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Award Number: DAMD17-99-1-9046

TITLE: Amplification of the Endothelin A Receptor Gene: A Potential Molecular Biomarker of Aggressive Prostate Cancer in African Americans

PRINCIPAL INVESTIGATOR: Joel B. Nelson, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21287-2411

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TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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______________________________
Joel Nelson, M.D. 5/4/00
Observations in our laboratory support the hypothesis that the endothelin axis is important in prostate carcinogenesis and progression, and that this pathway may be uniquely active in African American (AA) men, by virtue of potential amplification and overexpression of the endothelin receptor subtype A (ET\textsubscript{A}). It is our hypothesis that the ET\textsubscript{A} receptor is amplified in this patient population, and this contributes to a more aggressive disease course. In preliminary studies, Southern blot analysis of tumor DNA obtained from 5 African-American individuals was performed, using an ET\textsubscript{A} cDNA probe: in every case, concentrations of ET\textsubscript{A} DNA were greater in the tumor tissue (lymph node metastasis) compared to the corresponding normal tissue. The aims of this study were to determine whether the ET\textsubscript{A} gene is amplified and differentially expressed in PCA from AA men, and if increased ET\textsubscript{A} expression results in a more aggressive clinical course.

We found evidence for increased ET\textsubscript{A} DNA in 20% of the samples studied, but we did not find convincing evidence for increased ET\textsubscript{A} protein expression, using two complementary techniques. It is certainly possible a larger data set would include clear cases of ET\textsubscript{A} overexpression at the DNA, RNA and protein levels.
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[Signature]

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**Introduction:**

Observations in our laboratory support the hypothesis that the endothelin axis is important in prostate carcinogenesis and progression, and that this pathway may be uniquely active in African American (AA) men, by virtue of potential amplification and overexpression of the endothelin receptor subtype A (ET\textsubscript{A}). It is our hypothesis that the ET\textsubscript{A} receptor is amplified in this patient population, and this contributes to a more aggressive disease course. In preliminary studies, Southern blot analysis of tumor DNA obtained from 5 African-American individuals was performed, using an ET\textsubscript{A} cDNA probe: in every case, concentrations of ET\textsubscript{A} DNA were greater in the tumor tissue (lymph node metastasis) compared to the corresponding normal tissue. The aims of this study were to determine whether the ET\textsubscript{A} gene is amplified and differentially expressed in PCA from AA men, and if increased ET\textsubscript{A} expression results in a more aggressive clinical course.

**Body:**

**Phase 1: Acquisition of Samples and Reagents (month 1)**

In the initial phase of this project, we identified 10 normal/tumor pair samples from the prostate cancer tissue bank at the Brady Urological Institute for analysis. It was decided to first study a rather small and manageable set of samples to work out the details of the proposed analysis. Frozen sections (n=30) were obtained from each sample for immunohistochemistry and autoradiography. Additional sections were cut from the frozen tissue for DNA isolation. Genomic DNA was extracted using standard techniques. It was decided to not purchase an ET\textsubscript{A} BAC clone until a tumor demonstrating ET\textsubscript{A} amplification and overexpression was detected as a positive control.
**Phase 2: Preliminary Experimentation Studying each tissue sample at multiple levels for \(ET_A\) expression (month 2-5)**

As stated above, FISH analysis was not to be performed until a positive control, an \(ET_A\) over expressing tumor, could be identified.

Southern blot analysis was performed using the isolated genomic DNA extracted from normal/tumor pairs, digested with MspI, equally loaded and resolved on agarose gels. Following transfer to membranes, the blots were probed with an \(ET_A\) cDNA probe. We found evidence of increase \(ET_A\) DNA in two of the samples compared to their corresponding controls.

In stead of performing in situ hybridization, we acquired an \(ET_A\)-specific rabbit polyclonal antibody which was applied to each of the normal tumor pairs. In parallel to the \(ET_A\) staining, we studied expression of the other endothelin receptor, \(ET_B\). Negative controls included omission of the antibody, nonspecific rabbit IgG, and preimmune rabbit serum. Positive controls included the media of blood vessels. As expected, we found expression of \(ET_A\) predominantly in the stromal component of the prostate gland with little to no expression in most of the benign epithelium. Areas of prostate cancer were also generally negative, although some \(ET_A\) staining was observed in areas of prostatic intra epithelial neoplasia (PIN). Interestingly, higher \(ET_B\) was noted in basal cells lining benign glands, but \(ET_B\) expression was lost in prostate cancer. Unfortunately, we also observed positive staining using preimmune rabbit serum in many of the areas read as positive (in the stromal component). At the time of this writing, we are adjusting antibody concentrations to limit this background.

On each section, autoradiography was also performed. Several attempts were required to obtain autoradiographs directly in the slides which were also stained with haematoxylon. Frozen
sections (20 μM) are incubated in one of the following solutions: total ET-1 binding (0.1 nM. 125I-ET-1(label)), nonspecific binding (label + 1 μM. ET-1), total ETb binding (label + 1 μM. BQ-123 or A-127722) and total ETA binding (label + 0.1 μM. Sarafotoxin S6C or 1 μM. BQ-788).

Rather than use the low resolution method previously described, we decided to use a high resolution emulsion technique. Similar to the findings using immunohistochemistry, we found the highest ETA binding in areas of stroma: blood vessels provided a reliable internal control. We also observed ETA binding in areas resembling PIN. We did not find increased ETA binding in areas of infiltrating prostate cancer, even in the samples with increased ETA DNA from the Southern blot analysis.

In summary, although we found evidence for increased ETA DNA in 20% of the samples studied, we did not find convincing evidence for increased ETA protein expression, using two complementary techniques. It is certainly possible a larger data set would include clear cases of ETA overexpression at the DNA, RNA and protein levels. We found the amount of work required to fully analyze these 10 samples took nearly the entire time allotted. Despite an absence of data to support the initial hypothesis, we believe further investigation of ETA expression in prostate cancer is warranted.

Phase 3: Data Analysis and Development of Grant Proposal Aims and Experiments (Month 6)

During the performance of this 6 month project, the DOD announced and submission was required for proposals directed towards prostate cancer. Given our preliminary data at the time of that request, we submitted a proposal to the DOD requesting additional time and funding to complete, in part, the studies outlined in the career development award. At the time of this
writing, the funding status of that proposal is unknown.

**Key Research Accomplishments**

- Increased $E_{T_A}$ DNA was observed in 20% of normal/tumor samples studied
- There was not a corresponding increase in $E_{T_A}$ protein expression in prostate cancer using immunohistochemistry and autoradiography
- $E_{T_A}$ protein expression was increased in areas of PIN

**Reportable Outcomes**

The only reportable outcome (drawing from the list provided) is the submission of a proposal (to the DOD) to study this question in more depth, with more samples, and over a longer time period.

**Conclusions**

We found evidence for increased $E_{T_A}$ DNA in 20% of the samples studied, we did not find convincing evidence for increased $E_{T_A}$ protein expression, using two complementary techniques. It is certainly possible a larger data set would include clear cases of $E_{T_A}$ overexpression at the DNA, RNA and protein levels. We found the amount of work required to fully analyze these 10 samples took nearly the entire time allotted. Despite an absence of data to support the initial hypothesis, we believe further investigation of $E_{T_A}$ expression in prostate cancer is warranted. Finally, the magnitude of the proposed project certainly exceeded the time allotted to such an
undertaking: put bluntly, in hindsight, this project could not have been performed in 6 months and requires a longer commitment of time and funding.

References

None

Appendices

None

Final Reports

There are no publications or meeting abstracts resulting from this preliminary project from any of the personnel receiving pay from the research effort (Joel B. Nelson, William Isaacs, Hong Zheng).
September 21, 1999

Commander
U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, MD 21702-5012

RE: Final Report for Award Number DAMD17-99-1-9046

Dear Commander:

Enclosed please find a final report for the above named award. All the information included in
this report is unpublished and distribution should be limited to the review by the Commander and
appropriate staff. None of the information contained within this report should be made public.

Thank you for your patience and support.

Sincerely,

Joel B. Nelson, M.D.

Enclosures
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FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for Information Management