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TITLE: Assessing EphA2 and Ephrin-A as Novel Diagnostic and Prognostic Biomarkers of Prostate Cancer

PRINCIPAL INVESTIGATOR: Carvell Tran Nguyen, MD PhD

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, OH 44109-1998

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14. ABSTRACT This study seeks to evaluate EphA2 and ephrin-A1 as novel biomarkers of prostate cancer (PCa) diagnosis and/or prognosis. We are recruiting men at high risk for PCa who are undergoing prostate biopsy and prostatectomy at our institution. We will correlate their levels of EphA2 and ephrin-A1 mRNA as well as staining of phosphorylated pS897-EphA2 to the presence of PCa, the aggressiveness of PCa as determined by traditional clinical predictors, and race. Completion of the studies will achieve the following: 1) Novel biomarkers to improve the ability to distinguish between indolent and aggressive PCa; 2) More accurate prediction of disease outcomes to facilitate optimal treatment selection for each patient; 3) Elucidation of the biological mechanisms behind the PCa health disparities that affect minority men. During this last study period, we have enrolled 20 additional male patients into our study. We have optimized the RNA isolation protocol and have been able to obtain excellent quality RNA from 90 patients. Complementary DNA was then made from all 90 patient RNA samples and we have performed RT-PCR analyses on 52 patients thus far. The sample size remains small but preliminary data suggest differential expression by race of some members of the Eph family of tyrosine kinases: lower EPHA4 and higher EPHB1 expression among black men with PCa as well as lower EPHA1 expression in black men without cancer. We have also successfully created viable mouse prostate organoid cultures, with plans to establish prostate organoids using primary human prostate tissue derived from prostatectomy specimens.					
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1. **INTRODUCTION:**

We seek to evaluate EphA2 and ephrin-A1, components of a receptor tyrosine kinase signalling pathway, as novel biomarkers of prostate cancer (PCa) diagnosis and/or prognosis. More accurate biomarkers are particularly important to African American men who are disproportionately affected by aggressive PCa and demonstrate a worse prognosis. We will recruit men at high risk for PCa who are undergoing prostate biopsy at MetroHealth Medical Center. Prostate tissue cores obtained from the biopsy procedure will be used for RNA extraction as well as protein staining. We will measure the levels of EphA2 and ephrin-A1 mRNA as well as staining of phosphorylated pS897-EphA2, which is a pro-oncogenic variant of the protein. These values will then be correlated to the presence or absence of PCa, the aggressiveness of PCa as determined by traditional clinical predictors, and race. Eventually, we will validate these data by performing the same analyses on full prostate specimens from enrolled patients who undergo radical prostatectomy. Completion of the proposed studies will achieve the following: 1) Novel biomarkers to improve our current ability to distinguish between indolent and aggressive PCa to determine which patients would benefit from therapy; 2) More accurate prediction of disease outcomes to facilitate optimal treatment selection for each patient; 3) Elucidation and potential countering of the biological mechanisms behind the PCa health disparities that affect African American men.

2. **KEYWORDS:** prostate cancer, biomarkers, racial disparity, health outcomes

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**
- **Specific Aim 1: Determine whether distinct levels and activity of EphA2 and ephrin-A distinguish benign from malignant prostate tissue and/or correlate with cancer aggressiveness.**
 - Major Task 1: Characterize EphA2 and ephrin-A expression levels in benign and malignant prostate tissue: 70% complete
 - Major Task 2: Characterize level of phosphorylated EphA2 in benign and malignant prostate tissue: 0% complete
 - Major Task 3: Correlate EphA2 and ephrin-A mRNA expression and/or protein staining with cancer aggressiveness: 0% complete

- **Specific Aim 2: Test the hypothesis that distinct expression and activity profiles of EphA2 and ephrin-A differentiate PCa in AA versus EA patients**
 - Major Task 1: Characterize EphA2 and ephrin-A expression levels between AA and EA men diagnosed with prostate cancer: 70% complete
 - Major Task 2: Characterize staining levels of phosphorylated EphA2 in AA versus EA men with prostate cancer: 0% complete
- **Specific Aim 3: Investigate whether EphA2 and ephrin-A are independent prognostic factors of PCa behavior and progression.**
 - Major Task 3: Correlate expression and activity levels of EphA2 and ephrin-A with clinical outcomes from a prospective cohort of PCa patients: 50% complete
- **What was accomplished under these goals?**
 - Specific Aim 1, Major Tasks 1-3: I have continued the recruitment of men undergoing prostate biopsy at MetroHealth Medical Center (MHMC) and collect prostate tissue cores for RNA extraction and analysis. To date, we have enrolled an additional 20 men into the study for a total of 107.
 - In order to obtain RNA from biologic tissue, a method of tissue homogenization needed to be chosen. We chose to use a tissue homogenizer which combines shear and mechanical forces to release genetic material from cells. Each prostate biopsy core was homogenized in cell lysis buffer and RNA was isolated using silica-based membrane technology provided in the Qiagen miRNA spin mini RNA isolation kit. RNA quantification was then performed using the Take3 (Biotek) micro-volume plate reader. Good quality RNA was determined by absorbance at 260/280nm of 2.0. If this standard was not met, the RNA was re-isolated from a second biopsy core. Next, 1ug of RNA was used to make complementary cDNA using the High-capacity cDNA kit from Applied Biosystems (ABI). To date, cDNA has been made for 90 prostate biopsy samples. Next, along with the EPHA2 and EFINA1 genes, we chose additional genes to analyze in the EPH family including EPHA1, EPHA3, EPHA4, EPHA5, EPHA6, EPHA7, EPHB1, EPHB2, EPHB4, EPHB6, EFRINA5, and EFRINB2 which have been shown to be differentially expressed in prostate cancer. Taqman probes (ABI) for each gene were used to run real-time PCR gene expression analysis on the ABI 7500 real-time PCR machine. All 14 genes have been run on a total of 52 patients to date. Descriptive data: 25 patients with prostate cancer,

27 patients with benign pathology. Broken down by race, 21 African-Americans (AA) with prostate cancer, 18 AAs with benign pathology. Three Caucasians (CA) with prostate cancer, 8 CAs with benign pathology. An exact Wilcoxon Rank Sum test was used to compare groups. Overall, there were no statistically significant differences in gene expression between prostate cancer and benign samples. Although a few genes do show a trend toward significance, the sample size is still small.

- Specific Aim 2, Major Tasks 1-2: Looking at the data stratified by race, a few genes do reach statistical significance. An exact Wilcoxon Rank Sum test was used to compare groups. Comparing AAs to CAs with prostate cancer, statistically significant gene expression differences are seen for EPHA4 ($p < 0.05$) and EPHB1 ($p < 0.05$) with a lower and higher expression of these genes in AAs respectively. Similarly, looking at AA vs CAs with benign pathology, a statistically significant difference was seen in EPHA1 ($p < 0.05$) with a decrease in expression of this gene in AAs.
- Specific Aim 3, Major Tasks 1-3: We are continuing enrollment of patients into our proposed prospective cohort of men who undergo radical prostatectomy at MHMC. Out of the 107 men thus far enrolled into the study at the pre-diagnostic stage (i.e., before prostate biopsy), 30 men have been diagnosed with PCa and have undergone radical prostatectomy. From these 30 patients, we have obtained benign and malignant prostate tissue specimens. We are beginning the process of isolating RNA from these prostatectomy specimens and will perform the same analyses from Specific Aims 1 and 2.
- Specific Aim 3, Major Tasks 4: 3D-organoid culture systems have been established to support long-term expansion of primary tissues of interest to serve as models for mechanistic studies. In effect, organoid cultures can accurately replace mouse models as cells are in a native 3D environment while at the same time allowing for more efficient genetic manipulation. Thus, we sought to establish organoid cultures from mouse prostate following Clevers et al. protocol (Clevers et al., Nature 2016). First, we sacrificed wild-type mice and dissected out the prostate gland. Following Clevers et al. protocol, the prostate tissue was minced/digested and taken through a series of steps to isolate prostate cells. A special growth media was concocted according to the Nature protocol and the cells were plated in Matrigel. Successful organoid cultures were established and passaged. With proof of principle that prostate organoid cultures could

be established in our laboratory, we will soon attempt to establish human prostate organoid cultures from benign and malignant prostatectomy tissue samples obtained for Specific Aim 3, tasks 1-3.

▪ **What opportunities for training and professional development has the project provided?**

- I continue to attend weekly lab meetings with Dr. Wang's group to discuss progress of my project as well as offer input into and derive insight from other related projects currently ongoing in the laboratory. Once a month, at minimum, I meet with Dr. Wang to discuss my progress and to plot future directions for the research as well as consider the type and timing of future grant applications. I also attend the Prostate Cancer Working Group Seminar Series and Journal Club, a monthly meeting of local prostate cancer researchers sponsored by the Cleveland Clinic. At this meeting, prostate cancer research scientists, both local and national, are invited to present their innovative work in the field, share their ideas, and discuss collaborative projects. I am also a member of the MetroHealth Cancer Center Oncology Research Committee, a group which meets monthly to discuss ongoing and prospective clinical trials at my institution. I am responsible for reviewing the trials related to urologic malignancies, include prostate, bladder, and kidney cancer. Since the last report, I have started a Prostate Cancer support and survivorship program for our patients at MetroHealth. I have also continued to give educational talks to the community and local colleges about prostate cancer and racial disparities.

▪ **How were the results disseminated to communities of interest?**

- Nothing to report.

▪ **What do you plan to do during the next reporting period to accomplish the goals?**

- The goals during the next reporting period include completion of gene expression analysis of the remainder of the 107 prostate biopsy samples as well as any additional samples that are collected during that time. We will also turn our attention to performing gene expression analysis on prostatectomy tissues and correlate this with that of the biopsy data. Another key goal during the next reporting period will be to establish human prostate organoid cultures for genetic manipulation and analysis.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
 - Nothing to report.
- **What was the impact on other disciplines?**
 - Nothing to report.
- **What was the impact on technology transfer?**
 - Nothing to report.
- **What was the impact on society beyond science and technology?**
 - Nothing to report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
 - Because we were initially having issues with isolating RNA from the prostate cores of sufficient quantity and quality, we had started a parallel project where we hoped to use the organoid cell culture technique to expand our genetic material pool. As described above, this has yielded positive results and we anticipate transitioning to human prostate organoid cell cultures in the next few months.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
 - As noted in last year's report, the study was suspended for the first quarter of 2016 due mainly to my use of the wrong version of the informed consent document (I was using consent forms that had the correct content but did not have the IRB's stamp of approval and expiration). I also failed to have patients initial the section of the consent form asking their preference regarding use of their tissue samples in future studies. Under IRB guidance and re-training, I have corrected the deficiencies in my consenting process and now also utilize a research study coordinator to help with the consenting process and ensure adherence to the regulations. I am now in the process of completing the reconsenting for the first 65 patients that were incorrectly consented.
- **Changes that had a significant impact on expenditures**
 - Nothing to report
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
 - Nothing to report
- **Significant changes in use or care of human subjects**

- Nothing to report

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
 - Nothing to report
- **Books or other non-periodical, one-time publications.**
 - Nothing to report
- **Other publications, conference papers, and presentations.**
 - Nothing to report
- **Website(s) or other Internet site(s)**
 - Nothing to report
- **Technologies or techniques**
 - Nothing to report
- **Inventions, patent applications, and/or licenses**
 - Nothing to report
- **Other Products**
 - Nothing to report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Carvell Nguyen
Project Role:	Principal investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	Dr. Nguyen has overseen the project and has been involved in the following facets of the project: patient recruitment, establishing and maintaining patient databases, molecular assays, data analysis, planning and performance of prostate biopsy, performance of radical prostatectomy, and patient follow-up.

Funding Support:	
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Name:	Bing-Cheng Wang
Project Role:	Co-investigator/mentor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.2
Contribution to Project:	Dr. Wang has provided scientific expertise, logistical support, and project feedback to the PI.
Funding Support:	NIH/NCI

Name:	Albert Kim
Project Role:	Research assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Dr Kim took over from Ji Zheng and has been responsible for much of the benchwork, including RNA purification, RT-PCR analysis, and processing and storage of the tissue specimens.
Funding Support:	

Name:	Cindy Newman
Project Role:	Research coordinator

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Ms.Newman has helped the PI with the clinical and logistical aspects of the project, including patient recruitment and consenting, transport of tissue samples between OR and the laboratory, and data collection/entry.
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

- Nothing to report

- **What other organizations were involved as partners?**

- Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**

- Nothing to report

9. **APPENDICES:**

- Nothing to report