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14. ABSTRACT We have been studying a gene family termed SIBLINGs (for small integrin binding ligand N-linked glycoproteins) whose members include bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein-1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE). Our Specific Aims are to describe the distribution of serum-based measurements of SIBLINGs among (a) normal individuals, (b) individuals with benign prostatic disease, (c) individuals with clinically defined prostate cancer, and (d) longitudinal samples from individuals with prostate cancer before and after treatment; and to establish serum-based measurements which maximize sensitivity and specificity of SIBLINGs as markers for prostate cancer detection as well as for prostate cancer progression and response to treatment. Although the laboratory is still blinded to staging and progression data at this point in time, some significant observations can be made. The distribution of serum levels of BSP and DSPP suggest they have utility for prostate cancer detection. Whether used separately or as an adjunct to PSA screening, the preliminary data indicates that measurement of SIBLINGs will have a significant effect on current prostate cancer management.					
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusions.....	10
References.....	10
Appendices.....	

Introduction

Prostate cancer is the leading cancer diagnosed among men in the United States. Detection is currently based on symptom presentation, physical examination including a digital rectal exam (DRE), measuring serum levels of prostate-specific antigen (PSA) and biopsy. The DRE can not detect certain tumors (that are nonpalpable or physically inaccessible) and PSA levels are elevated in certain non-cancerous conditions (acute prostatitis and benign prostatic hyperplasia). PSA measures have a high rate of false positive test results (the PSA is elevated but no cancer is present). False positives are associated with additional medical procedures, significant financial costs and mental stress. In addition both DRE and PSA can't detect early tumors and are sometimes uninformative in terms of predicting disease progression. Biopsies performed for confirmation of abnormal test results or to follow disease progression or response to treatment can have side-effects that impact profoundly upon the quality of life.

Our hypothesis is that serum levels of a gene family we have been studying are an informative marker for prostate cancer detection and progression. Members of this gene family, termed SIBLINGs for Small Integrin Binding LIgand N-linked Glycoproteins) are induced in different cancers (1) have been shown to bind and modulate matrix metalloproteinase (MMP) activity through both the activation of the latent proenzyme and reactivation of tissue inhibitor of matrix metalloproteinase (TIMP)-inhibited MMP (2). MMPs have a well defined role in tumor angiogenesis, progression and metastasis (3). The biological activity of SIBLINGs and MMPs is consistent with a role for SIBLINGs in early tumor progression. This biological plausibility suggests that the levels of these proteins in blood may be used as not only as adjuncts to conventional detection of prostate cancer, but also as serological markers for prostate cancer progression. A confounding facet of prostate cancer is the variable nature of progression (growth rate, metastasis, etc.) and the absence of non-invasive markers that consistently track with progression. The characterization of novel serum markers whose levels may correlate with disease progression will have a profound effect on current prostate cancer management. The work has the potential to benefit individuals with prostate cancer across the spectrum from early detection to disease progression monitoring and modulating therapy. This is a pre-clinical, translational study that will lay the groundwork for future large scale clinical trials.

Body

Overview:

As of the end of the second year of this grant, Tasks 1 and 2 are almost complete. We have yet to be unblinded as to staging data for all of the samples. We have been trying to resolve issues with the stability of the immunoassay for one of the SIBLINGs, dentin matrix protein-1 (DMP1) (see below). We have also utilized commercially available tissue banks to use monoclonal antibodies against SIBLINGs to look at their expression by immunohistochemistry (see below).

Statement of Work:

The tasks outlined in the original Statement of Work were to:

Task 1. To determine the utility of serum SIBLING (BSP, OPN, DMP1 and DSPP) levels in detecting cancer of the prostate (Months 1 - 8):

- a. Using competitive ELISAs, measure the distribution of BSP, OPN, DMP1 and DSPP in 200 normal individuals free of prostate cancer.
- b. Using competitive ELISAs, measure the distribution of BSP, OPN, DMP1 and DSPP in individuals with prostate cancer.

- c. Using competitive ELISAs, measure the distribution of BSP, OPN, DMP1 and DSPP in 200 individuals with benign prostatic disease.
- d. Determine sensitivity, specificity, positive and negative predictive values as well as receiver operating characteristic (ROC) curve analyses.

Task 2. To determine the utility of serum SIBLING (BSP, OPN, DMP1 and DSPP) levels in predicting prostate cancer progression (Months 9 – 22):

- a. Using competitive ELISAs, measure BSP, OPN, DMP1 and DSPP in baseline samples from 200 prostate cancer patients with clinically characterized stage and progression state.
- b. Using competitive ELISAs, measure BSP, OPN, DMP1 and DSPP in longitudinal samples collected yearly after initial diagnosis of prostate cancer in 200 patients.
- c. Test for clinical association between serum SIBLING levels and tumor grade, stage and progression.

Task 3. To determine the utility of serum SIBLING (BSP, OPN, DMP1 and DSPP) levels in assessing response to treatment. (Months 23 - 36).

- a. Using competitive ELISAs, measure BSP, OPN, DMP1 and DSPP in longitudinal samples from 200 prostate cancer patients undergoing treatment.
Treatment: androgen-deprivation therapy (gonadotropin-releasing hormone peptide analogues) with a three year follow-up and serum samples drawn at baseline and every six months (1,400 samples total).
- b. Test for statistical association between serum SIBLING levels and prostate cancer progression after treatment.

Progress:

Study Design. Intrinsic to our study design is that the laboratory is blinded to staging and progression data on samples until all samples have been analyzed. The plan is to complete DMP1 ELSA analyses, “lock down” the raw data and results and only then will the study be unblinded.

Sample recruitment. During the first two years of the project, we have obtained a total of 220 normal serum samples and 400 serum baseline samples from subjects diagnosed with prostate cancer, 400 longitudinal samples from subjects with prostate cancer (from six month follow-up sampling), and 150 serum samples from individuals with benign prostatic disease.

Immunoassays. The laboratory has operational competitive enzyme-linked immunosorbent assays (ELISAs) for quantitatively determining the levels of bone sialoprotein (BSP), osteopontin (OPN), and dentin sialophosphoprotein (DSPP). In addition we have a competitive ELISA for a fourth SIBLING, termed MEPE (for matrix extracellular glycoprotein), but we have not utilized it for extensive analysis as the levels in sera were no different between normal donors and subjects with prostate cancer. A fifth SIBLING, termed DMP1 (for dentin matrix protein 1) has had some issues with the stability of the assay (see below). We have applied the robust and functional BSO, DSPP and OPN assays to the analysis of prostate cancer serum samples and compared the distribution to that from normal subjects. The assays have all been completed and general comparisons on the distribution of the values and receiver operator characteristic (ROC) curves determined (Figure 1). While there was overlap between the high end of normal BSP levels with the low end of the prostate cancer group, the area under the ROC curve was a significant 0.95 (panels a and b). The SIBLING DSPP continues to exhibit the greatest difference between normal sera and sera derived from prostate cancer subjects (panels c and d). Although there is a fair amount of overlap between high-end normal OPN levels and low end prostate cancer sera OPN levels, the determined AUC is 0.81 (panels e and f).

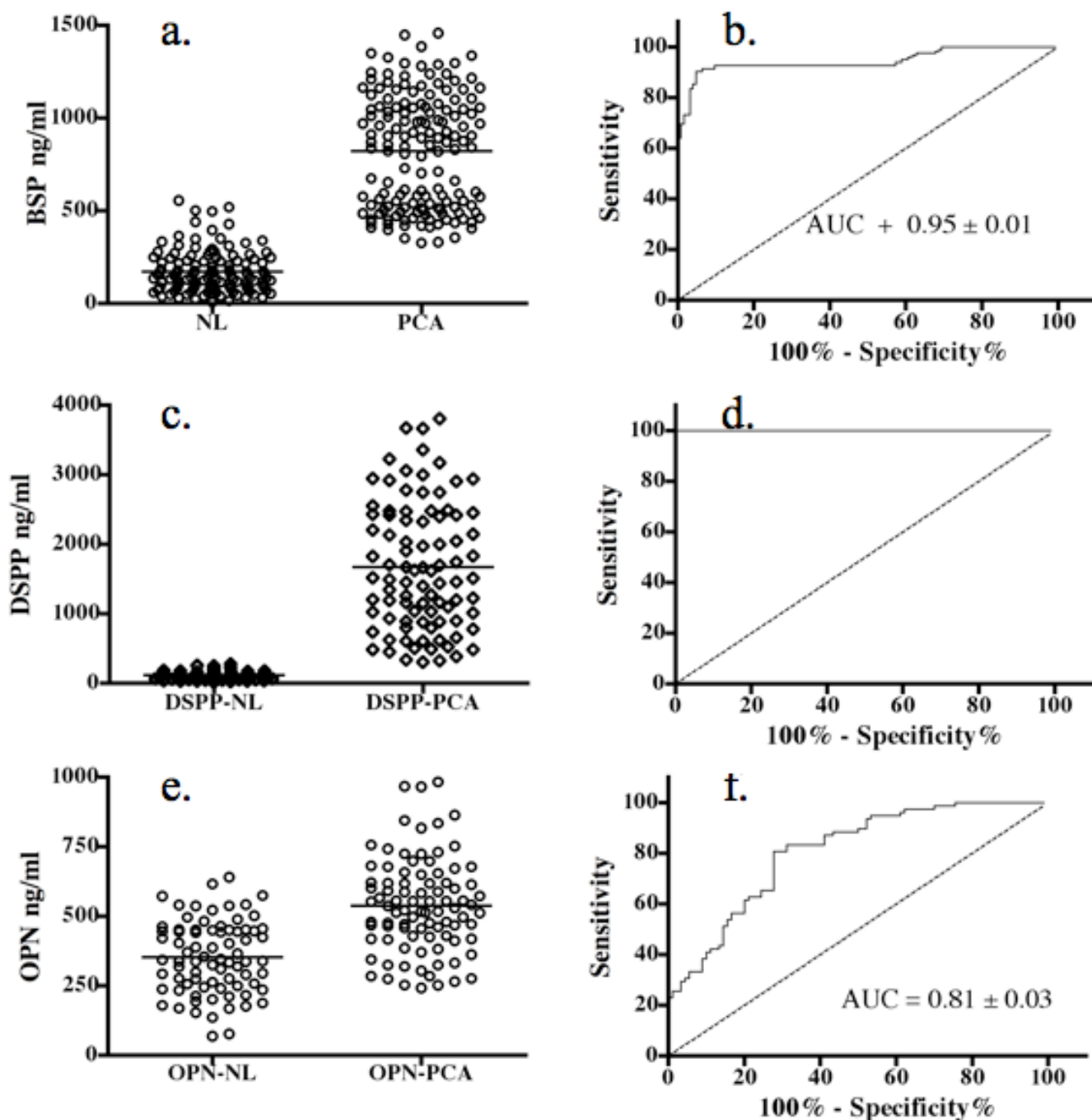


Figure 1. Serum levels of SIBLINGs in prostate cancer sera. Serum levels of (a) bone sialoprotein (BSP), (c) dentin sialophosphoprotein (DSPP) and (e) osteopontin (OPN) in samples from subjects with diagnosed prostate cancer (PCA) and normal subjects (NL) were quantified using competitive ELISAs following sample extraction and clean-up [(4)]. Receiver Operator Characteristic plots were determined for (b) BSP, (d) DSPP and (f) OPN.

Because we have been refining the ELISAs, we also undertook, in the second year of this grant, a study of the stability of the assays over time. When samples analyzed during the first year were reanalyzed a year later, the values determined were found to be highly correlated and comparable (Figure 2).

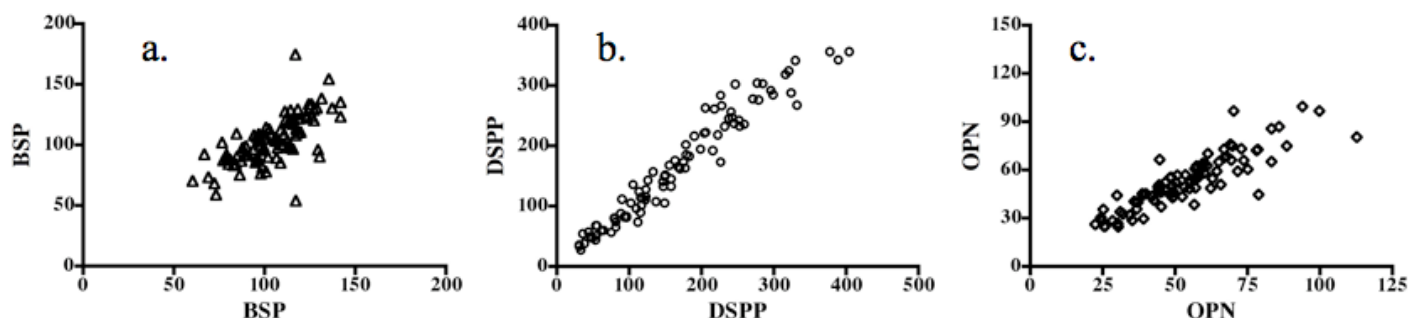
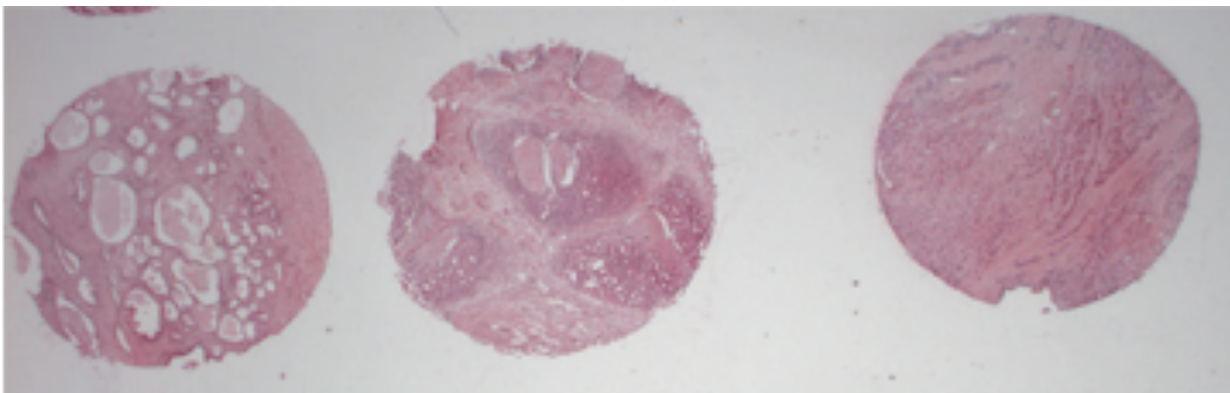


Figure 2. SIBLING ELISA stability. The same serum samples were analyzed for (a) BSP, (b) DSPP and (c) OPN by ELISA with the repeat assays run 1 year apart. The x-axis values are for the assays run during the first year of the grant and the y-axis values are for the second year. Between the first and second year analyses, a switch in 96-well plate supplier was made. The values between the two assays performed at different times are comparable and correlated, suggesting that the changes in the immunoassay mechanics did not alter the results.

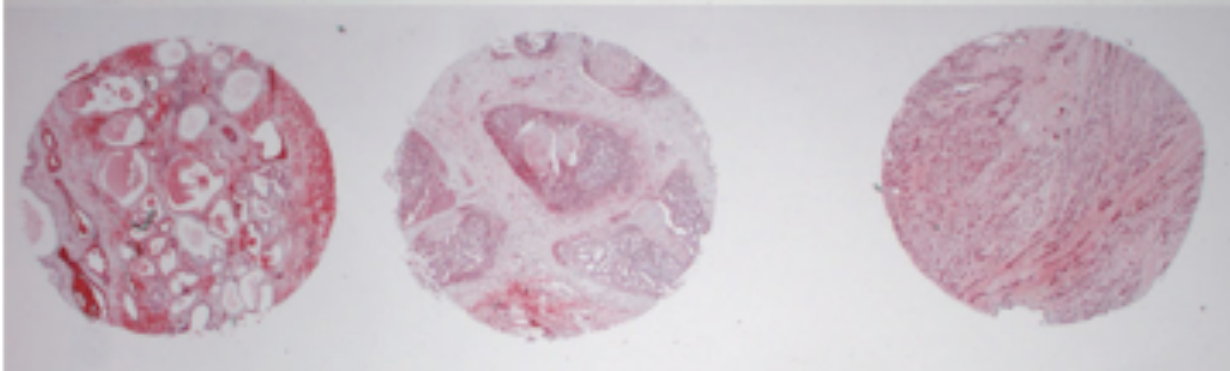
A major issue has arisen concerning the current DMP1 competitive immunoassay. The assay has not been stable since the change in coating plate chemistry (We had to switch microtiter plate manufacturer's from Greiner Bio-One high binding plates to Costar ELISA/RIA high binding plates. This was necessitated by a change in Greiner's manufacturing process that altered the surface charge/properties of their plates that adversely effected SIBLING protein binding. We rescreened a number of manufacturer's 96 well plates to obtain binding profiles and standard curves closes to those obtained with the "old" plates.) Since the change in Greiner's plate preparation methodology we have not been able to identify plates that DMP1 can reproducibly be coated on. At this point, we are pursuing the sandwich-based ELISA for DMP1 using for capture a monoclonal antibody and a polyclonal antibody (LF151) as the second half of the sandwich. While we fine tune this assay, we have delayed the 'unblinding' of the codes (staging) of the samples. We anticipate implementing and completing the DMP1 sandwich ELISAs by early 2007. We will then proceed with the analysis for associations between SIBLING levels and tumor grade, stage and progression.

We have utilized the monoclonal antibodies that we have developed against specific SIBLINGs to screen commercially available human prostate tissue biopsies. The monoclonal antibodies are LFMAb 25 for BSP, LFMAb31 for DMP1, LFMAb21 for DSPP and LFMAb14 for OPN. The positive immunohistochemical staining of the biopsies for SIBLINGs is consistent with the serum data. (Figure 3).

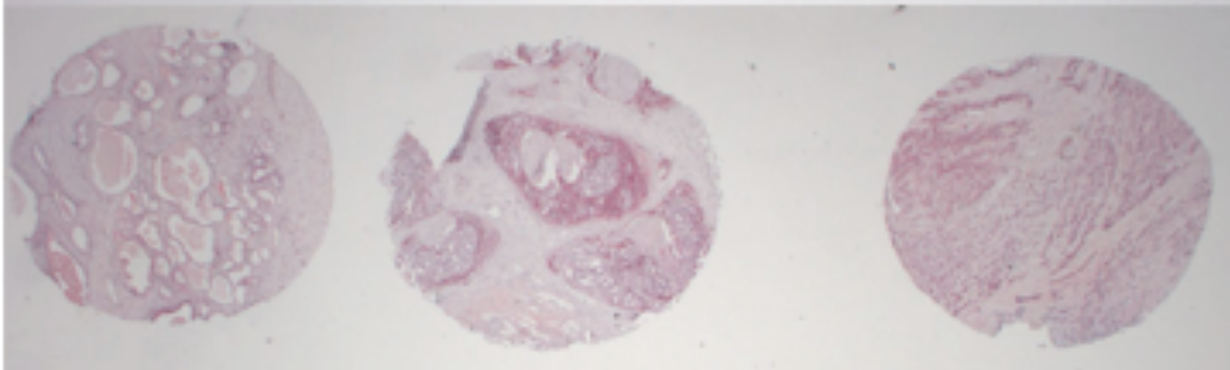
BSP



DMP



DSPP



OPN

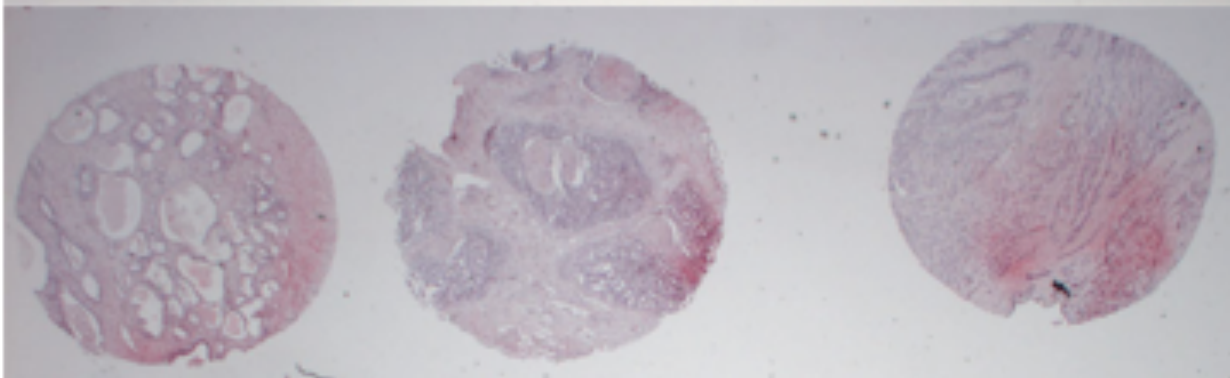


Figure 3. Serial sections of biopsies from prostate cancer subjects were immuno-reacted with monoclonal antibodies against the SIBLINGs BSP, DMP1, DSPP and OPN. The brownish-red color indicates positive immuno-reactivity. The ability of the monoclonal antibodies against SIBLINGs to work in tissue sections suggest that the use of these monoclonal antibodies in sandwich-based ELISAs will enable future parallel studies where the levels of SIBLINGs in serum is correlated with prostate tumor expression of SIBLINGs (by immunohistochemistry) and with prostate cancer disease – where sera and tissue come from the same subjects.

Key Research Accomplishments

Assay Development:

- Competitive ELISAs
completed for BSP, OPN and DSPP.
- Assay stability
Reproducible results
- Sandwich ELISA for DMP1
Stability testing underway

Assay Application:

- Competitive ELISAs of normal and prostate cancer sera completed for BSP, DSPP and OPN
1,170 samples analyzed so far.

Results of note so far:

- DSPP has highest sensitivity and specificity.

Recent data indicates that DSPP in serum is not intact, but exists as two distinct fragments that arise from proteolytic cleavage of the protein. The competitive ELISA (which uses a polyclonal antibody) against DSPP may be giving such a high discrimination (between normal and prostate cancer) because the assay measures both the N-terminal and C-terminal fragments. This raises some interesting areas for future research, including: what is the enzyme that appears to be induced in prostate cancer that cleaves the glutamic acid rich DSPP protein, what is the discriminatory power of a DSPP assay that uses monoclonal antibodies to capture and measure specific fragments, and do the intact protein and fragments have distinct biological activities?

Reportable Outcomes

- Invited Presentations:
 - “MMP activation by SIBLINGS” Gordon Research Conference on Small Integrin-Binding Proteins, September 18th, 2005 Big Sky, MT.
 - “The Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family, protease activation and tumor progression. November 18th, 2005; University of Liège, Belgium.
 - "What do bone proteins have to do with tumor progression, inflammation and wound healing?" Johns Hopkins Bayview Medical Center Research Conference, February 16th, 2006; Baltimore MD.
- Funding Received
 - National Cancer Institute, NIH; R01 CA113865; “Small Integrin-Binding Proteins and Tumor Progression.” The goal is to study the biological activity of SIBLINGS in regulated angiogenesis and metastasis in an MMP-dependent manner using both in vitro and in vivo animal model systems.

Conclusions

Significance

Prostate cancer is the leading cancer diagnosed among men in the United States. Detection is currently based on symptom presentation, physical examination including a digital rectal exam (DRE), measuring serum levels of prostate-specific antigen (PSA) and biopsy. The DRE can not detect certain tumors (that are nonpalpable or physically inaccessible) and PSA levels are elevated in certain non-cancerous conditions (acute prostatitis and benign prostatic hyperplasia). PSA measures have a high rate of false positive test results (the PSA is elevated but no cancer is present). False positives are associated with additional medical procedures, significant financial costs and mental stress. In addition both DRE and PSA can't detect early tumors and are sometimes uninformative in terms of predicting disease progression. Biopsies performed for confirmation of abnormal test results or to follow disease progression or response to treatment can have side-effects that impact profoundly upon the quality of life. If further work confirms BSP and DSPP as markers of disease progression will have a significant effect on current prostate cancer management. The work has the potential to benefit individuals with prostate cancer across the spectrum from early detection to disease progression monitoring and modulating therapy. This is a pre-clinical, translational study that will lay the groundwork for future large scale clinical trials.

Plans

Before the data and results are “locked down,” we need to complete the analysis of DMP1 levels in both normal and prostate cancer groups. We will then be unblinded to the staging and progression data and complete our analyses.

References

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