Small Integrin-Binding Proteins as Serum Markers for Prostate Cancer Detection

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Abstract Purpose: The small integrin-binding ligand N-linked glycoprotein (SIBLING) gene family includes bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), matrix extracellular phosphoglycoprotein (MEPE), and osteopontin (OPN). Previous studies have separately reported elevated expression of BSP, OPN, or DSPP in prostate tumor paraffin sections. We hypothesized that SIBLINGs may be informative serum markers for subjects with prostate cancer.

Methods: Expression levels of SIBLINGs in biopsies of normal tissue and tumors from prostate were determined by cDNA array and by immunohistochemical staining with monoclonal antibodies. Competitive ELISAs for measuring total BSP, DSPP, MEPE, and OPN were applied to a test group of 102 subjects with prostate cancer and 110 normal subjects and a validation group of 90 subjects.

Results: BSP, DMP1, DSPP, and OPN exhibited elevated mRNA expression and protein levels in biopsies. BSP, DSPP, and OPN were elevated in serum from prostate cancer subjects, with serum DSPP exhibiting the greatest difference, yielding an area under the receiver operator characteristic curve value of 0.98. Serum BSP and OPN levels were significantly elevated only in late stages, whereas DSPP was significantly elevated at all stages. Optimal serum value cutoff points derived for BSP, OPN, and DSPP were applied as a validation test to a new group of 90 subjects and DSPP yielded a sensitivity of 90% and a specificity of 100%.

Conclusion: Of the SIBLING gene family members, DSPP appears to be a strong candidate for use in serum assays for prostate cancer detection. (Clin Cancer Res 2009;15(16):5199–207)

Prostate cancer is the leading cancer diagnosed among men in the United States (1). Detection is currently based on symptom presentation, physical examination (including a digital rectal examination), measuring serum levels of prostate-specific antigen (PSA), and biopsy. PSA measures have a significant rate of false-positive test results (the PSA is elevated but no cancer is present) that often result in additional medical procedures, significant financial costs, and mental stress (2, 3). Furthermore, neither digital rectal examination nor PSA detect early tumors and they are sometimes uninformative in terms of predicting disease progression. Biopsies done for confirmation of abnormal test results, to follow disease progression, or response to treatment can have side effects that affect profoundly the quality of life (4).

We have been studying members of a gene family (termed SIBLINGs for small integrin-binding ligand N-linked glycoproteins) that are aligned head-to-tail in tandem on human chromosome 4 and share the following: an abundance of acidic amino acids; the integrin-binding tripeptide, RGD; similar post-translational modification motifs (e.g., casein kinase phosphorylation and various glycosylation events); and at least one site of controlled proteolysis (5). SIBLINGs are normally expressed in skeletal tissues as well as metabolically active ductal epithelial cells (6); however, one or more are often induced in many different cancers (7). To date, at least three SIBLINGs [bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), and osteopontin (OPN)] have been shown to bind and modulate specific matrix metalloproteinase (MMP) activities through both activation of the latent proenzyme and reactivation of tissue inhibitor of MMP–inhibited MMP (8, 9). MMPs have a well-defined role in tumor angiogenesis, progression, and metastasis (10). The biological activities of SIBLINGs and MMPs are consistent with a role for SIBLINGs in...
Translational Relevance

Prostate cancer is the leading cancer diagnosed among men in the United States. The development of informative serum marker(s) to augment prostate-specific antigen (PSA) measures would provide clinicians with greater information to enable assignment of individuals with prostate cancer to groups at different risks for cancer progression. A new marker that tracks with PSA might not be expected to yield any new information, whereas a marker with a high degree of sensitivity and specificity that tracks with disease but not PSA (such as the small integrin-binding ligand N-linked glycoprotein dentin sialophosphoprotein) might be more informative. The work has the potential to benefit individuals with prostate cancer across the spectrum from early detection to disease progression monitoring and modulating therapy.

early tumor progression. This biological plausibility suggests that the levels of these proteins in blood may be used not only as adjuncts to conventional detection of prostate cancer but also as serologic markers for prostate cancer progression (5). A confounding facet of prostate cancer is the variable nature of progression (growth rate, metastasis, etc.) and the absence of noninvasive markers that consistently track with progression. The characterization of novel serum markers with levels that correlate with disease progression has the potential to benefit individuals with prostate cancer across the spectrum: from early detection to monitoring disease progression and response to therapy.

Materials and Methods

Sample recruitment. Normal subjects (n = 110) were obtained under institutional review board–approved protocols from the Johns Hopkins Bayview Medical Center General Clinical Research Center. The Johns Hopkins Bayview Medical Center General Clinical Research Center normal group was obtained from an existing serum bank using samples from which all patient identifiers were removed. For this study, inclusion criteria as a normal serum donor included measures within the normal range for fasting glucose (<100 mg/dL), thyroid-stimulating hormone (0.5-2.1 mIU/mL), and body mass index (20-25 kg/m²) as well as a fasting for glucose (<100 mg/dL), thyroid-stimulating hormone (0.5-2.1 mIU/mL), and body mass index (20-25 kg/m²) as well as a physical assessment by a physician. Exclusionary criteria included a previous history of hypertension, heart disease, diabetes mellitus, renal or hepatic dysfunction, cancer, or any chronic inflammatory condition (e.g., rheumatoid arthritis). Prostate cancer sera (n = 102) and tissue biopsies from subjects recently diagnosed with prostate cancer, before initiation of treatment, were obtained through commercial biorepositories (East Coast Biologics, Promedex, and LifeSpan BioSciences). An additional group of 90 serum samples (30 normal and 60 with prostate cancer), with PSA values already determined, was obtained through the Department of Urology at Johns Hopkins University for assay validation testing.

SIBLING probes and prostate cancer array analysis. A cancer-profiling array (Clontech) containing normalized cDNA from tumor and corresponding normal tissues from four individual patients with prostate cancer was employed to screen for SIBLINGs as described previously (7). Briefer, several cancer-profiling arrays were hybridized in ExpressHyb hybridization solution (Stratagene) with 32P-labeled cDNA probes for specific SIBLINGs as per the manufacturer’s instructions. Washed membranes were hybridized with 32P-labeled cDNA probes for specific SIBLINGs as per the manufacturer’s instructions. Washed membranes were quantified by exposure to phosphorimager screens for up to 24 h and the exposed screen was analyzed on a Molecular Dynamics PhosphorImager (GE Healthcare Bio-Sciences) using the manufacturer’s ImageQuant program. The labeled DNA probes for human OPN and BSP were cDNA inserts released from OP-10 and B6-5g plasmids, respectively (11, 12); for human DMP1, a ~1.4-kbp coding region of exon 6 was employed (13). For the matrix extracellular phosphoglycoprotein (MEPE) probe (1.4 kbp) and dentin sialophosphoprotein (DSPP) probe (2.64 kbp), the labeled probes were the same cDNA inserts that were in making the recombinant protein (7, 14).

Antibodies. The production of monoclonal antibodies raised against specific SIBLINGs has been described previously (15) and are available from Santa Cruz Biotechnology. The polyclonal antibodies against BSP, DMP1, MEPE, and OPN used in enzyme immunoassays and Western blotting have been characterized previously. A polyclonal antibody for DSPP was developed as follows: recombinant human dentin sialoprotein (DSP) fragment of DSPP (corresponding to amino acids T132-D373) was made by PCR of this portion of exon 4 from human genomic DNA using a high-fidelity DNA polymerase and oligonucleotides that introduced an in-frame Ndel site at the 5′ end and an added stop codon plus BamHI site at the 3′ end of the PCR product. The purified PCR product was digested with Ndel and BamHI and ligated into prepared pET-15b (Novagen/EMD Biosciences) bacterial expression vector. The protein made in Escherichia coli was purified on His-Bind resin (Novagen), dialyzed against water, and freeze-dried, and four injections of ~250 μg protein each were made in rabbit LF-151 using Animal Care & Use Committee–approved protocols at an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility. Bleeds were taken every 2 weeks to produce the antisera used.

Immunohistochemistry methods. Universal block and antibody diluent were from DAKO and biotinylated anti-mouse secondary antibody (Vector BA-2000), alkaline phosphatase streptavidin Vector-AP (AK-5000), and chromogen substrate Vector Red (SK-5100) were obtained from Vector Laboratories and were employed at dilutions suggested by the manufacturer. The production of monoclonal antibodies raised against specific SIBLINGs has been described previously (15) and are available from Santa Cruz Biotechnology. The polyclonal antibodies against BSP, DMP1, MEPE, and OPN used in enzyme immunoassays and Western blotting have been characterized previously. A polyclonal antibody for DSPP was developed as follows: recombinant human dentin sialoprotein (DSP) fragment of DSPP (corresponding to amino acids T132-D373) was made by PCR of this portion of exon 4 from human genomic DNA using a high-fidelity DNA polymerase and oligonucleotides that introduced an in-frame Ndel site at the 5′ end and an added stop codon plus BamHi site at the 3′ end of the PCR product. The purified PCR product was digested with Ndel and BamHI and ligated into prepared pET-15b (Novagen/EMD Biosciences) bacterial expression vector. The protein made in Escherichia coli was purified on His-Bind resin (Novagen), dialyzed against water, and freeze-dried, and four injections of ~250 μg protein each were made in rabbit LF-151 using Animal Care & Use Committee–approved protocols at an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility. Bleeds were taken every 2 weeks to produce the antisera used.

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Fig. 1. SIBLING mRNA expression levels in normal prostate and prostate tumor biopsies. A cancer-profiling array was hybridized with 32P-labeled cDNA probes for SIBLINGs. The arrays contained samples from paired normal (NL) and prostate tumor (PCA) tissue mRNA from individual subjects. The hybridized probe for BSP, DMP1, DSPP, MEPE, and OPN was visualized by PhosphorImager (A). The image was then quantified by ImageQuant software whereupon the average relative expression levels in normal prostate (NL) and prostate tumor (PCA) were determined (B). Mean ± SD of the four samples.
manufacturer. Tissue slides were prepared from commercially available human prostate biopsies. In brief, after being fixed in formalin and embedded in paraffin wax, 4 μm sections of tissue were prepared and placed on charged microscope slides. Slides were dried in a tissue-drying oven for 45 min at 60°C. Antigen retrieval was carried out after deparaffinization and rehydration by heating slides in 0.01 mol/L sodium citrate (pH 6.0) at 99°C to 100°C for 20 min. Slides were removed from heat and let stand at room temperature in the same buffer for 20 min. Slides were rinsed in TBS, 0.05 mol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl containing 0.05% Tween 20 (TBS-T) and placed in universal protein block for 20 min at room temperature. Slides were then incubated for 45 min at room temperature with monoclonal antibodies developed against specific SIBLINGs. Antibodies LFMb25, LFMb31, LFMb21, LFMb33, and LFMb14 were used at 10, 10, 0.625, 5.0, and 2.5 μg/mL to detect the levels of BSP, DMP1, DSPP, MEPE, and OPN, respectively. After rinsing in TBS-T, the sections were treated for 30 min at room temperature with biotinylated anti-mouse secondary antibody, rinsed with TBS-T, and incubated with alkaline phosphatase–conjugated streptavidin for 30 min. After another rinse in TBS-T, color was developed for 20 min with Vector Red alkaline phosphatase substrate, a substrate system that produces a pink/red reaction product. Sections were briefly counterstained with Vector hematoxylin, dehydrated, and coverslipped. Micrographs were taken using an Axioplan2 microscope, AxioCam MRm camera using AxioVision software (Carl Zeiss Vision).

**ELISA sample preparation.** SIBLINGs present in human serum are complexed with complement factor H (16, 17). Taking advantage of the properties of SIBLINGs that they are unstructured (18) and are highly negatively charged, the binding complexes in serum can be disrupted by denaturants to enable subsequent total SIBLING levels to be measured (19). Briefly, serum samples are denatured, disulfide bonds are reduced to disrupt complexes and subjected to strong anion exchange chromatography on small disposable columns to remove proteins and reagents that interfere with antibody binding, and the eluted samples are analyzed by competitive ELISA for each total SIBLING level (19, 20). About 10% of OPN (that is presumably not bound to complement factor H) can be measured in plasma by directly assaying the sample with no prior chaotropic treatment. Appropriate plasma samples to measure this “free” OPN in a subset of 40 normal and prostate cancer patient samples were also taken for analysis by competitive ELISA.

**ELISA procedures.** Greiner high-binding plates were coated with 20 ng/mL recombinant human BSP or OPN, 10 ng/mL MEPE, or 25 ng/mL DSPPxRep [lacking the dentin phosphoprotein (DPP) repeat domain] overnight in 50 mmol/L sodium carbonate (pH 8.0). Recombinant human BSP and OPN were expressed and purified as described previously (8). Recombinant human MEPE was generated and isolated as described previously (20). hDSPPxRep lacking most of the carboxyl-terminal repetitive domain was made as described (14). Samples and standards were incubated for 2 h with shaking at room temperature with primary antibody. Primary antibodies used were a 1:200,000 dilution of LF-100 rabbit polyclonal antibody (for BSP), a 1:100,000 dilution of LF-124 rabbit polyclonal antibody (for OPN), a 1:200,000 dilution of LF-155 rabbit polyclonal antibody (for MEPE), or a 1:100,000 dilution of LF-151 rabbit polyclonal antibody (for DSPP) in TBS-T in polypropylene 96-well plates. During the 2 h incubation, antigen-coated plates were blocked with protein-free blocking buffer in TBS (Thermo Fisher Scientific). Antigen-coated plates were then rinsed three times with TBS-T and the antibody-sample solution was added to the wells. After incubation for 1 h at room temperature with shaking, the plates were washed three times with TBS-T. A secondary antibody of peroxidase-labeled goat anti-rabbit antibody conjugate (human serum adsorbed; Kierkegaard & Perry) at 1:2,000 was then added and the plates were incubated at room temperature for 1 h. After three washes with TBS-T,
substrate (3,3′,5,5′-tetramethylbenzidine microwell peroxidase substrate; BioFX Laboratories) was added, and after a 20 min incubation, the color reaction was reacted with stop solution. Absorbance was read at 450 nm and the data were analyzed using the program AssayZap (BioSoft). Commercial ELISAs to measure total PSA were obtained from ALPCO Diagnostics and the manufacturer’s protocol was followed.

**SDS-PAGE and Western blot.** Serum samples from normal and prostate cancer subjects were reduced, resolved by SDS-PAGE, and transferred to nitrocellulose membrane, which were processed for Western blotting as described previously (8). Primary antibody (LF-151 or LFMb21) was used at a 1:2,000 dilution, whereas secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase) was employed at a dilution of 1:10,000. Following removal of the secondary antibody solution, the membrane was washed and incubated with SuperSignal West Dura Substrate (Thermo Fisher Scientific). The chemiluminescent signal was captured, digitized, and analyzed using a Kodak GEL Logic 2200 Imaging System (Carestream Health).

### Results

**SIBLING mRNA expression.** Previous studies have separately reported elevated expression of BSP, OPN, or, most recently, DSPP in prostate tumor paraffin sections. In this study, the expression levels of all five SIBLINGs by prostate tumors were determined using a commercial cancer array containing normalized cDNA separately made from tumor and normal tissue obtained from the same subject. Patient cDNA array was hybridized with 32P-labeled probes for BSP, DMP1, DSPP, MEPE, and OPN, the amount of hybridized probe was quantified, and the expression levels between normal and tumor tissues were compared (Fig. 1). Each patient’s “normal” prostate tissue expressed BSP, DMP1, DSPP, and MEPE message only at very low levels. Relative to normal tissue expression, the average levels of the tumor-rich areas were elevated at least 9-fold for BSP, DMP1, and DSPP, whereas MEPE was not significantly elevated. OPN was elevated an average of only 4-fold in tumor extracts due mostly to the apparently higher level of expression of this SIBLING in each patient’s “normal” prostate tissue.

**SIBLING immunohistochemistry.** The observation of elevated SIBLING mRNA expression in array samples from subjects with prostate cancer led to the screening of SIBLING proteins in tumor tissue by immunohistochemistry. Serial sections of biopsies from five prostate cancer subjects were reacted separately.
with monoclonal antibodies against BSP, DMP1, DSPP, MEPE, and OPN. The typical results from a single patient can be seen in Fig. 2. Positive immunoreactivity, as indicated by the red color, was observed for BSP, DMP1, DSPP, and OPN. Consistent with the mRNA expression data, immunoreactive staining for MEPE was not detectable in any of the prostate cancer biopsy sections. In all tumors studied, BSP and OPN immunoreactive staining was visible in the cytoplasm of tumor cells, with diffuse staining of adjacent normal tissue and matrix. DMP1 and DSPP immunoreactive staining was much more localized to the tumor cells in all of the patient samples.

**SIBLINGs in serum.** Serum samples from a large normal group and a group with prostate cancer were obtained. Samples from the prostate cancer group were obtained at diagnosis and before treatment. For the normal group (n = 110), the average age was 65 ± 10 years and body mass index values were 23.1 ± 2.6 kg/m². For the prostate cancer group (n = 102), the average age was 63 ± 5 years and body mass index values were 25.0 ± 4.1 kg/m². Competitive ELISAs for quantitatively determining the levels of total BSP, DSPP, MEPE, and OPN were developed and applied. (Unfortunately, the ELISA for quantifying DMP1 has recurrent issues with the stability of the recombinant DMP1 protein standard.) The mean levels of BSP, OPN, and DSPP protein were all elevated in the cancer patients. The mean values for the normal group versus the cancer group were 114 ± 63 versus 222 ± 98 ng/mL for BSP, 242 ± 122 versus 1,500 ± 500 ng/mL for DSPP, 93 ± 19 versus 80 ± 11 ng/mL for MEPE, and 353 ± 130 versus 537 ± 170 ng/mL for total OPN, respectively. For both BSP and total OPN, there was significant overlap between the distributions of normal and cancer patient serum levels (Fig. 3). Mean values in prostate cancer sera were elevated 2-fold for BSP, 6-fold for DSPP, and 1.5-fold for total OPN, whereas MEPE showed no difference between cancer and normal patient sera.

OPN exists in both complement factor H–bound and “free” forms in plasma and several studies have measured OPN directly.
in plasma without pretreatment of the sample with reducing or chaotropic agents to free the protein from factor H (21–28). A subset of our normal and prostate cancer subjects (n = 40 per group) had blood specimens processed appropriately for measuring free OPN in plasma. The average level of free OPN was 45 ± 20 ng/mL in normal samples and 127 ± 80 ng/mL in prostate cancer samples (Fig. 3D). Free OPN accounted for 13% of total OPN in normal samples and 23% of total OPN in prostate cancer samples. Receiver operator characteristic curve profiling of serum SIBLINGs yielded area under the curve values that were very significant for DSPP and revealed that free OPN had a better discriminatory power than total OPN (Fig. 3E-H). Cutoff values maximizing sensitivity and specificity for the SIBLINGs were determined to be 142 ng/mL for BSP, 500 ng/mL for DSPP, 358 ng/mL for total OPN, and 45 ng/mL for free OPN.

The prostate cancer serum training set samples were evenly distributed between organ-confined (pT2) where cancer is local-
to compare values (Fig. 6A-C). The validation set spanned from stage pT1c to pT2b, so late-stage disease was not present. Thus, BSP exhibited no increase and the potential negative correlation of DSPP with late-stage disease could not be addressed. Stratifying subjects by both serum SIBLING and PSA values revealed that DSPP enabled the clearest distinction between the two groups (Fig. 6D-F). It is of note that the six lowest lying samples visible in the DSPP prostate cancer group were also from the patients with the lowest values in both OPN and BSP.

**Discussion**

Prostate cancer is the most common type of cancer in men in the United States after skin cancer. Indeed, prostate cancer accounts for over a quarter of all cancers in men and the risk increases with age (1). There has been an increase in the reported incidence of prostate cancer since the early 1980s most likely due to an increased use of screening using the PSA test (30), although the effectiveness of screening for prostate cancer remains controversial (31).

SIBLINGs, through their interactions with multiple binding partners such as proteases and cell surface receptors, have biological plausibility to be playing an active role in tumor progression (5). Different cancer types exhibit different patterns of individual SIBLING expression (7) as well as serum SIBLING protein levels (19). Several separate studies have investigated BSP and OPN levels in prostate cancer. Increased BSP expression in prostate carcinoma has been associated with tumor progression and poor prognosis (32). Induced OPN expression and a positive correlation between OPN expression and poor prognosis have also been observed (33, 34). Both BSP and OPN protein levels in serum were found to be elevated in prostate cancer (21). Relative levels of DSPP as measured by immunohistochemistry were associated with the pathologic stage and the Gleason score of the tumors (35). The distributions of DMP1 and MEPE in prostate cancer, however, have not been reported previously.

The expression of SIBLINGs by prostate (and breast) carcinoma prompted the hypothesis that osteotropic cancer cells express osteomimetic properties that favor “seeding” in the skeleton by improving their adhesion, proliferation, and/or survival in bone (36). The expression of these “bone” proteins by prostate tumor cells do not necessarily target cell metastasis to

**Fig. 6.** Serum values for SIBLINGs at specific prostate cancer stages and PSA levels in the validation study group. The distributions of SIBLING values between normal (n = 30) and prostate cancer stages pT1c (n = 34), pT2a (n = 13), and pT2b (n = 12) were compared for (A) BSP, (B) DSPP, and (C) total OPN using Tukey’s box-and-whiskers plots as in Fig. 4. ***, P < 0.001, unpaired t test. Samples from normal donors and subjects with prostate cancer were plotted as a function of PSA and (D) BSP, (E) DSPP, and (F) total OPN. Dotted line, cutoff value for each SIBLING; open symbols, values for normal subjects; solid symbols, values from subjects with prostate cancer.
bone. It is also possible that transcription factors that regulate SIBLINGS (e.g., RUNX2) produce a mesenchymal phenotype that finds bone a fertile soil for survival. Furthermore, SIBLINGS and their partner MMPs (when known) have recently been shown to be expressed in normal metabolically active ductal epithelial cells of salivary glands (15) and the kidney (37). Normal sweat gland expressed all but MEPE (6). Therefore, it is no longer necessary to invoke an epithelial-to-mesenchymal conversion hypothesis when considering SIBLING expression in epithelially derived tumors.

In the current study, the total serum levels of BSP and OPN were found to be significantly elevated only in late-stage prostate cancer. Whereas free OPN levels in plasma increased significantly from pT2 to lymph node–positive/venous/skeletal–positive stages, the positive association of both free (plasma) and total (serum) OPN levels with serum PSA levels may be indicative of reactive processes of inflammation and not necessarily direct tumor pathology (38, 39). Whereas there was significant overlap between the levels of both BSP and OPN in sera of normal and prostate cancer patients, the serum DSPP levels uniquely showed minimal overlap.

DSPP was initially characterized through the isolation of one of its cleavage products from dentin, DPP (40). Subsequent cloning of the gene revealed that the transcript encodes the amino-terminal DSP and DPP (31). DSP has been hypothesized to be an important factor in dentinogenesis, the processing of DSP into additional fragments by other proteases (44, 45). MMP-20, and kallikrein-related peptidase 4 has been reported (43) and up-regulation of both MMP-2 and kallikrein-related peptidase 4 in prostate cancer has been observed (44, 45). Observations of our current study are consistent with SIBLING levels being associated with tumor progression and suggest that SIBLINGS, particularly DSPP, may be developed as informative serum biomarkers. The lack of correlation of DSPP serum levels with PSA values suggests that the two markers might complement each other. Indeed, as shown in the validation group, segregating samples as a function of both PSA and DSPP (Fig. 6) removed overlap between the normal and cancer groups, thereby increasing sensitivity and specificity. The current study also provides evidence in support of future studies screening a large patient population as well as testing baseline value association with disease outcome and response to treatment to determine the utility of this new serum biomarker for prostate cancer detection.

Disclosure of Potential Conflicts of Interest

N.S. Fedarko and L.W. Fisher are co-inventors of a NIH/DHHS patent (U.S. Patent No. 6,995,0187) and as such have ownership interests.

References
