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TITLE: Pilot Comparison of Stromal Gene Expression among Normal Prostate Tissues and Primary Prostate Cancer Tissues in White and Black Men

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14. ABSTRACT

This critical hypothesis development project is still underway, and we have requested a deadline extension without additional funding to allow us to complete the work. The two sources of the delay are 1) histological analysis of all tissues available for study took longer than expected but is now complete and 2) we have been working (using other sources of funding) to get a lab database ready to handle the data in way that will allow the data generated to be optimally useful for prostate cancer research. This work has taken longer than expected, but is nearly ready. In order to complete the project as soon as possible, and to extend its utility, our laboratory has entered into a collaboration with another laboratory and our goal is have a manuscript ready by Fall 2006.

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Introduction

In a one-year Exploration: Hypothesis Development project, we propose to explore the hypothesis that one can detect important differences in stromal cell RNA expression using Affymetrix U133 Plus 2 genechip technology, through laser microdissection of stromal cells from a uniquely informative set of prostate samples. We will prioritize identified gene expression differences biostatistically and biologically, and will test the validity of these differences in a large set of tissue microarrays and by real-time rtPCR.

Body

Please note that we did not have available the specific feedback from the reviewer in the revised progress report submitted in March 2006. We now have this, and we have spent three days pulling together what is needed to fully respond to it here.

We should add that additional progress has been made since February 2006 and is included in this updated report.

We want to be clear that for this hypothesis development award, no SOW was required for the submission and none was provided. The reviewer mentions an SOW and we just want to be clear that there was none.

This is hypothesis development using a hypothesis development award, and as I'm sure the CDMRP agrees, the ability to properly frame the hypothesis is **critical** to success, especially with the necessarily relatively sparse data we will be able to generate with the resources provided (using expensive but routine transcript analysis technology).

We don't want to go into the transcript analysis phase (which is also time consuming, but relatively routine) with anything but the best possible samples. Anything less than this is a waste of taxpayer's funds and will deviate from the mission of the CDMRP.

First, we would like to respond to the reviewer's critique. The reviewer stated that the following are the steps required to complete the project:

(1) prepare slides and perform stromal cell PALM laser microdissections and isolation of RNA;

(2) perform RNA amplifications;

(3) quantify RNA expression in central zone and peripheral zone stromal cells isolated from normal prostate tissue and in pericancerous stroma;

(4) perform biostatistical analysis;

(5) validate the top 10 candidate differentially expressed genes; and (6) publish the results of the study.

Steps 2-5 are time and resource-consuming but fairly routine, Step 1 is what makes this study unique and worthwhile, and unfortunately is vastly oversimplified. Here are the steps we have actually taken, and are in still working on:

Here are the Actual Steps we have taken so far and the status of each:

1. Identify and circle regions of unequivocal peripheral and central zone in available slides and blocks. This task was performed in collaboration with Dr. Angelo De Marzo, took several months, and is completed. We have formalin fixed, alcohol fixed, and frozen material from each of the normal prostates. We learned from this exercise that it is much easier to identify PZ and CZ on the formalin and alcohol-fixed tissues, and that it is difficult but not impossible with flash-frozen tissue. We also learned that we will have an easier time identifying zones if we do whole mount processing of future samples collected. We have reviewed the literature regarding transcript analysis from alcohol fixed and formalin fixed tissue as compared to frozen tissue, and we are planning to compare transcripts from alcohol and frozen tissue from microdissected material from the same case to determine what type of bias alcohol fixation may introduce, and whether it is legitimate to include both frozen and alcohol fixed tissues in the same experiment. We also learned from this exercise that in order for researchers to appropriately interpret data coming from the transcript comparison, we will need to actually define our dissection technique using anatomic references (distance from epithelium) and show what "stroma" was dissected in each case using our database technology (see next step). This is because in any zone, "stroma" varies in cellular content quite dramatically from the base of the epithelium to several microns below the basal area. In an attempt to improve our ability to differentiate regions of stroma, we experimented and performed several different special stains of sections of the normal tissues (Movat, Alcian Blue, Alcian Blue with hyaluronidase, and copper), and found that Movat Stain provides far superior distinction between the epithelial and stromal compartment compared to H&E, and very clear distinction between various elements of the stroma. The above work constitutes critical data collected by the project so far, all of which is requied for the project to succeed. Images of the same CZ area with H&E and Movat's stain taken expressly for this report (to exhibit an example of these data produced so far) is provided in Figure 1.

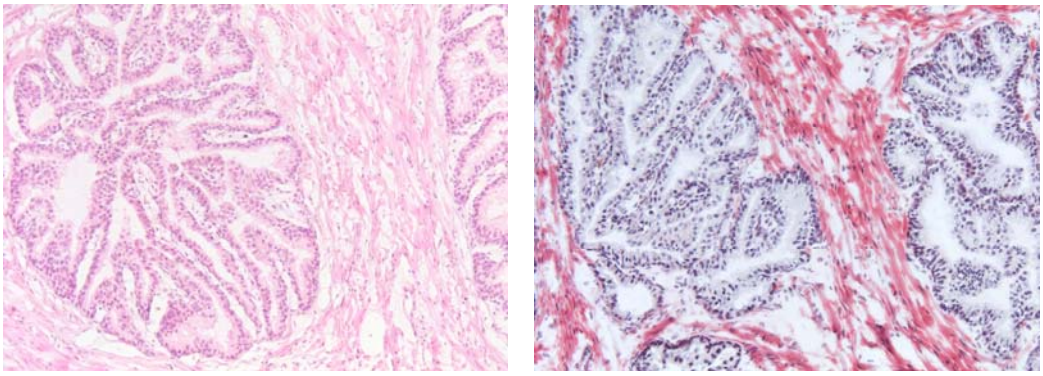

Fig. 1 10x images comparing H&E (left) and Movat's Staining of the same block of frozen normal prostate tissue to be used for microdissection. Movat's stain provides much better contrast between epithelial and stromal compartment, to be used as guide during microdissections

2. Create support for ability of researchers accessing our data to view images of what was dissected. This work has been supported to date by other funding sources, and is critical to the successful completion of the project. The reviewer of our rejected progress report asked:

"For example, what are the details of the "sophisticated laboratory database system?" Does it have proprietary components? Does it use commercially available software? What algorithms are used? How are the data integrated? What are the key features of the "highly advanced, unique laboratory translational research database system?" How was it improved

to allow the data to be "conveniently managed from one place?" What are the "critical changes" to the database system?

Our database application is web-based, and uses a SQL Server 2000 backend, and Adobe Cold Fusion MX7 frontend (off the shelf applications). It runs only in 128-bit encrypted secure-sockets layer (SSL), the same technology used to enable secure credit card transactions on the web. It is further protected by internal database encryption, complex username and password authentication, ip address restrictions, and firewalls. The databases are backed up nightly onto tape, and tape is kept offsite. The backend table structure and frontend scripts have all been developed in my laboratory, as no publicly available off-the-shelf code exists (to our knowledge) to support the type of integration this application supports. Because the application is web-based, researchers will not need to install or use proprietary code to be gain access to the results of this project. For this project, the database application has been improved to allow images to be associated with specific slides and specific laser microdissections, and is now being modified to allow whole-slide images to be attached to specific slides and related to specific transcript assay results. We call this applications LabmatrixV1, and its key features are summarized below:

LabmatrixV1 allows researchers to track study subjects' medical and phenotype data, biomaterials, a select set of "bench" laboratory and genomic data and administrative data such as supplies and equipment. All of this data is gathered in the context of curated reference data (a hierarchical list of human cell types, for example). When fully completed, LabmatrixV1 will allow researchers to perform queries, aggregate "locked" data for analysis, and allow the creation of figures and reports directly from the locked primary data. The result is a much more convenient, secure, and powerful way to manage and promote creativity in a single lab, or multiple labs working together.

LabmatrixV1 Modules

As illustrated in Figure 1, LabmatrixV1 is composed of an integrated set of Primary Modules supporting key phenotypic and molecular data capture and review, and Laboratory Support

Figure 2: Logon page to Laboratory Database System critical to success of project. Areas in Biomaterials and Images have been modified for this project

Modules allowing custom configuration and supporting key scientific metadata. The main modules are presented below.

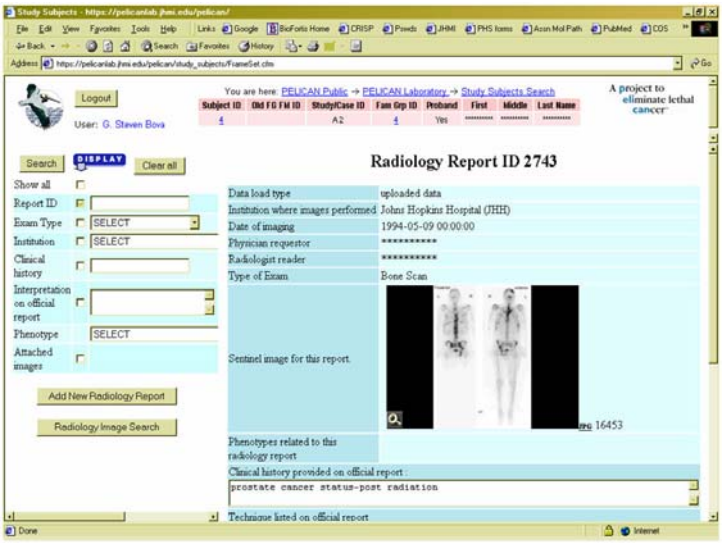

Figure 3: Screen from Study Subjects portion of application, critical to identifying and recording appropriate phenotypes.

Study Subjects. In the Study Subjects module all types of clinical, family history, pedigree, and subject phenotype data are recorded and searched. LabmatrixV1 users can link subjects and specific phenotypic data to disease terms directly from the UMLS (Unified Medical Language System), licensed from the National Library of Medicine. Users can collect, view and manipulate all types of clinical data and reports, including radiology images. Users can track consent forms, authorizations, contacts, build custom surveys, and to-do lists for each subject, with appropriate standard data references (e.g. Ancestry terms, relationship to proband terms, etc). Survey questions, for example, are drawn from a library of questions within a curated reference database. Security and confidentiality are maintained through complex password, role-based access,user-customized ip (internet protocol) address based restrictions, and system features such as use of secure sockets layer for all data transmissions.

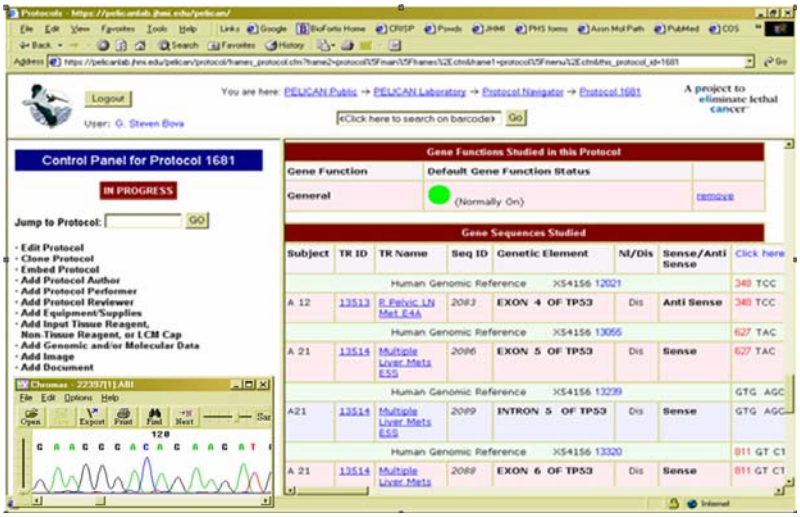

Figure 4: Sample Genomic Sequencing Record from Protocols module. This section will be used to record RNA isolation, amplification, and Affymetrix data when generated.

Biomaterials The Biomaterials module is where critical information on tissue samples, tissue microarrays, body fluids, cell lines, DNA, RNA, and protein samples are managed in relation to the protocols used to create them and their physical location in refrigerators and freezers. Samples are organized according to their anatomic and cell type source using a reference hierarchy, and maintained using barcoding technology. Critical changes have been made to this portion of the database application to allow the project to go forward. This has been done up til now with funds from other projects.

Protocols Protocols is a hierarchically-organized workflow module designed to manage the day-to-day bench activities of the laboratory. It is an easy-to-use, powerful, and flexible method for recording the input and output of specific work in the laboratory, such as the isolation of new DNA samples from tissues or body fluids, or the generation of sequence, comparative genomic hybridization, cDNA microarray, or immunostaining data. Protocols

are simple to create through a user-friendly interface, and serve as a record of today's lab activity as well as a reference source for general methods used in the lab. Protocols provides the user with a convenient place to easily record methods and results (including images) accurately in real time, eliminating the need for time-consuming and error-prone rewriting of methods and results when a series of experiments is complete and ready for analysis and publication. This area has been used to support other types of transcription data in the past, and will be used for transcription data from the current study.

Workflow-Based Data Review: Functional Genomic Status Summarization As currently configured, any user-created LabmatrixV1 protocol wherein new Study Subject-based molecular data is obtained is required to go through a semi-automated review process. Users listed as reviewers (reviewer 1, reviewer 2, etc) are notified on the LabmatrixV1 logon screen with a link to the protocol in need of review. The review function proceeds through three stages: 1) final review of accuracy and completeness of data recorded in protocol 2) automated or semi-automated review and reduction of the annotated molecular data. For example, in the case of genomic sequence, each experimentally-derived study subject sequence is compared to a user-provided genomic reference (from public genome databases currently) and the user is prompted to annotate mutations using a standard mutation annotation methods. 3) The third and final phase of review of molecular-data producing protocols is to place the newly obtained, annotated data in a novel "virtual" functional genomic context.

Scientific Query Builder (SQB) In the Scientific Query Builder, researchers construct queries of the full dataset collected in their laboratory or included in third party sources, allowing them to aggregate data that they wish to preserve and export from LabmatrixV1 for publication of individual result reports, full scientific manuscripts, or patent applications.

Equipment and Supplies/Contacts The Equipment and Supplies module provides the ability to efficiently produce and maintain inventories of available equipment and supplies, facilitated by user-customizable lists of known equipment, supplies and reagents. It also allows laboratories to track costs, locations, expiration dates, product details and service contracts for individual items.

Images/ConfigurationTools The Images module provides a separate search engine for images in the various LabmatrixV1 modules. This includes radiology images uploaded through Study Subjects, laser capture micro-dissection and other histology images recorded in Biomaterials, and gel images recorded in Protocols.

Laboratory Administrator The Laboratory Administrator sub-module allows the laboratory's Administrator to add new LabmatrixV1 users within the laboratory, to conveniently administer role-based access to specific modules and submodules within the application, and to create and change the encryption key for the laboratory.

To allow access to study data including images and transcript profiles, a study web page will be built which will allow read only access to appropriate areas of the above application. This page will be built when the transcript profiles have been generated.

3. We performed test dissections using Arcturus and PALM technology, and decided that the more recent Arcturus Veritas technology suits the needs of this study best. We also found that the dissections took longer than anticipated. To allow the project to be completed sooner rather than later, we have entered into a collaboration with the laboratory of Dr. Michael Emmert-Buck of the National Cancer Institute, where the dissections will be peformed. In addition to allowing the dissections to be completed sooner, this colllaboration will allow the project to be extended to include both epithelium and stroma from the same samples. Dr.

Emmert-Buck's lab will collaborate with my lab using the database application described above to complete the project. We will also explore the possibility of inclusion of appropriately controlled ethanol-fixed tissues in the study if PZ and CZ cannot be identified in all cases using frozen tissue alone.

4. After the dissections are performed, RNA will be isolated, and transcript analysis will be performed by routine Affymetrix assay, biostatistical analysis and validation will be performed, and results will be submitted for publication.

Key Research Accomplishments

-Identified regions to be dissected
-Prepared database application to accept data from study
-Re-evaluated microdissection technologies available
-Entered into collaboration to complete study sooner and to make study more relevant by including epithelium

Reportable Outcomes: None so far. Project is still underway.

Conclusions: The project was delayed to allow necessary detailed, time-consuming analysis of tissues to be dissected and to allow database application to be prepared to handle data from project so that these data can be made available over the web upon completion of the project. We are working as hard as we can with resources available to complete the project as soon as possible. Our goal is to have a manuscript ready by Fall 2006.

References: None so far

Appendices: None