 1) and compared to manual counting of mast cells in each TMA spot (Fig. 2). The image analysis algorithm for quantification of mast cells performed very well against manual counting, with an R^2 = 0.95 (Fig. 2).

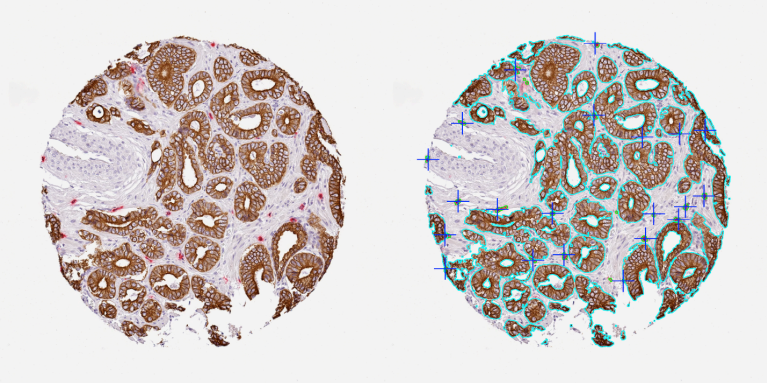

Figure 1. Use of the PIP software for immune cell image analysis. Digital image analysis using PIP (PIP is for Image Processing), a software framework integrating whole slide imaging, virtual microscopy, and ImageJ based analysis algorithms. **Left,** representative TMA spot double stained for mast cell tryptase (red) and CK8 (brown). **Right,** identification of mast cells (blue crosses) and epithelial area (outlined in green) using PIP software.

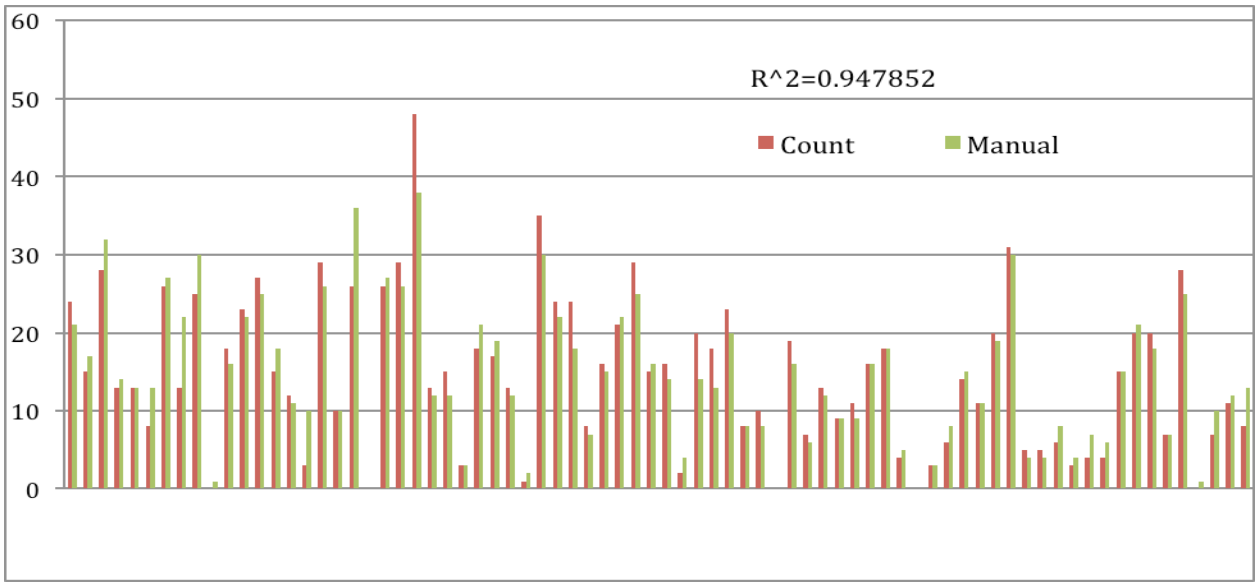

Figure 2. Comparison of mast cell numbers in TMA spots counted manually (green) or with the PIP software (red).

To date, the entire recurrence TMA set (16 blocks, 16 TMA slides) have been double stained for tryptase and CK8 and these slides have been scanned with the Aperio ScanScope **(Task 5 items d and e for one marker completed in Year 2)**. We are currently analyzing the images for mast cell numbers and total epithelial area with the PIP software. We have also optimized double stains for CK8 and CD4, CD8, CD20, CD68, and FoxP3 (Fig. 3). The test TMA for the recurrence TMA set has been stained with all of these different double stains for immune cell types and the slides have been scanned with the Aperio ScanScope. All of these TMAs have been manually counted for the respective immune cell types and we are currently optimizing image analysis algorithms for each of the different cell types.

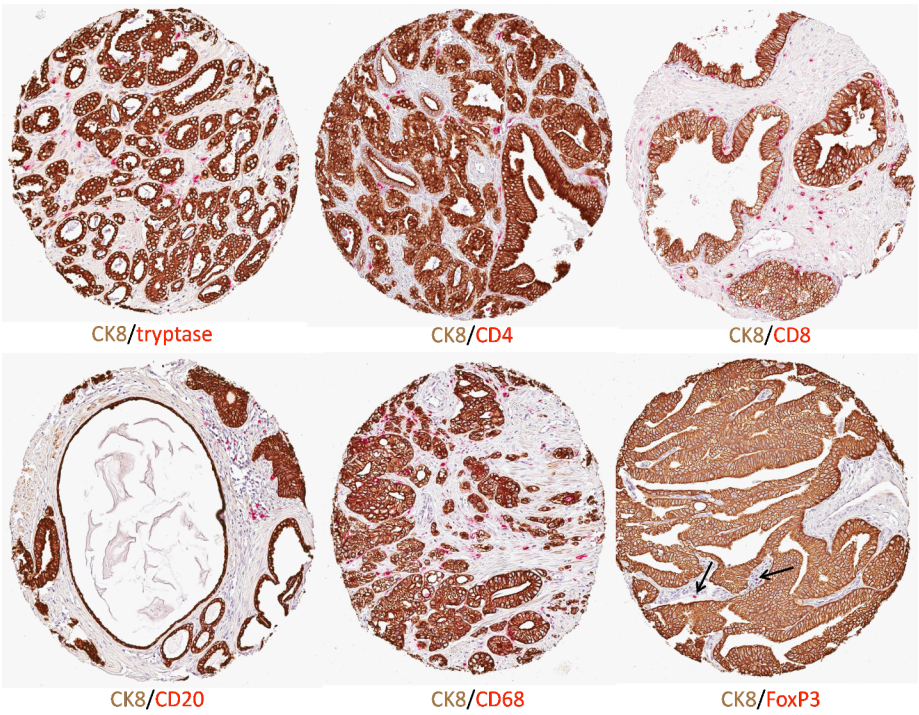

Figure 3. Examples of TMA spots stained via IHC with double stains for CK8 (in brown, for quantification of total epithelial area) and red staining for tryptase (mast cells), CD4 (CD4+ T cells), CD8 (CD8+ T cells), CD20 (B cells), CD68 (macrophages), or FoxP3 (regulatory T cells).

Next steps are to perform the review of the H&E stained images for the recurrence set for the prevalence and extent of inflammation and focal atrophy, stain all of the TMA sections for the remaining markers, image, and then use PIP to count the number of cells staining positive.

KEY RESEARCH ACCOMPLISHMENTS:

We performed a preliminary statistical analysis, while still blinded to prostate cancer case-control status, to characterize the prevalence and extent of inflammation and focal atrophy in the PCPT-SELECT linked data. 54.6% of the slides have at least one biopsy core with inflammation. Of the slides with inflammation, 96.3% have chronic inflammation; 16.3% of these also had concurrent acute inflammation. The percentage of cores per slide with inflammation is 36.5%. The mean total core area with inflammation is 3.9%. Using the consensus method of Nickel (BJU Int 2001;87:797-805), the mean extent of chronic inflammation was 0.65 (possible range is 0 to 3) and among slides with at least 1 core with inflammation, 1.18. For focal atrophy, 48.3% of the slides had a least one biopsy core positive. Of the slides with focal atrophy, 94.5% had simple atrophy. The mean total core area with focal atrophy was 9.2%. Note that the full analysis by case-control status and handling the repeated measures for each man will be performed by John Barber, with supervision by Drs. Platz and Joshu, as his first major project with our team.

As described above, we have successfully implemented an automated image analysis algorithm for the recurrence TMAs. We documented that the counts for IHC-positive cells are comparable to manual assessment for one of the immune cell types (mast cells).

REPORTABLE OUTCOMES: Through this work, we developed a resource for prostate cancer researchers: a new cohort derived from the linkage of the PCPT and SELECT trials. This cohort consists of men who were negative for prostate cancer on PCPT end-of-study biopsy and who then enrolled in SELECT. Linking these 2 cohorts is the ONLY epidemiologically sound approach for prospectively testing the association of tissue markers in men without an indication for biopsy or surgery with prostate cancer incidence – at this time and in the foreseeable future. Access to this linked resource is via SWOG (http://swog.org/Visitors/Biorepository/).

CONCLUSION: None to date, as consistent with the Statement of Work.

REFERENCES: None

APPENDICES: None

SUPPORTING DATA: None