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14. ABSTRACT We have undertaken a proteomic approach to identify proteins altered in both cancer and benign cells near the cancers. This study takes advantage of the "field effect" of the cancer, the malignancy associated changes in normal cells to discover cancer altered protein that could be more abundant in serum or urine since they would come from both benign and cancer cells. We have set optimal conditions for laser capture microdissection (LCM) of prostate epithelia and extraction of protein from them for the mass spectroscopic (MS) analysis of resultant peptides and protein identification through available soft ware programs. We changed the control approach to our study since cystoprostatectomy prostate tissues did not become available, but we were given cross sections of prostates. Whereas we originally had planned to use a pool of cystoprostatectomy prostate epithelia as a control in each iTRAQ™ run, we now are able to have controls internal to the individual prostate specimen; i.e., benign distant to cancer and benign prostatic hyperplastic (BPH) tissues vs cancer and benign near cancer tissues. In addition, use of new state of the art equipment in the Mass Spectroscopy and Proteomics Core Facility of the University of the Minnesota and implementation of the 8iTRAQ™ labeling method will increase the data acquisition capability of our studies.					
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Introduction:

Two major challenges in treatment of prostate cancer (CaP) are the lack of specificity in diagnosis using prostate specific antigen (PSA) and inability to distinguish aggressive and latent prostate cancers in biopsies. Success in addressing these needs is dependent upon discovery of new biomarkers that are specific for diagnosis of cancer and classification of the tumor. The search for new biomarkers has been extensive and although a number of promising proteins have been identified, they have not yet proven acceptable. We have undertaken a different focus in this search, and that is a proteomic approach to identify proteins altered in both cancer and benign glands near the cancers. This is to take advantage of the “field effect” of the cancer, the malignancy associated changes in normal cells, to discover cancer altered proteins that could be more abundant in serum or urine since they would come from both benign and cancer cells, and/or be Gleason grade related. Our study design is that we collect prostate tissues (cross section of the gland) from men undergoing radical prostatectomy in which we select areas that are cancer, benign close to the cancer, benign distant to the cancer, and benign prostatic hyperplastic (BPH). Frozen sections from these specific areas, or laser capture microdissected epithelia from them, are extracted, cleaved with trypsin, differentially iTRAQ™ labeled, subjected to two dimensional liquid chromatography (2DLC), and peptides identified through mass spectroscopy (MS). There are 8 different iTRAQ™ labels within a 2DLC-MS run (can label 8 different tissue area samples), and the ratios of the iTRAQ™ labels for a given protein will allow us to determine if that protein is greater in cancer or cancer and benign near vs benign distant and/or BPH. Thus, through this approach we expect to identify proteins elevated in cancer and benign glands near cancer that would warrant further analysis as cancer biomarkers.

Body:

With the initiation of funding 15/05/2007, we contacted the Pathology Service of the Minneapolis VA Medical Center (MVAMC) to begin collecting deidentified prostate tissue samples from prostatectomy specimens (for Tasks 1 and 2, and Task 3, item 1). A total of 16 prostatectomy specimens, 9 of which contained cancer, were collected during the year 15/05/2007 and 14/05/2008. Ms Konjit Betre, a person with experience in the histology and immunohistochemistry of human prostate, was hired at 50% effort for this project. Ms Betre or Dr. Wilson collect prostate specimens from Surgical Pathology, cut them into pieces appropriate for cryostat sectioning, make a diagram of the prostate cross section and location of each tissue piece, and then freeze the pieces at -70 °C on dry ice. Ms Betre cuts frozen sections, stains them with hematoxylin and eosin, and she and Dr. Wilson review them with Dr. Stephen Ewing, Chief of Pathology Service at the MVAMC. Areas of cancer and their Gleason grade, high grade prostatic intraepithelial neoplasia (PIN), BPH, and other histologic characteristics are noted and recorded.

Our initial goal had been to collect tissues from cystoprostatectomy specimens for establishing laser capture microdissection (LCM) parameters and to use as control tissues in our studies (Tasks 1 and 2). The prostates of cystoprostatectomy specimens in the first 8 months were small and the Pathology Service would not give us tissues from them for fear of compromising their clinical evaluations. However, rather than receiving a tissue piece from the posterior region of the prostate and one more anterior to it as described in our original proposal, we were given cross sections of prostates. This permitted us to examine areas of cancer, benign glands near the cancer, benign glands distant to the cancer (often on opposite side of the prostate), and benign prostate hyperplasia (BPH) in the same patient specimen. This then permitted us to have benign distant and BPH as control tissues within the same prostate for comparison to proteins from cancer and benign near cancer areas, which is a decided advantage over our initial approach which was to use a pool of cytoprostatectomy tissue in each iTRAQ™ 2DLC-MS run as a control. This changes our use of control tissue extracts as listed in Task 3, item 2. Note that our expectations when writing the proposal was that we would get small pieces of tissue from two areas within the prostate and LCM would be the only practical approach to collect cancer and benign near tissue epithelia. This change allows us to use frozen sections from each of these tissue areas for extraction for proteomic analysis and for comparison to LCM of epithelia (much more time consuming) from the same areas. The former gives a more comprehensive (epithelia plus stroma) and speedy examination of the tumor field effect, whereas the latter a more epithelium specific analysis.

The control studies out lined in Task 1 were undertaken with the first tissue specimen available. The thickness of frozen sections and the effect of the Arcturus stain were examined with respect to quality of LCM isolated

tissues.⁵ One persistent problem with benign glands was attachment of strands of apparent connective tissue from the stroma with the epithelia when the LCM capture caps were lifted from the slide. Use of frozen sections at 7 micron thickness minimized this, but did not totally eliminate it. Different means of extracting proteins from the LCM captured cells on the caps, as well as from total frozen sections, were compared. It was determined that extraction with a triethylammonium, bicarbonate, SDS (sodium dodecylsulfate), Triton X-100, and protease inhibitor buffer gave a similar yield of protein as PPS Silent Surfactant (3-[3-(1,1-bisalkyloxyethyl)pyridin-1-yl]propoane-1-sulfonate); the former was chosen because it was much less expensive and was not time sensitive as PPS Silent Surfactant (must be used within 12 hrs).

The lack of availability of cystoprostatectomy prostate tissue samples has also affected our progress in Task 2. Specifically, the comparison of proteins in the secretion with the epithelium is problematic. The secretion tends to contract to the epithelium when the frozen section dries on the slides as evidenced in hematoxylin and eosin staining. Although information on proteins in prostate secretion and of normal prostate function is desirable, it is not the major thrust of this proposal. The variability in protein expression of benign tissues (Task 2, item 1) can now be directly obtained through the introduction of the 8 iTRAQ sytem (see below). The protein composition of prostate secretion (Task 2, item2) can be obtained by proteomic study of expressed prostate secretions of men without evidence of cancer (a goal of a different project in our laboratory).

Two developments that would considerably improve the technical aspect or our project were to occur in the first 6 months of our project and we delayed initiation of MS analysis of samples. These were the introduction of new state of the art equipment in the Mass Spectroscopy and Proteomics core facility at the University of Minnesota and the availability of the 8iTRAQTM labeling system (in contrast to the 4iTRAQTM labeling system used previously by us and included in the original proposal). The former utilizes a Tempo LCMALDI powered by Eksigent from Applied Biosystems/MDS SCIEX to apply fraction drops from a C-18 reverse phase column to MALDI targets (384 or more fractions) and analyze them in a 4800 TOF/TOF Analyzer (TOF, time of flight). This system has greater sensitivity (requires less sample) and faster per sample run than the Applied Biosystems Q-Star MS available when we wrote the original proposal. This meant we would need less LCM captured cells per sample, which is important because LCM is so very labor intensive. The latter meant we could compare 8 different samples in the same MS experiment, i.e., 4 prostate tissue areas from 2 patients in the same run. The 4800 TOF/TOF Analyzer and the 8 iTRAQ labeling method were to be operational for us in October, 2007, and after several delays for technical reasons, they became available to us in April, 2008. However, this delay in the MS analysis of samples was less problematic because it coincided with an extended personal leave of Ms Betre.

A delay in progress towards our goals came about because it became necessary for Ms Betre to return to Ethiopia, her native country, because of family related issues. Her leave of absence was initially to be 5 weeks from the middle of January, but due to delays in the legal system there, her stay was unexpectedly extended incrementally to 3 months.

The first experiment of Task 3, item 2, examining proteins expressed in cancer, benign adjacent, benign distant, and control pooled cystoprostatectomy sample was initiated with identification and preparation of frozen sections for analysis. MS protein identification data are not available at the time of this report. The control in the experiments as indicated above is to use benign distant and BPH tissue areas as control samples.

Key Research Accomplishments:

- Collection of prostatectomy tissue specimens.
- Establishment of number of cells for LCM preparation of iTRAQ MS proteomic study.
- Preparation of frozen section tissue extracts for use in the new advanced technical MS and 8iTRAQ methods for study.

Reportable Outcomes:

No abstracts, manuscripts, etc. have been prepared from data from this study at this time.

Conclusions:

We have set the conditions for optimal LCM capture of prostate epithelia. However, the nature of prostatectomy specimens we receive has changed the approach to the study; we received no cystoprostatectomy specimens, but we obtained cross sections of prostates. Whereas we originally had planned to use a pool of cystoprostatectomy prostate epithelia as a control in each 4iTRAQ run, we are now able to have controls internal to the individual prostate specimen; i.e., benign distant and BPH contrasted with cancer and benign near tissue. In addition, implementation of the 8iTRAQ labeling method will increase the data acquisition capability of the study since we can do 2 prostate specimens in the same MS run.

Appendices:

None

Supporting Data:

None