Elevated Serum Bone Sialoprotein and Osteopontin in Colon, Breast, Prostate, and Lung Cancer[1]

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ABSTRACT

Purpose: **Histological studies have shown that the two sialoproteins, bone sialoprotein (BSP) and osteopontin (OPN), are induced in multiple types of cancer. We have recently found that these proteins are bound in serum to complement factor H and that the complex must be disrupted to generate free protein to measure their total levels. We hypothesized that measuring total BSP and OPN levels would provide informative markers for the detection of cancer.**

Experimental Design: **As a proof of concept study, serum from patients with diagnosed breast, colon, lung, or prostate cancer ($n = 20$ for each type) as well as normal serum ($n = 77$) were analyzed using competitive ELISAs developed for BSP and OPN. Sensitivity, specificity, as well as positive and negative predictive values were determined for each sialoprotein and cancer type. The relationship between sensitivity and specificity was profiled by receiver operating characteristic curves.**

Results and Conclusions: **Determined values for serum BSP in ng/ml were 285 ± 19 for prostate, 373 ± 19 for colon, 318 ± 18 for breast, 155 ± 11 for lung cancer sera, and 154 ± 13 for normal sera. Values of OPN in ng/ml were 653 ± 39 for prostate, 449 ± 22 for colon, 814 ± 53 for breast, 724 ± 33 for lung, and 439 ± 30 for normal sera. The assays provide a high degree of sensitivity and specificity that enables the detection of colon, breast, prostate, and lung cancer.**

INTRODUCTION

The family of secreted proteins, which we term SIBLINGs[4] (1), contains the integrin-binding tripeptide Arg-Gly-Asp (RGD), as well as several conserved phosphorylation and *N*-glycosylation sites. All of the genes cluster on chromosome 4 and are expressed in the skeleton. At this time, there are at least five known members of the SIBLING family of glycoproteins: BSP, OPN, dentin matrix protein-1, dentin sialophosphoprotein, and matrix extracellular phosphoglycoprotein (1). Recent observations in paraffin sections have shown that BSP and OPN were expressed by multiple malignant tissues. BSP was expressed in primary breast cancers (2–4), prostate cancer (5), lung cancer (6), thyroid cancer (7), malignant bone disease (8), and neoplastic odontoblasts (9). OPN was expressed in breast cancer (4, 10, 11), as well as in prostate cancer (12), thyroid cancer (13), and skin cancer (14).

BSP expression in paraffin sections was associated with poor survival in breast cancer (15) and with bone metastases development (3). Similarly, BSP expression in clinically localized human prostate cancer had prognostic value (5, 16). Using a serum assay that does not detect all of the BSPs in circulation, Diel *et al.* (17) showed that 7.4% (29 of 388 patients) had BSP levels greater than two SDs above their normal cohort. Interestingly, of those 29 patients, 17 were reported to progress to bone metastases. By comparison, 89% of the sections from 454 breast cancer patient tumors were positive for BSP expression. Using the same serum assay as Diel *et al.* (17), Woitge *et al.* (18) showed that 73% of patients with multiple myeloma had elevated levels of serum BSP. Similarly, OPN expression was associated with clinical severity and tumor progression in lung cancer (19), invasive breast cancer (11, 20), gastric cancer (21), and perhaps ovarian carcinoma (22). In these cases, expression levels were determined retrospectively in pathological specimens at the level of either message expression or antibody reactivity.

Experimental evidence suggests that OPN may play multiple roles in promoting tumor progression, including inhibiting macrophage function and enhancing growth or survival of metastases (23); altering adhesion via, or signal transduction from, vitronectin receptors (24); increasing cell migration and inducing urokinase type plasminogen activator expression (25); stimulating proliferation through integrin-mediated intracellular calcium signaling (26); and directly stimulating prostate cancer cell growth and progression (27). We have found that expression of

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[4] The abbreviations used are: SIBLING, small integrin-binding ligand *N*-linked glycoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen; CEA, carcinoembryonic antigen; HPLC, high-performance liquid chromatography; TBS, Tris-buffered saline; TBS-T, TBS with Tween 20; TMB, 3,3′,5,5″-tetramethylbenzidine; ROC, receiver operator characteristic; PPV, positive predictive value; NPV, negative predictive value; IRB, institutional review board.

BSP and OPN promotes human breast cancer and myeloma cell survival *in vitro* (28). These observations suggested that members of the SIBLING gene family may provide usefulness as surrogate end point markers for the presence of cancers.

MATERIALS AND METHODS

Human Subjects. Sera from clinically defined colon, breast, prostate, and lung cancer and from normal patients were obtained from a commercial serum bank (East Coast Biologicals, Inc., North Burwick, ME) with IRB approval. All patient identifiers were removed prior to shipment. However, PSA and CEA values did accompany the sera. Sera were obtained before surgery and under informed consent. For all groups from East Coast Biologicals, $n = 20$. Additional control sera were obtained from the Johns Hopkins Bayview Medical Center General Clinical Research Center. This larger normal group was obtained from an existing serum bank using samples that had been unlinked from their donors.

Production of Recombinant BSP and OPN. We have developed competitive ELISAs for measuring BSP and OPN using adenovirus-produced recombinant BSP or OPN. Adenoviral constructs were generated, selected, and propagated as described previously (28).

Serum Sample Preparation. Serum samples (100 μl) were diluted 1:10 in a 50% formamide-40-mM phosphate buffer (pH 7.4) and were reduced with 2 mM DTT at 100°C for 5 min to disrupt the binding of BSP or OPN by complement factor H in serum. Residual reducing agent and formamide were removed by strong anion exchange column chromatography (ToyoPearl QAE resin; TosoHaas, Montgomeryville, PA) using 300 μl of resin in a Bio-Spin disposable chromatography column (Bio-Rad Laboratories, Hercules, CA). After loading the 1.0-ml sample, the column was washed with 6× column volumes of TBS solution [0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl] containing 0.05% Tween 20 (TBS-T). BSP and OPN were eluted with TBS-T containing 1.0 M NaCl.

Competitive ELISA Procedure. Samples (100 μl) were taken for analysis using a modified competitive ELISA. Greiner high-binding plates (USA Scientific, Inc., Ocala, FL) were coated with 20 ng/well BSP or OPN overnight in 50 mM carbonate buffer (pH 8.0). The standard curves for BSP and OPN were constructed using recombinant protein at concentrations of 0.0, 1.95, 3.91, 7.8, 15.76, 31.25, 62.5, 125, 250, 500, 1000, and 2000 ng/ml Samples and standards (100-μl volume) were incubated for 2 h with shaking at room temperature with 100 μl of a 1:200,000 dilution of LF-100 antibody (for BSP) or a 1:100,000 of LF-124 antibody (for OPN) in TBS-T in polypropylene 96-well plates (USA Scientific, Inc.). During those 2 h, the antigen-coated plates were blocked with TBS + 5% powdered milk. Antigen-coated plates were then rinsed three times with TBS-T, and the antibody-sample solution was added to the wells. After a second incubation for 1 h at room temperature with shaking, the plates were washed three times with TBS-T. A secondary antibody of goat antirabbit peroxidase-labeled antibody conjugate, human serum adsorbed (Kirkegaard & Perry, Gaithersburg, MD) at 1:2000 was then added, and the plates were incubated for 1 h. After three washes with TBS-T, substrate (TMB microwell peroxidase substrate; BioFX Laboratories) was added and after a final 20-min incubation, the color reaction was stopped by the addition of 25 μl of 1 N H_2SO_4. Absorbance was read at 450 nm, and the data were analyzed using the program AssayZap (BioSoft, Cambridge, United Kingdom).

Sensitivity, Specificity and ROC Analysis. Sensitivity (*Se*) was defined as the incidence of true positive results when the assays were applied to patients known to have cancer and was calculated using:

$$Se = \frac{TP}{TP + FN} \quad \text{(A)}$$

where TP = true positive and FN = false negative. Specificity (*Sp*) was defined as the incidence of true negative results when the assays were applied to subjects known to be free of cancer and was determined using:

$$Sp = \frac{TN}{TN + FP} \quad \text{(B)}$$

where TN = true negative and FP = false positive. The percentage of patients known to have cancer that were correctly classified as positive by the assay was:

$$PPV = \frac{TP}{TP + FP} \times 100 \quad \text{(C)}$$

The percentage of healthy donors correctly classified as negative by the assay was:

$$NPV = \frac{TN}{TN + FN} \times 100 \quad \text{(D)}$$

Depending on the cutoff value, a range of sensitivity and corresponding specificity values were obtained for BSP and OPN. The PPV (frequency of cancer patients in all patients with a positive test result) and NPV (frequency of noncancer or control subjects in all subjects with a negative test result) were determined for various serum level cutoff values of SIBLINGs.

The relationship between specificity and sensitivity was profiled by ROC curves of BSP and OPN for different cancer types. These were generated by determining possible cutoff values, calculating *Se* and (1 − *Sp*) for each cutoff value, and plotting. These values and plots were generated using a Microsoft Excel macro-analysis tool pack.

HPLC BSP Assay. Fifty μl of serum from a normal donor or from a cancer patient prior to surgery was added to 50 μl of fresh formamide and 10 μl of 100 mM DTT. The sample was heated in a sand-filled heating block (100°C) for 5 min, microfuged for 1 min at 15,000 × *g*, and injected onto a 1-ml ToyoPearl TSK QAE column that was preequilibrated with 0.05 M sodium phosphate/50% formamide (pH 7.4). Using a flow rate of 2 ml/min, the column was washed for 5 min in the same low-salt buffer, and then the salt was linearly increased to 1 M NaCl over 25 min and then to 2 M NaCl in the following 15 min. Fractions were collected every minute. One hundred μl of each fraction was placed in a corresponding well of a 96-well microtiter plate (Greiner high-binding plate) for coating overnight at 4°C. Plates were washed three times (5 min each) with ≥200 μl/well TBS-T and then exposed to 100 μl of rabbit antiserum

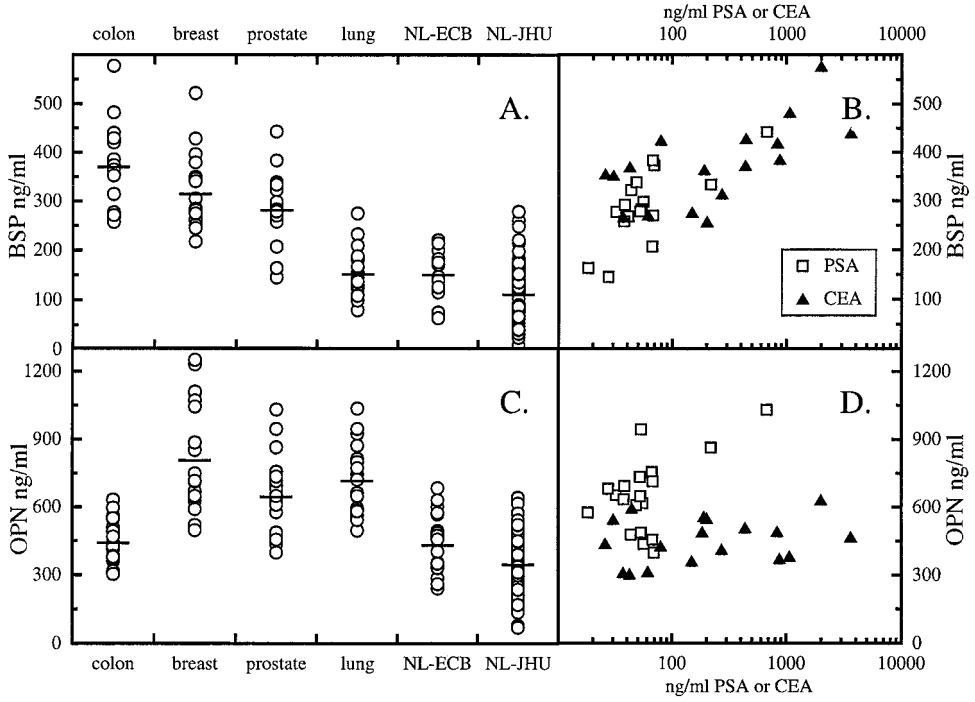

Fig. 1 Preliminary competitive ELISA screen of serum BSP and OPN levels. Sera were obtained from clinically defined colon, breast, prostate, and lung cancer and normal patients (East Coast Biologicals, Inc.) as well as from defined normal patients (Johns Hopkins Bayview Medical Center General Clinical Research Center) under informed consent with IRB approval. For all cancer groups, $n = 20$, for normal sera obtained from East Coast Biologicals (*NL-ECB*), $n = 20$; and for the normal sera obtained from Hopkins (*NL-JHU*), $n = 77$. Mean serum values for BSP (*A*) and OPN (*C*) were determined by duplicate analyses using the competitive ELISA. Serum values of PSA and CEA in prostate cancer and colon cancer patients, respectively, were plotted *versus* BSP (*B*) and OPN (*D*) values to compare relative correlation.

Table 1 Sensitivity, specificity, and predictive values for BSP and OPN at selected cutoff values[a]

	Colon cancer n = 20			Breast cancer n = 20			Prostate cancer n = 20			Lung cancer n = 20			NL-ECB[b] n = 20	NL-JHU n = 77
Age, yr	64 ± 2			64 ± 1			63 ± 1			69 ± 2			42 ± 3	61 ± 2
BSP, ng/ml	373 ± 19			318 ± 18			285 ± 19			155 ± 11			154 ± 13	122 ± 7
Cutoff, ng/ml	200	225	250	200	225	250	200	225	250	200	225	250		
Sensitivity, %	100	100	100	100	94.4	88.9	87.5	81.3	81.3	15.0	10.0	5.0		
Specificity, %	88.3	94.8	96.1	88.3	94.8	96.1	88.3	94.8	96.1	88.3	94.8	96.1		
PPV	66.7	81.8	85.7	66.7	81.0	84.2	60.9	76.5	81.3	25.0	33.3	25.0		
NPV	100	100	100	100	98.6	97.4	97.1	96.1	96.1	80.0	80.2	79.6		
OPN, ng/ml	448 ± 22			814 ± 53			653 ± 39			724 ± 33			439 ± 30	353 ± 15
Cutoff, ng/ml	400	450	500	400	450	500	400	450	500	400	450	500		
Sensitivity, %	65.0	45.0	30.0	74.1	100	95.0	94.7	89.5	73.7	100	100	95.0		
Specificity, %	55.7	69.1	84.5	55.7	69.1	84.5	55.7	69.1	84.5	55.7	69.1	84.5		
PPV	23.2	23.1	28.6	31.7	40.0	55.9	29.5	36.2	48.3	31.7	40.0	55.9		
NPV	88.5	85.9	85.4	88.5	100	98.8	98.2	97.1	94.3	100	100	98.8		

[a] Reported error is SE.
[b] NL-ECB, normal sera from East Coast Biologicals, Inc.; NL-JHU, normal sera from The Johns Hopkins University, Bayview Medical Center.

against human BSP (1:2000, LF-100) in TBS-T for 1 h at room temperature. Wells were washed as above and then exposed to 100 μl of 1:2000 horseradish peroxidase-conjugated goat anti-rabbit IgG (human serum adsorbed; Kirkegaard & Perry) in TBS-T for one h at room temperature. After a final three washes, color was developed using TMB for 10–30 min at room temperature. Color development was stopped and analyzed as above.

RESULTS

The recent characterization of a serum-binding protein for BSP and OPN suggested that for optimum measurement of BSP and/or OPN serum levels, the binding complex would need to be disrupted (28). We have developed a protocol by which the serum complex was disrupted by heating and reduction in a formamide-containing buffer. The chaotropic buffer and residual reducing agent were removed by passage of the sample over anion exchange resin. BSP and OPN were retained on the column and, after washing with 6× column volumes with TBS-T, were eluted with TBS-T containing 1.0 M NaCl. An aliquot of the eluate was then taken for analysis by competitive ELISA. Spiking serum samples prior to reduction and analysis by competitive ELISA led to an average yield of 84% based on four different trials. The coefficient of variance for repetitive measurements on the same serum sample varied between 15 and 20%. The source of this variance was tracked to the column

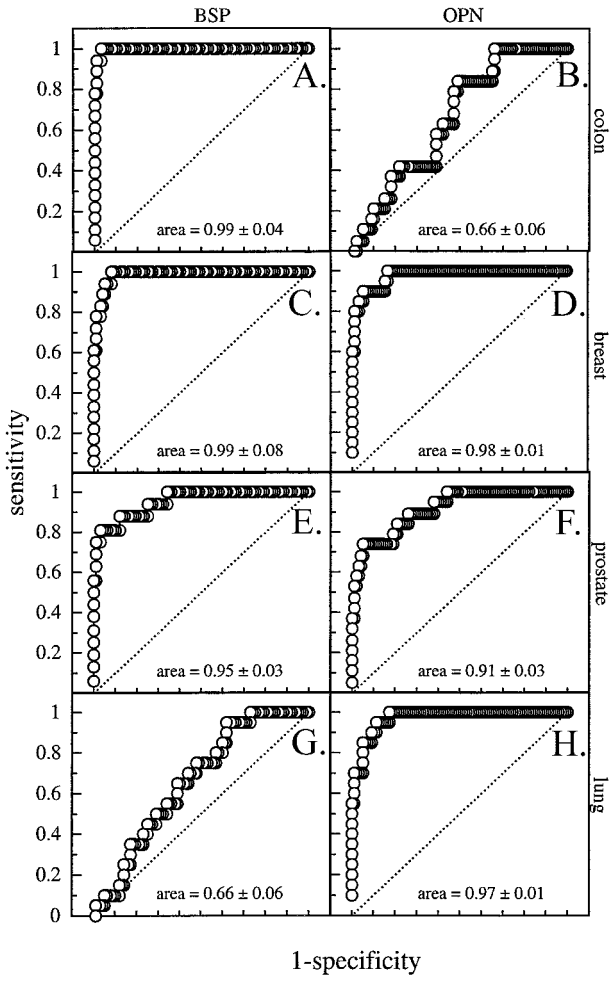

Fig. 2 ROC curves of BSP and OPN immunoassays in discriminating patients with various cancers from control, noncancer patients. Sensitivity, a measure of the number of true positives, and the quantity (1 − specificity), the number of false positives, are calculated at various cutoff values for BSP (*A*, *C*, *E*, and *G*) and OPN (*B*, *D*, *F*, and *H*) in colon (*A* and *B*), breast (*C* and *D*), prostate (*E* and *F*), and lung (*G* and *H*) cancer patients. The area under the curve for a perfect discriminatory test would be 1.0. *The dashed diagonal line*, marks values of sensitivity and (1 − specificity) for which no discrimination can be made by the test.

chromatography step. Repeated measures of postreduction and column samples gave rise to a coefficient of variance of 11%.

The screening of BSP and OPN levels in normal- and cancer-patient-derived sera revealed distinct patterns for each cancer type (Fig. 1). Because of a difference in mean age of donors (Table 1), the normal serum obtained from the same commercial source as the cancer sera was segregated from the larger normal serum pool. The normal serum group obtained from East Coast Biologicals was not significantly different from the larger normal population in terms of either BSP or OPN values. BSP levels were significantly elevated in colon, breast, and prostate cancer sera, whereas its levels in lung cancer sera were no different from that in normal serum (Fig. 1*A*; Table 1). It was of note that the levels of BSP in serum by the current assay were in the 100-to-500-ng/ml range, whereas previous reports based on a different 2-day assay by Seibel *et al.* (8) had reported serum levels of BSP in the 10-to-20-ng/ml range. The RIA developed by Dr. Karmatschek, which was briefly marketed commercially (Immunodiagnostik Inc., Heidelberg, Germany) has proved to be problematic in terms of stability and variable results.[5] Problems with stability and variability could be accounted for by the inability of their assay to detect total serum BSP (which is bound in serum in a complex with complement factor H and must be disrupted for accurate measurement). When the current competitive BSP ELISA was carried out directly on serum (with no sample heating, reduction, or column chromatography) and incubated with primary antibody for 24 h at 4°C (as were the conditions for the Immunodiagnostik assay), BSP was detected in the 10-to-15-ng/ml range.

OPN levels were also significantly elevated in multiple cancer sera (Fig. 1*C*; Table 1). For OPN, levels were highest in breast, followed by lung and then prostate cancer sera. OPN levels in colon cancer sera were not significantly different from those in normal sera. The levels of OPN in plasma in a group of normal and breast cancer patients had been previously reported (29). In that study, plasma samples were analyzed directly (with no sample heating, reduction, or column chromatography) and values of 47 and 142 ng/ml for control and patients with known metastatic breast carcinoma, respectively, were found. Those reported values were 6- to 10-fold lower then the observed results of the current immunoassays.

The difference between cancer and normal sera values for BSP and OPN determined by the current assays were ~2-fold, which was consistent with previous studies that used immunoassays that would measure only free BSP or OPN (8, 29, 30). Total BSP and OPN were, to a degree, mirror images of each other. BSP levels were highest in colon cancer serum, whereas OPN levels were low. Conversely, high OPN levels in lung cancer sera were matched by low levels of BSP in lung cancer sera. On the basis of the current data, it was not evident that one could distinguish between colon, breast, or prostate cancer by determining serum levels of BSP alone. Measuring OPN provided a second marker that allowed colon and lung cancers to be separated out.

Because of the nature of this preliminary screening and the IRB-approved protocol under which it took place, information on disease severity, course, tumor load, and so forth, was not available. However, the results from other diagnostic markers in the serum of certain patients were available. PSA levels and CEA levels were obtained for prostate and colon cancer sera, respectively, and were analyzed for correlation with BSP and OPN values (Fig. 1, *B* and *D*). Because of the wide distribution of PSA and CEA, values were plotted on a logarithmic scale, and correlation was determined statistically using a Spearman rank correlation test. This nonparametric test yielded a significant correlation for BSP and PSA (Spearman r value of 0.67; $P \leq 0.01$), whereas the other markers were not significantly correlated by this statistical test.

[5] M. Karmatschek and M. J. Seibel, personal communication.

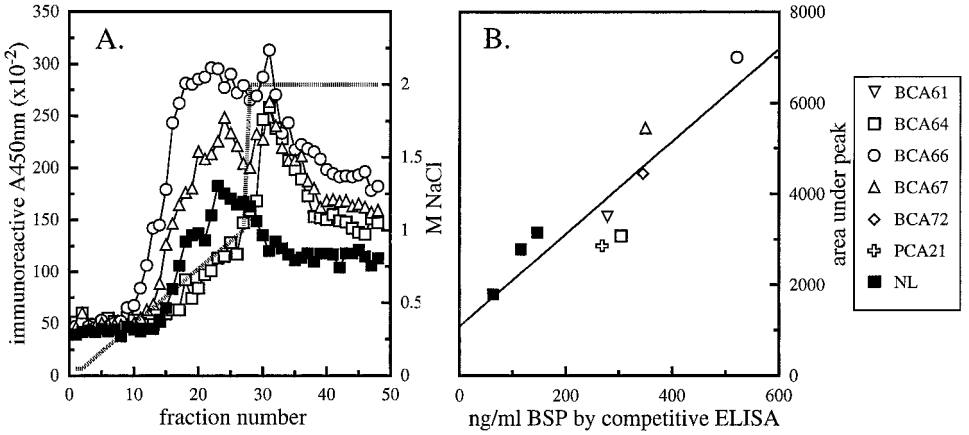

Fig. 3 HPLC *versus* competitive ELISA. Sera from selected breast and prostate cancer patients as well as from normal subjects were heated and reduced in the chaotropic formamide buffer and resolved by strong anion exchange HPLC. Fractions from the column were analyzed for BSP by direct ELISA analysis (*A*) and the area under the curve (AUC) of the resulting peak was determined. AUC values were then compared by regression analysis to ng/ml values determined by the competitive ELISA on separate aliquots of the same serum (*B*).

We used the results from our screening to calculate sensitivity (incidence of true positive results when the assays were applied to patients known to have cancer), specificity (incidence of true negative results when the assays were applied to subjects known to be free of cancer), and predictive values at specific BSP and OPN cutoff values. Depending on the cutoff value, a range of sensitivity and corresponding specificity values were obtained for each particular cancer. The PPV (frequency of cancer patients in all patients with a positive test result) and NPV (frequency of noncancer or control subjects in all subjects with a negative test result) were determined for various serum level cutoff values of BSP and OPN (Table 1). The relationship between specificity and sensitivity was profiled by ROC curves of BSP and OPN for each cancer (Fig. 2).

To verify that the measurements made by the competitive ELISA reflect actual serum levels of BSP or OPN, a second separate method of analysis was performed. Serum was heated and reduced as before, but the sample was injected onto a strong anion exchange HPLC column. A linear salt gradient with a rapid ramp to high salt concentration was used to elute the BSP. Aliquots of collected fractions were transferred to 96-well plates and analyzed by direct ELISA for BSP immunoreactivity (Fig. 3*A*). A series of HPLC analyses of serum from a number of breast cancer donors, a prostate cancer donor, and multiple normal donors were analyzed for their area under the curve to determine the relative amount of BSP detected in each serum sample by this second method. The area under the curve values were plotted *versus* ng/ml values of BSP determined by the competitive ELISA on separate aliquots of the serum (Fig. 3*B*). There was a significant correlation between area under the curve values and BSP ng/ml ELISA values for normal and cancer sera combined ($P \leq 0.001$).

DISCUSSION

The prevention of cancer would be greatly facilitated by the availability of a sensitive and specific screening method that could detect the cancer. One approach to developing markers for the detection of cancer has been to identify specific cellular products that correlate with the appearance of a specific neoplasm. Although specificity can be targeted by determining individual neoplastic profiles, there are steps and pathways shared by many different neoplasms that arise from different tissue types. To survive, nascent neoplasms must overcome proliferation blockades, growth restriction, physical barriers, and host defense systems. Markers that arise from mutations in cell cycle checkpoint regulators (*e.g.*, p53) may be considered paradigms of such generalized markers.

We have been studying a family of proteins that correlate with multiple cancer types in tissue sections. A biological role that these gene family members play during neoplastic expression may involve tumor cell evasion of the humoral surveillance system (28). BSP, which was shown earlier to be elevated in tumor sections from 89% of breast cancer patients (17), was seen in our new assay to be elevated two SDs above the normal mean in 18 (90%) of 20 patients in our cohort. This contrasts with earlier results using an assay that did not disrupt the complex with factor H, and among which, less than 10% of patient sera were significantly elevated in BSP (17). Furthermore, although colon cancer patients have not yet been identified as having elevated levels of BSP in tumor sections, the data presented in this report clearly shows that serum BSP is elevated in those patients. This suggests that BSP and OPN may be useful markers for cancers beyond those currently described in the literature. Although some cancer types (breast and prostate) were high in both BSP and OPN, lung and colon cancer exhibited high levels of one marker with low levels of the other. Analysis of a single SIBLING family member yielded reliable results on the detection of cancer, although the specific type of cancer was not definable from serum levels of BSP or OPN alone. Determining the serum levels of both SIBLING proteins also yielded very high degrees of sensitivity and specificity. For example, the results of the two screens enabled unequivocal

differentiation of colon from lung and from breast and prostate cancer.

This proof of concept study was designed to test whether serum levels of SIBLINGs were predictive of cancer. SIBLING proteins (*i.e.*, BSP and OPN) were found to be elevated in certain cancer types but not in others. Given the elevated levels in multiple neoplasm types, the diagnostic specificity of BSP and OPN for distinct cancer types *versus* any cancer needs to ascertained. This will be addressed by determining serum levels of these SIBLINGs in a series of much larger clinically defined populations among which tumor stage and load are known.

The future analysis of sera derived from cancer patients before and after surgery and/or treatment will also define the responsive nature of these markers. Although it has been proposed that the SIBLINGs BSP and OPN may be associated with metastasis of breast cancer to bone (3, 6, 15, 29, 31, 32), it seems prudent to first describe the distribution of serum levels initially in simply the absence or presence of the disease (cancer). Studies of bone metastatic tumors and their SIBLING levels will be a natural future outgrowth of the present studies. In conclusion, BSP and OPN, separately or in combination can, when dissociated from complement factor H, be used to detect the presence of a number of different tumors.

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