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TITLE: Molecular markers for prostate cancer risk stratification from multiple ultrasound-guided biopsies.

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Molecular markers for prostate cancer risk stratification from multiple ultrasound-guided biopsies.

We hypothesize that adding molecular markers, including recurrent patterns of genomic DNA rearrangement, mRNA and miRNA levels, and DNA point mutations, to current standard practice will significantly enhance risk stratification to appropriately guide treatment decisions. This is a pilot project to establish the feasibility and initial validation of a methodology that could serve as a bona fide clinical trial assay in future studies, while at the same time expanding our basic knowledge regarding the initiation, progression, and metastasis of the disease by charting the genetic relationships among individual cancer cells and the clonal cancer cell populations that develop into tumors.
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INTRODUCTION

Prostate cancer is characterized by a combination of gene mutations and genomic rearrangements that can be observed as copy number variations or CNV. The goal of the project is to examine prostate cancer biopsies from a series of individuals being treated by our co-PI and collaborator, Dr. Herbert Lepor at the Langone Center of NYU and set up a protocol that could be used in a trial to test whether cellular analysis could form the basis of an improved prognostic tool. This first objective is to develop a method for collecting individual cells from core biopsies in a fashion that preserves them in a manner suitable for genomic CNV profiling. The second objective is to begin the actual analysis. As described in the body of this report, Objective 1 has been accomplished and we have begun our work on Objective 2.

BODY

We based this proposal on our hypothesis that markers for risk stratification could be optimized if molecular methods, including single cell genomic profiling could be applied to fresh biopsy tissue while still preserving the biopsy core for standard fixation and histopathology, thus permitting a direct comparison of the information available from standard and molecular prognostics.

It is our objective to execute a pilot project that would establish an operationally feasible method for obtaining single cells or nuclei from multiple prostate biopsies in real time in order to examine the potential value of prognostic information derived from a variety of molecular techniques in combination with existing pathology. Our progress against the milestones in our proposal is shown in the annotated version of the SOW below, followed by a detailed description of each accomplishment.

Annotated SOW from proposal

Objective 1 Year 1
Exfoliating cells from biopsy cores. The goal is to develop a protocol that can be used routinely for obtaining cells from fresh biopsy cores. Testing will be done on the first cases to see how many cells are released by the ‘washing’ protocol. Cell number can be estimated by FACS and by the total amount of genomic DNA released from the pellet.

1.1 Months -6-0: Obtain IRB approval and write informed consent document (Lepor).

1.2 Month 1: Recruit first patient and perform first core ‘washing’ protocol, evaluate path report (Alexander, Lepor).

1.2 MILESTONE: ACCOMPLISHED Receive approval from pathologist that washed cores are satisfactory (Lepor).

1.3 Month 1: Evaluate exfoliated cell suspensions as to suitability for RNA, DNA and nuclear preparation for FACS sorting (CSHL group).

1.3 MILESTONE ACCOMPLISHED Satisfactory exfoliated cell suspensions.

1.4 Months 2-5: Recruit 4 more cases, and perform ‘washing’ protocol, checking for approval from pathologist each time (Alexander, Lepor).


1.5 Months 6-12: Recruit at least 15 cases for procedure and preserve cell suspensions until evaluated (Alexander, Lepor).

MILESTONE MODIFIED as described below. Cells isolated from minimum of 20 cases.
Objective 2. Begins In Year 2

Performing molecular assays on cell suspensions. This includes single nucleus sequencing surveys at low read density, followed by higher read density on selected samples or cells where lineage relationships need to be established, such as in comparisons of different multifocal sites in the same case. This objective also includes the expression assays for PTEN/PHLLP and comparison of single cell data with that from the bulk DNA preparation.

2.1 Months 1-2: Sort nuclei, amplify DNA, create sequencing libraries for cells from first 12 cores. Sequence 12-24 cells from each core sample (Alexander, Hicks).

MILESTONE: ACCOMPLISHED Obtain satisfactory copy.

Summary of progress on Objective 1. We have made very strong progress on the goals of Objective 1, achieving all procedural and methodological milestones. As outlined in the SOW, the first 5 months of the project were concerned with first, obtaining essential approvals for the project and second, a series of trial runs of the biopsy washing protocol to minimize disruption of the normal pathology procedure.

During the trial period, the washing protocol was modified to maximize the number of exfoliated cells collected per core needle biopsy washing while maintaining the integrity of the tissue biopsy for downstream histological evaluation. After collection, the washings were transferred to CSHL where isolated nuclei were sorted (see Figure 1) then processed for single nucleus sequencing.

Figure 1: Histogram. Isolated nuclei from a core needle biopsy washing that was scored as a Gleason 7 (4+3) by the pathologist. Gates were set at 2C (diploid) and >2C (aneuploid) with ~25-50 single nuclei collected per biopsy washing. Sorted sample is NYU001 Gleason7 (Sector 6_Right Apex).

By month 6 of year 1 we had begun to perform actual genomic copy number profiling on single cells from actual biopsies. It became clear that the profiling data, some of which is presented below, was leading us toward selectively studying more early stage patients rather than taking all patients in sequence. Thus, we altered the plans for Objective 1.5 which had been to accumulate washings from 15 patients before proceeding to single cell analysis. Instead we began a more Bayesian approach whereby we perform single cell analysis on one or two patients and choose subsequent patients based on the results of the accumulating data. Thus, by month 12 of year 1 we had performed CNV analysis of 5 cases with varying Gleason scores as described in the summary for Objective 2.

Summary of progress on Objective 2.

To date, we have collected prostate biopsy washings from ~10 patients and have performed SNS on half of these clinical specimens ranging from benign to low and high-risk disease as listed in Table1.
Table 1: Clinical data on cases selected for SNS

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Pretreatment PSA (ng/mL)</th>
<th>PCA3</th>
<th>Gleason score of initial TRUS-guided prostate biopsy</th>
<th>Cores (pos/total)</th>
<th>Gleason score of Radical prostatectomy pTMN</th>
<th>Accessory Findings</th>
</tr>
</thead>
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<tr>
<td>NYU_001</td>
<td>64</td>
<td>6.08</td>
<td>8.7</td>
<td>7 (4+3)</td>
<td>10/15</td>
<td>7 (3+4) pT3b</td>
<td>EPE, SVI, PNI, HGPIN</td>
</tr>
<tr>
<td>NYU_002</td>
<td>72</td>
<td>1.6</td>
<td>neg</td>
<td></td>
<td>2/14</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>NYU_003</td>
<td>47</td>
<td>3.51</td>
<td>5</td>
<td>HGPIN</td>
<td>0/15</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>NYU_004</td>
<td>75</td>
<td>19</td>
<td>benign</td>
<td></td>
<td>9/17</td>
<td>ND</td>
<td>PNI</td>
</tr>
<tr>
<td>NYU_005</td>
<td>64</td>
<td>4.06</td>
<td>65</td>
<td>8 (4+4)</td>
<td>7/20</td>
<td>ND</td>
<td>PNI</td>
</tr>
<tr>
<td>NYU_006</td>
<td>56</td>
<td>3.19</td>
<td>ND</td>
<td>benign</td>
<td>0/18</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>NYU_007</td>
<td>65</td>
<td>10.6</td>
<td>ND</td>
<td>6 (3+3)</td>
<td>1/15</td>
<td>------</td>
<td>HGPIN</td>
</tr>
<tr>
<td>NYU_008</td>
<td>60</td>
<td>4.5</td>
<td>ND</td>
<td>6 (3+3)</td>
<td>2/12</td>
<td>ND</td>
<td>EPE, SVI, PNI, ALI, HGPIN</td>
</tr>
</tbody>
</table>

Note: PSA = Prostate Specific Antigen, DRE = Digital Rectal Exam, PCA3 = Prostate Cancer Antigen 3, TRUS = Transrectal ultrasound, EPE = Extra Prostatic Extension, SVI = Seminal Vesicle Invasion, PNI = Perineural Invasion, ALI = Angiolymphatic Invasion, HGPIN = High Grade Prostatic Intraepithelial Neoplasia, ND = Not Determined.

Single cell genomic profiling of approximately 2400 single nuclei from 70 prostate biopsy washings has been completed. For interactive data visualization and interpretation, we have constructed a novel integrated single cell genomics viewer (SCGV). The data are displayed within the SCGV as a combined heatmap and dendrogram with menu bars of enhanced options, all featured in an intuitive and user-friendly interface. Thus, it is possible to simultaneously view hundreds of genomic profiles of the nuclei sampled from multiple regions within the prostate gland. Many features of genomic complexity, such as degree and frequency of genomic instability, clonal emergence, heterogeneity among clones, ploidy violations, overlap with common patterns of CNV and clone migration/trafficking, which cannot be readily observed by standard histopathology, are easily identified by our methodology and analysis.

The heatmap is a matrix where chromosomes are ordered in rows from 1-22 followed by X and Y (on the left side of SCGV image) and the individual copy number profiles of nuclei are arranged in columns. Legends along the right side of the SCGV image describe the color-coding scheme for both copy number and selected sectors while the ploidy legend is set to a grayscale. A color bar along the bottom of the heatmap identifies the sector of origin for each nucleus and beneath it is a grayscale bar denoting ploidy for that nucleus. Within the heatmap, white denotes a genomic region that is copy number 2 while blue and red specify regions of deletion and amplification, respectively. The numeric scale along the left side of the dendrogram represents the cluster merging distance where height is relative to distance such that a node with the maximum height represents a cluster with the largest distance between its members.

Our preliminary studies demonstrate strong agreement between single cell data and histopathology. Single cell genomic analyses of several hundred cells from cases of benign, low and high grade disease reveal a detailed picture of heterogeneity and clonality as shown in SCGV images in figures 2 through 4.
Figure 2: Single cell genomic viewer. The combined heatmap and dendrogram for case NYU001 Gleason 7 (4+3) showing copy number alterations across genomes for 739 nuclei profiles from prostate biopsy washings from 14 sectors. Also included in this case were CD326 (+) and CD326 (-) (EpCAM) cells isolated from patient's urine collected post-DRE. Individual profiles or groups of profiles can be visualized by clicking, producing detailed and whole genome copy number profiles (as in Figure 5) with adjustable zoom levels to examine any subgenomic region in detail (as in Figure 6).

Figure 3: Single cell genomic viewer. The combined heatmap and dendrogram for case NYU002 (mostly benign but with evidence of HGPIN) showing copy number profiles across genomes for 679 nuclei profiles from prostate biopsy washings from 13 sectors. Also included in this case were CD326 (+) and CD326 (-) (EpCAM) cells isolated from patient's urine collected post-DRE.
**Figure 4:** Single cell genomics viewer. The combined heatmap (showing copy number profiles) and dendrogram for a benign case with 317 nuclei profiled from washings from 13 sectors.

**Figure 5:** Alignment of 3 genome plots from NYU001 Gleason 7 case. Genome plots of normalized bin counts (gray line) and segmentation (blue line) corresponding from top to bottom to Sectors: #3 Right Lateral Apex (top and middle), #6 Right Apex (bottom). Both sectors were scored as Gleason 7 (4+3).
Figure 6: Magnified view of chromosomes 5 through 13 of 3 genome plots from NYU001 Gleason7 (4+3). Profiles show deletions on Chr5q11.2-q12.1 (frequent LOH, breakpoints in prostate cancer), amplification chr8 (myc), deletions on chrom 10 and chromosome loss on 13 (RB).

Additionally we have compared data from bulk nuclei to single nuclei and initial results suggest that bulk analysis is not as sensitive as single cell analysis. Aberrant profiles of cancer clones are dampened by aggregation with benign prostatic epithelial cells and stroma as shown in Figure 7.

Figure 7: Copy number profiles of bulk nuclei versus single nucleus. Genome plots from NYU001 Gleason 7 case (Sector 6). Bulk sample is an aggregate of approximately 100 nuclei.

We have demonstrated that we can obtain useful data from SNS of washings from core biopsies. For SNS, we profiled ~25-50 nuclei per biopsy washing. The integrated profile of the NYU001 Gleason 7 case shown in Figure 2 shares many of the genomic features observed in the advanced cases (data not shown). From our preliminary studies it’s clear that what the pathologist sees as neoplastic foci correlates to what we observe at the genome level in terms of the emergence of clones with genomic complexity, aneuploidy and instability. Our initial data suggests that SNS provides a more complete and quantitative characterization of early and advanced stage prostate cancer than that which can be garnered by conventional pathology thereby improving prostate cancer risk stratification.
KEY RESEARCH ACCOMPLISHMENTS

1. We proved that washing the biopsies will yield sufficient cells for genomic profiling and that the cells yield sufficient DNA for sequencing and CNV analysis.
2. We profiled all of the biopsies from five clinical specimens ranging from benign to low and high-risk disease. Early stage analysis indicates that genome complexity as measured by CNV profiling is proportional to standard pathology grading.

REPORTABLE OUTCOMES

The Key Research Accomplishments described above will form the basis for a publication in the near future, however, there is additional mathematical and bioinformatics analysis required to determine what can be concluded.

CONCLUSIONS

We conclude from this first year of work that we have developed a feasible method for obtaining cells from prostate biopsies and that the data from the CNV profiles of these cells is reproducible and are likely to be interpretable in the way that we proposed. It is too early to predict whether the long term goal of developing a prognostic tool will be achieved. We are well on the way, however, to developing the tools necessary for achieving that goal.

REFERENCES - none

APPENDICES - none