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TITLE: Prostate Cancer Progression and Serum SIBLING (Small Integrin Binding N-Linked Glycoprotein) Levels

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> 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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## **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.* The original study design requires the laboratory to be blinded to the staging and progression data on serum samples until all samples have been analyzed for the levels of the four SIBLINGs (BSP, DMP1, DSPP and OPN). As noted in the previous annual report, in parallel to the serum-based studies, we began immunohistochemical studies of the expression pattern of all four SIBLINGs in prostate cancer tumor biopsies.

*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. 67 different prostate tumor biopsies have been obtained for immunohistochemistry.

*Immunoassays.* Competitive enzyme-linked immunosorbent assays (ELISAs) for quantitatively determining the levels of bone sialoprotein (BSP), osteopontin (OPN), dentin sialophosphoprotein (DSPP), matrix extracellular phosphoglycoprotein (MEPE) are operational. Another SIBLING, termed DMP1 (for dentin matrix protein 1) has proven recalcitrant to analysis by standard competitive ELISA (see below). We have applied the robust and functional SIBLING immunoassays 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 (Table I). The SIBLING DSPP continues to exhibit the greatest difference between normal sera and sera derived from prostate cancer subjects.

Table I. SIBLING levels in normal and prostate cancer sera.

	Normal (ng/ml)	Prostate cancer (ng/ml)	ROC AUC
BSP	114 ± 63	348 ± 58	0.85
DSPP	42 ± 15	167 ± 91	0.98
MEPE	93 ± 19	80 ± 11	0.49
OPN	353 ± 130	537 ± 169	0.81

A major issue continues to be the current DMP1 competitive immunoassay. The assay has not been stable since the change in coating plate chemistry (noted in the previous progress report). Since the change in Greiner's plate preparation methodology we have not been able to identify plates that DMP1 can reproducibly be coated on. In the past year, we pursued developing sandwich-based ELISA for DMP1 using for capture a monoclonal antibody and a polyclonal antibody as the second half of the sandwich. The monoclonal antibodies that we screened did not work in the ELISA format. We have obtained antibodies against DMP1 from other researchers and are screening them for their suitability for immunoassays of serum levels. With a revised DMP1 immunoassay, we will proceed with the analysis for associations between SIBLING levels and tumor grade, stage and progression in the next year.

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. All four SIBLINGs were stained positive in the biopsies for SIBLINGs (Figure 1). This data is novel and consistent with the serum data. Curiously, DSPP (whose serum levels showed the highest elevation) did not exhibit the highest degree of immunoreactive tissue staining. A set of 67 different prostate tumor biopsies have been analyzed for SIBLING immunohistochemical staining .

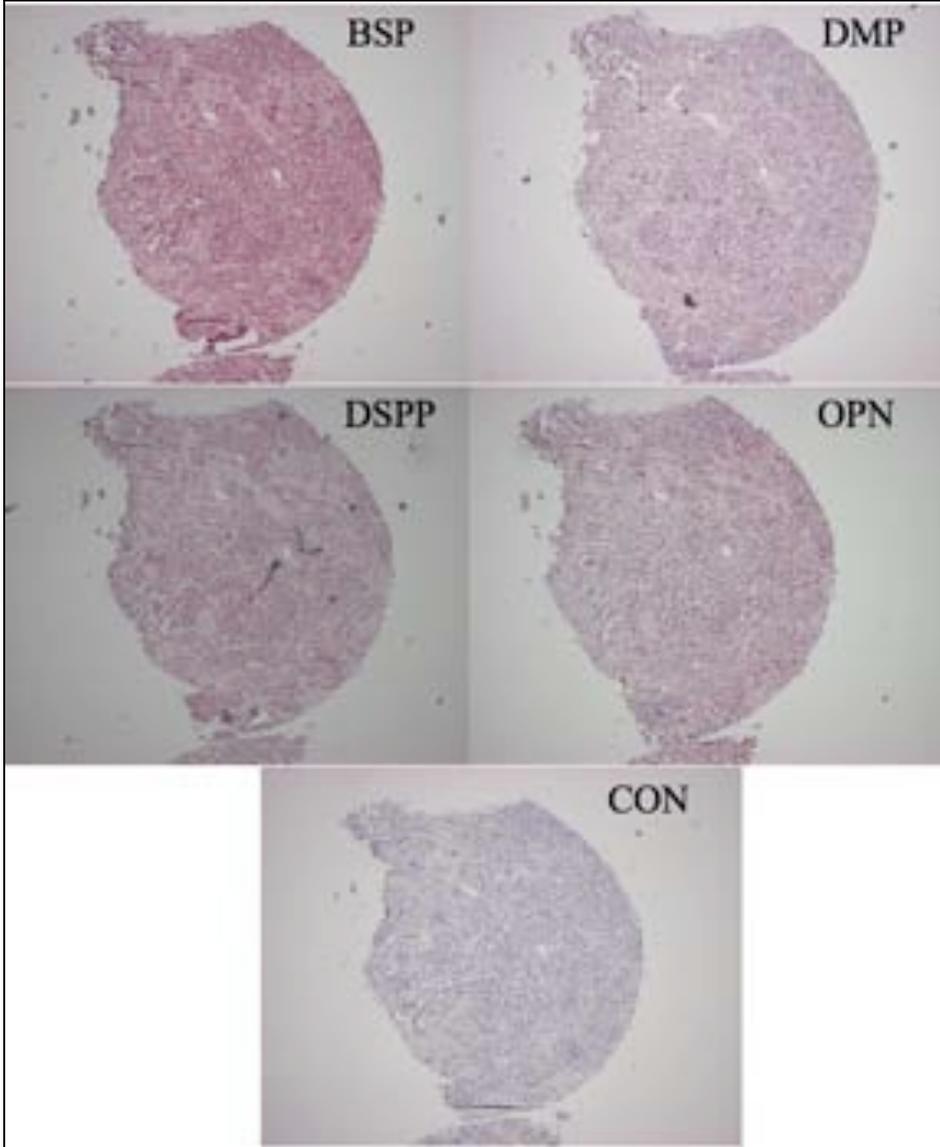


Figure 1. Serial sections of biopsies from prostate cancer subjects were immunoreacted with monoclonal antibodies against the SIBLINGs BSP, DMP1, DSPP and OPN. The pinkish-red color indicates positive immuno-reactivity. Control sections indicate the degree of background staining for the immunohistochemical assays. In the 67 biopsies analyzed, the highest degree of staining was for BSP, followed by OPN and DSPP. DMP1 exhibited string staining in a subset of biopsies. We are currently pursuing correlating the pathological characteristics of the biopsy samples with the staining pattern.

Because of the potential of DSPP serum levels to be diagnostic for prostate cancer, we have investigated the SIBLING's structural characteristics in serum. DSPP is synthesized as a chimeric protein, composed of three parts: dentin sialoprotein (DSP), dentin glycoprotein (DGP) and dentin phosphoprotein (DPP, also known as phosphophoryn). Aliquots of a large number of normal and prostate cancer sera were resolved by SDS PAGE and the resolved proteins transferred to nitrocellulose membranes which were probed with a polyclonal antibody raised against DSPP (Figure 2, below). The polyclonal antibody, which reacts to multiple epitopes across the DSPP molecule, recognizes multiple different molecular weight immunoreactive bands in prostate cancer sera, while normal sera shows no similar bands. The major bands apparent in the prostate cancer sera migrate at  $M_r$  100 kDa, 80 kDa, 65 kDa, 30 kDa, and 16 kDa.

Although we have yet to complete amino acid sequencing of these bands, the molecular mass of DSPP components have been analyzed using dentin as a tissue source (5,6). In that tissue, intact DSPP has a molecular mass of over 200 kDa. The known molecular sizes for the components of DSPP are 98 kDa (DPP), 50 kDa (DSP-DPG), 30 kDa (DSP), and 16 kDa (DGP). It may be that DSPP is processed in the local prostate tumor environment to fragments of DSP, DPP and DGP by the action of as yet unidentified proteases. The multiple bands recognized by the polyclonal antibody would also suggest why the competitive serum assay yields such elevated levels (relative to normal serum) – the ELISA detects multiple forms of DSPP.

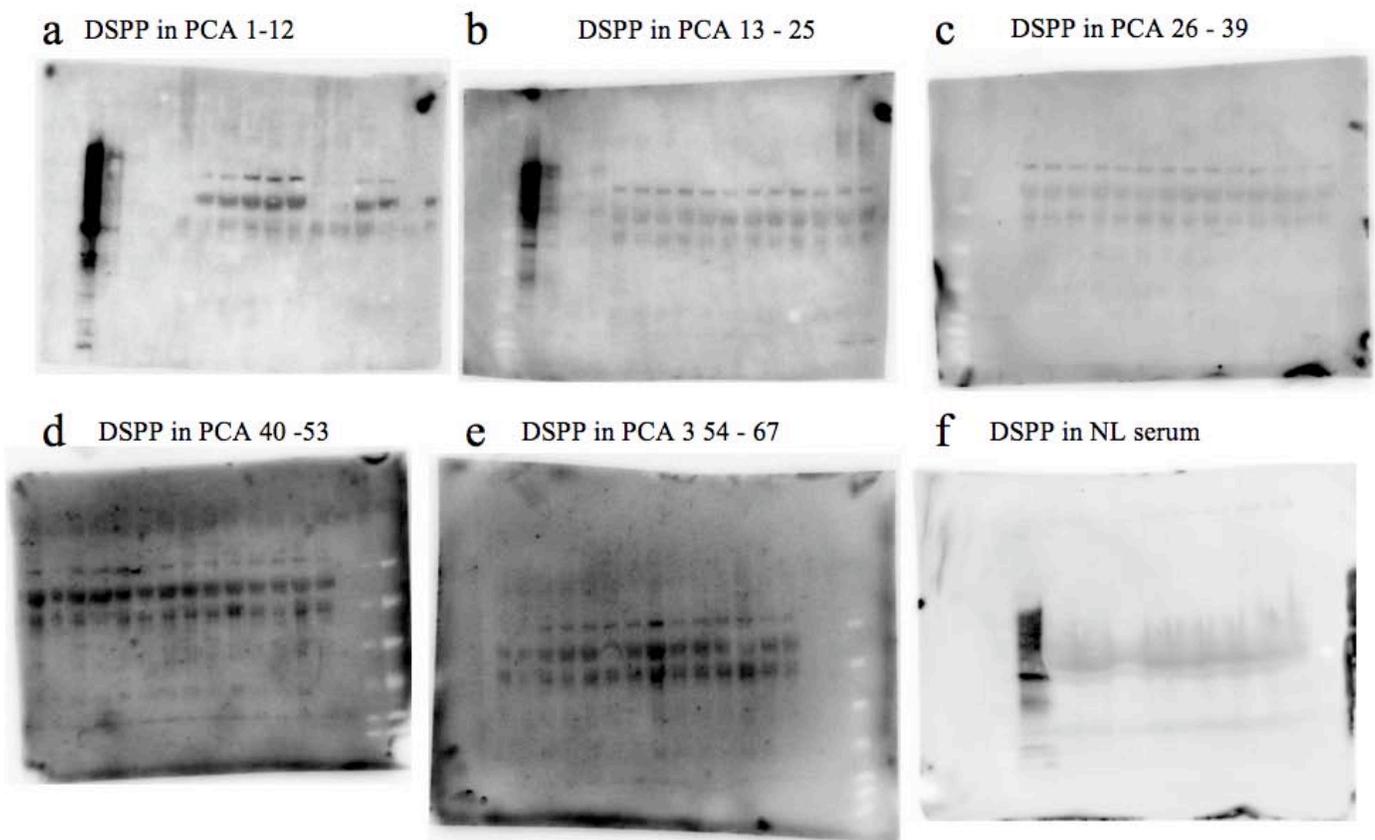


Figure 2. Western blot analysis of serum for immunoreactive DSPP. Serum samples were resolved by SDS-PAGE 4 to 20% acrylamide gradient gels following sample reduction. An anti-DSPP polyclonal antibody was employed as the primary antibody. Sera derived from 67 prostate cancer patients consistently exhibited robust staining and a three band pattern of (panels a – e) of immunoreactive material. In contrast, normal serum (panel f) stained lighter at an equivalent exposure time and did not exhibit the pattern of fragments.

We are pursuing, during this next year of our requested no cost extension, the writing of manuscripts describing the correlation studies (following unblinding of the samples). These studies include the testing for the correlation of baseline serum SIBLINGS with disease stage, for association with disease outcome, and for association with response to treatment. In addition, we will continue research to identify DSPP binding partner (its counterpart MMP) as well as identify the protease acting on DSPP in prostate cancer (that appears to not be operant in normal prostate/sera).

## Key Research Accomplishments

### Assay Development:

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

### Assay Application:

- Competitive ELISAs of normal and prostate cancer sera completed for BSP, DSPP, MEPE and OPN  
1,300 samples analyzed so far.

### Results of note so far:

- SIBLINGs show a distinct staining pattern in prostate tumor biopsies.
- Of the SIBLINGs, DSPP exhibits the highest elevation in serum derived from subjects with prostate cancer.
- DSPP did not correlate with PSA values (while BSP and OPN do).
- DSPP exists as three distinct fragments in prostate cancer sera, while in normal serum the fragments are below the limit of detection.
- 

## Reportable Outcomes

- Invited Presentations:
  - - “SIBLINGs as Serum Markers for Prostate Cancer.” Gordon Research Conference on Small Integrin-Binding Proteins, August 8th, 2007 Biddeford, ME.
    - “Small Integrin Binding Proteins as Serum Markers for Prostate Cancer Detection.” Innovative Minds in Prostate Cancer Today (IMPACT), Department of Defense Prostate Cancer Research Program. September 8, 2007, Atlanta, GA.
- Manuscripts in preparation:
  - Jain, A., Fisher, L.W., and Fedarko, N.S. SIBLING serum levels in prostate cancer.

- Kavathia, N., Spencer, M., Jain, A., and Fedarko, N.S. The SIBLING osteopontin and markers of apoptosis and immune activation in prostate cancer.

## 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*

The results so far indicate that a large scale clinical study is the next logical step, where the SIBLINGs BSP and DSPP will be targeted for measurement in a sufficiently large population.

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Small Integrin Binding Proteins as Serum Markers for Prostate Cancer Detection. Alka Jain & Neal S. Fedarko. Johns Hopkins University, Baltimore, MD, 21224

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. Both the DRE and PSA tests suffer from drawbacks, including an inability to detect early disease and are sometimes uninformative in terms of predicting disease progression. We have been studying a gene family, termed SIBLINGs for Small Integrin Binding Ligand N-linked Glycoproteins) which are normally restricted in expression to skeletal tissue, but are also induced in different cancers. At least three SIBLINGs have the capacity to bind to and modulate the activity of specific matrix metalloproteinases. Because matrix metalloproteinases play a central role in tumor growth and progression, we hypothesize that SIBLINGs may have an active role in prostate cancer development and furthermore that SIBLING levels in serum may be informative marker for prostate cancer detection. We have developed competitive ELISA tests for quantitatively determining the levels of four SIBLINGs: bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), matrix extracellular phosphoglycoprotein (MEPE), and osteopontin (OPN). We have applied the assays to the analysis of 110 prostate cancer serum samples (mean age  $63 \pm 5$  years) and compared the distribution to that from 110 normal subjects (mean age  $65 \pm 10$  years). The mean values for the normal population were  $114 \pm 63$  ng/ml for BSP,  $42 \pm 15$  ng/ml for DSPP,  $93 \pm 19$  ng/ml for MEPE, and  $353 \pm 130$  ng/ml for OPN. The mean values for the prostate cancer group were  $348 \pm 58$  ng/ml for BSP,  $167 \pm 91$  ng/ml for DSPP,  $80 \pm 11$  ng/ml for MEPE, and  $537 \pm 169$  ng/ml for OPN. Receiver operator characteristic (ROC) curves were also determined for each marker. 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 (AUC) was a significant 0.92. The SIBLING DSPP exhibit the greatest difference between normal sera and sera derived from prostate cancer subjects with an AUC of 98. Although there was a fair amount of overlap between high-end normal OPN levels and low end prostate cancer sera OPN levels, the determined AUC was 0.81. The markers with discriminatory power (BSP, DSPP and OPN) were compared to PSA values determined on the same subjects. BSP & OPN positively correlated with PSA levels, while DSPP did not.

Innovative Minds in Prostate Cancer Today (IMPACT), Department of Defense Prostate Cancer Research Program. September 8, 2007, Atlanta, GA.

Platform Presentation.