

Development of an Immunoassay for Serum Caveolin-1: A Novel Biomarker for Prostate Cancer¹

Salahaldin A. Tahir, Chengzhen Ren,
Terry L. Timme, Yehoshua Gdor,
Ron Hoogveen, Joel D. Morrisett, Anna Frolov,
Gustavo Ayala, Thomas M. Wheeler, and
Timothy C. Thompson²

Scott Department of Urology [S. A. T., C. R., T. L. T., Y. G., A. F., T. C. T.], Houston VA Medical Center [T. L. T., T. C. T.], Department of Medicine [R. H., J. D. M.], Department of Pathology [G. A., T. M. W.], Department of Molecular and Cellular Biology [T. C. T.], and Department of Radiology [T. C. T.], Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

Purpose: Caveolin-1 (cav-1), the major protein component of caveolae, plays an important role in multiple signaling pathways, molecular transport, and cellular proliferation and differentiation. The specific functions of cav-1/caveolae are highly cell and context dependent. We have previously shown that cav-1 expression is increased in metastatic human prostate cancer and that cav-1 cellular protein expression is predictive of recurrence of the disease after radical prostatectomy. Recently, we reported that cav-1 is secreted by androgen-insensitive prostate cancer cells, and we detected, by Western blotting, cav-1 in the high-density lipoprotein₃ fraction of serum specimens from patients with prostate cancer.

Experimental Design: Using rabbit polyclonal antibodies with specificity for cav-1, we developed a direct sandwich immunoassay for the determination of cav-1 in serum. A recombinant human cav-1 fusion protein was overexpressed and purified from 293 PE cells and used as a calibrator.

Results: The assay was highly specific and had a minimum detection limit of 0.017 ng/ml (mean + 3 SD of zero calibrator) and measuring range of up to 200 ng/ml. Intra-assay coefficient of variation was 2.29–6.74% and inter-assay coefficient of variation was 2.81–6.43% over the serum concentration tested 0.04–31.89 ng/ml. The recovery limit of cav-1 by the assay was 89.55–100.28%. The median serum cav-1 level in 102 prostate cancer patients with clinically localized disease (0.463 ng/ml) was significantly higher

than 81 healthy control men (0.324 ng/ml; $P = 0.0446$, Mann-Whitney test) or 107 men with benign prostatic hyperplasia (0.172 ng/ml; $P = 0.0317$, Mann-Whitney test).

Conclusions: Our results indicate that serum cav-1 has the power to differentiate between prostate cancer and benign prostatic hyperplasia patients and the potential to be an important biomarker for prostate cancer. Additional studies to test the potential of serum cav-1 as a diagnostic and/or prognostic marker in prostate cancer are warranted.

INTRODUCTION

Prostate cancer is the second leading cause of death and the most common cancer in men. African-American men have a much higher incidence of and mortality from prostate cancer than white-American men (1). The precise risk factors for prostate cancer are unknown with both genetic factors and environmental factors likely to be involved (2). In recent years, efforts to detect and treat prostate cancer have increased dramatically throughout the United States (1). The widespread use of serum PSA³-based screening of asymptomatic men resulted in a sharp (>2-fold) increase in the incidence of prostate cancer during the 1990s. PSA is a member of the human kallikrein family of serine proteases produced by the prostatic epithelium and the epithelial lining of the periurethral glands. Although PSA is selectively expressed in prostate, it is not prostate cancer specific, and serum PSA levels rise not only in prostate cancer but also in benign prostatic disease, physical trauma to the prostate, and other conditions affecting the integrity of the prostate gland (3). It is therefore not surprising that serum PSA levels lack the specificity to permit the accurate prediction of the pathological or clinical stage (4, 5). Because the standard treatments for prostate cancer are only useful for localized disease, they are entirely dependent on accurate staging for their effectiveness. Therefore, because of the inadequacies of serum PSA or any other commonly available modality to accurately predict pathological or clinical stage, the curative potential of standard localized treatment is limited. Indeed, over recent years, there has emerged a unique and significant patient population who have received localized therapy but who have recurred with a rising serum PSA, indicating either local recurrence and/or occult metastasis (6, 7). Additional biomarkers with specificity for virulent prostate cancer and/or metastatic disease are clearly needed.

Cav-1 is the principal structural protein of caveolae and functions in signal transduction and lipid transport. Cav-1 can also accumulate in cellular compartments other than the plasma

Received 6/5/02; revised 2/21/03; accepted 2/27/03.

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¹ This work was supported by National Cancer Institute Grants CA 68814, CA 50588, and SPOR P50-58204.

² To whom requests for reprints should be addressed, at Scott Department of Urology, 6560 Fannin Street, Suite 2100, Houston, TX 77030. Phone: (713) 799-8718; Fax: (713) 799-8712; E-mail: timothy@www.urol.bcm.tmc.edu.

³ The abbreviations used are: PSA, prostate-specific antigen; Cav-1, caveolin-1; BPH, benign prostatic hyperplasia; HDL, high-density lipoprotein; CV, coefficient of variation; TBS, Tris-buffered saline; 2-ME, 2-mercaptoethanol; HRP, horseradish peroxidase.

membrane such as the cytosol of skeletal muscle cells and keratinocytes, in modified mitochondria of airway epithelial cells, and in the secretory pathway of endocrine and exocrine cells (8). Through biochemical and molecular analyses of prostate cancer tissues and cell lines, we previously identified cav-1 as being overexpressed in metastatic prostate cancer (9). We also demonstrated that cav-1 is an independent prognostic marker for prostate cancer progression in lymph node-negative patients who have recurred after radical prostatectomy (10) and that there is a significant association of increased cav-1 in prostate cancer in African-American men *versus* white-American men (11). Recently, we showed that cav-1 up-regulation is associated with the development of androgen-insensitive prostate cancer (12) and that androgen-insensitive prostate cancer cells secrete biologically active cav-1 in a steroid-regulated fashion (13). We further demonstrated that the secreted cav-1 can stimulate viability and clonal growth in prostate cancer cells that do not express cav-1, and by Western blot analysis, we detected cav-1 in the serum HDL₃ fraction of prostate cancer patients (13).

We describe in this article, the development of a sensitive, reproducible, and specific immunoassay for the measurement of cav-1 in serum. We also show that cav-1 is significantly elevated in the sera of prostate cancer patients compared with those of BPH or controls.

MATERIALS AND METHODS

Reagents. All general laboratory reagents were purchased from Sigma or Bio-Rad unless otherwise stated. Protein concentrations were determined by the Bradford method (14), using BSA as a calibrator (Bio-Rad protein assay; Bio-Rad Laboratories).

Expression and Purification of Cav-1 Calibrator. A plasmid, termed phcavV5His, was constructed to express the human cav-1 cDNA as a fusion protein with a V5 and 6X histidine tag. The human cav-1 cDNA was previously isolated from a cDNA library we prepared from the human prostate cancer cell line PC-3. This cDNA is completely homologous with the cDNA for cav-1 in GenBank accession no. NM001753. The cDNA was amplified from a stock plasmid using oligonucleotide primers designed to add the restriction site *EcoRI* to the 5' end of the cDNA and *XhoI* to the 3' end of the cDNA. The sequence was carefully chosen to allow insertion into the plasmid pcDNA6/V5-HisB (Invitrogen) that was cleaved with *EcoRI* and *XhoI*. The insert was predicted to generate an in-frame fusion of the V5 epitope and six histidine amino acids at the COOH terminus of the expressed human cav-1 protein. The resultant plasmid was sequenced using as sequencing primers the T7 primer (on the 5' side polylinker of the hcav-1 insert) and a primer in the BGHpA region on the 3' side. The sequence and translation of the sequence confirmed that the correct fusion protein would be expressed.

The plasmid phcavV5His was transfected into 293 PE cells with Lipofectamine Plus reagent (Invitrogen) according to manufacturer instructions. The cells were collected 48 h after transfection into centrifuge tubes, and the medium was decanted and washed twice with ice-cold PBS. The washed cells were frozen and kept at -85°C . The cells from six 15-cm plates were frozen

and thawed twice and suspended in 12 ml of ice-cold Tris-HCl buffer [50 mM (pH 8.5) at 4°C] containing 5 mM 2-ME, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin, and 1 mM Na_3VO_4 . The suspension was sonicated until 80% of the cells were lysed. The sonicate was centrifuged at $10,000 \times g$ 20 min at 4°C and the supernatant collected. The hcav-1-V5-His protein was additionally purified by affinity chromatography using Ni-NTA resin (Qiagen). Briefly, the column containing 6 ml of 50% slurry Ni-NTA resin was equilibrated with buffer A [20 mM Tris-HCl (pH 8.5) at 4°C , 100 mM KCl, 5 mM 2-ME, 10% v/v glycerol, and 20 mM imidazole]. The crude supernatant was loaded on the column and kept for 20 min at 4°C . The column was then washed with 10 volumes of buffer A at a flow rate of 0.5 ml/min, followed by a wash with 2 volumes of buffer B [20 mM Tris-HCl (pH 8.5) at 4°C , 1 M KCl, 5 mM 2-ME, and 10% v/v glycerol]. The column was washed again with 2 volumes of buffer A. The bound cav-1-V5-His was eluted with buffer C [20 mM Tris-HCl (pH 8.5) at 4°C , 100 mM KCl, 5 mM 2-ME, 10% v/v glycerol, and 100 mM imidazole]. The eluted fraction was concentrated using Centriprep YM-10 centrifuge ultrafiltration apparatus (Amicon). Aliquots from each step of the preparation of the hcav-1-V5-His were analyzed by Western blotting using rabbit polyclonal cav-1 antibody (Santa Cruz Biotechnology) and V5-HRP-conjugated antibody (Invitrogen); the purified cav-1-V5-His had an electrophoretic mobility slightly slower than the cav-1 from cell lysates of the human prostate cancer cell lines DU145 or PC3. The purified protein concentration was measured by silver staining (Bio-Rad) a known volume of the preparation separated on SDS-PAGE and comparison to a standard curve plotted from the band intensity scanned using NucleoVision software (Nucleo Tech Corp) of at least three different concentrations of BSA standard (Pierce).

Western Blot Analysis. Serum lipoproteins were separated into lipoprotein subfractions by KBr density gradient ultracentrifugation after a modified method of Redgrave (15). Serum HDL₃ fractions collected from 1 ml of serum, were delipidated, dialyzed, and freeze-dried. The powder was redissolved in 0.2 ml of 10 mM decyl sodium sulfate in PBS, and 10 μl of each sample or calibrator were mixed with SDS sample loading buffer, boiled for 5 min, and separated by 12% SDS-PAGE. The gel was transferred electrophoretically to a nitrocellulose membrane, and blotted with rabbit polyclonal cav-1 antibody (Santa Cruz Biotechnology) overnight at 4°C , with shaking. After incubation with HRP-conjugated secondary antibodies (ICN Biochemicals, Inc.), the binding was detected by enhanced chemiluminescence with Super Signal (Pierce).

Protocol for Serum Cav-1 Assay. Two commercial affinity purified polyclonal rabbit cav-1 antibodies were chosen for a direct sandwich ELISA based on results in a direct ELISA assay using recombinant His-tagged cav-1. The capture cav-1 antibody used was generated from human recombinant cav-1 (Transduction Laboratories), and the detection antibody was HRP-conjugated rabbit polyclonal antibody raised against a peptide mapping at the NH_2 terminus of human cav-1 (Santa Cruz Biotechnology). Costar microplate wells were coated with 100 μl of cav-1 antibody (5 mg/l) in carbonate buffer (pH 9.6) and incubated overnight at 4°C . The wells were then blocked with 200 μl of TBS containing 1.5% w/v BSA and 0.05% v/v

Tween 20 for 2 h at room temperature and were washed three times with TBS. Serum samples, calibrators, and controls were added to the wells and incubated for 2 h at room temperature. All samples consisted of 50- μ l aliquots diluted with 50 μ l of TBS containing 0.5% v/v Tween 20. The wells were washed four times with 200 μ l of TBS, and 100 μ l of HRP-conjugated cav-1 (Santa Cruz Biotechnology) antibody diluted 1:200 in TBS containing 1.5% w/v BSA and 0.05% v/v Tween 20 was added. After incubation for 90 min at room temperature, the wells were washed four times with TBS, and 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate solution (Sigma) was added and incubated for 20 min at room temperature. The reaction was stopped by adding 50 μ l of 2 N H₂SO₄, and the absorbance was read at 450 nm with a microplate reader (SLT Spectra; SLT Lab Instruments).

Sample Specimens. Serum cav-1 was measured in three groups of subjects 50–70 years of age. The control group consisted of sera taken from 81 men (age median 59.5 years) with normal digital rectal examinations and serum PSA levels \leq 1.5 ng/ml over a period of 2 years. The BPH group consisted of 107 men (age median 61.1 years) with clinical BPH. The PSA value for only a limited number of these BPH patients were available at the time of clinical diagnosis. A group of 102 men (age median 60.8 years) with clinically localized prostate cancer had serum samples collected preradical prostatectomy. The patients in this group had a median PSA level of 5.5 ng/ml (range, 0.5–48.7 ng/ml), a relatively low percentage of extracapsular extension (33.7%), seminal vesicle invasion (4.9%), and positive margins (14.7%). The patients in this group also had negative lymph nodes. The sera were frozen at -85°C until the day of analyses. Statistical analysis was performed using SPSS 11.0 statistical software (SPSS, Inc., Chicago, IL).

Determination of Optimal Antibody Concentration.

The working concentrations of the antibodies were determined by chessboard titration (16) of both capture and detection antibody using constant concentration of recombinant cav-1-V5-His-tagged protein. This process involves the dilution of the two antibody solutions against each other to examine the activities inherent at all of the resulting combinations.

Calibrators, Controls, and Standard Curve. Calibrators were prepared by mixing a known quantity of purified recombinant cav-1-V5-His with the serum of a normal young male with an undetectable basal level of cav-1. This stock standard and samples of the zero control serum were aliquoted and stored at -85°C . On the day of analysis, a standard curve was constructed by measuring the absorbance of a serially diluted stock standard using the zero control serum as a diluent. All measurements of standards, positive controls, and unknown sera were done in triplicates, and the average was taken as a final reading. Three positive control sera (high, medium, and low) were identified and subsequently a large volume of the corresponding serum was aliquoted and stored at -85°C .

Because the assay uses commercially available coating and detection antibodies, we were careful in our selection of batches of antibody. We routinely maintained a sample of the previous lot which was tested in comparison to a new lot to be purchased. The new lot was tested using three concentrations of the standard His-tagged cav-1 preparation and the three positive serum controls (high, medium, and low). We only selected lots that had

absorbance readings that did not differ by $>5\%$ from the previous lot of antibody.

Specificity. The assay specificity was tested using protein lysates from three cell lines with known cav-1 content based on Western blotting. The specificity of the assay was also tested by competitive inhibition using a synthetic peptide (N-20) that specifically binds and blocks the detection antibody (Santa Cruz Biotechnology; HRP-cav-1 antibody). The peptide was incubated with the antibody for 30 min at room temperature before addition to the wells.

Analytical Validation. The sensitivity of the assay, (*i.e.*, the lowest detectable cav-1 concentration that could be distinguished from 0 using statistical criteria) was calculated as the mean absorbance value of triplicate measurements of the 0 standard plus three times the SD. The concentration in ng/ml was calculated from the slope of the standard curve.

The intra-assay variance was determined for four serum samples with different levels (high, medium, low, and very low) of serum cav-1 to determine the precision of the assay. The samples were assayed in eight replicates. The inter-assay precision was determined in triplicates for four (high, medium, low, and very low) cav-1 level serum samples measured on eight subsequent independent assays.

Analytical recovery was measured using either a positive control serum or a negative control serum with undetectable cav-1 spiked with a predetermined concentration of recombinant cav-1. These two serums were serially diluted with negative control serum as diluent then assayed in triplicate.

Comparison of Serum and Plasma Cav-1. Three dilutions of cav-1 calibrator were made in either serum or plasma from two healthy control individuals with previously determined undetectable serum cav-1. The standard immunoassay was performed in triplicate, and the mean cav-1 levels were compared. In addition we compared cav-1 concentrations in the serum and plasma of two prostate cancer patients known to have elevated cav-1 levels.

RESULTS

Detection of Cav-1 in HDL₃ Fraction of Serum. On the basis of our previous results indicating secretion of cav-1 by prostate cancer cells *in vitro* (13), serum samples from men with or without prostate cancer were screened for the presence of cav-1 by Western blotting. The sera were fractionated by KBr density gradient ultracentrifugation, and a quantity of the HDL₃ fraction equivalent to 50 μ l of serum was loaded. The band corresponding to cav-1 that migrates with an apparent molecular weight of M_r 22,000–24,000 was detected in 14 of 16 prostate cancer samples, whereas only 4 of 16 samples of the control showed detectable level bands (Fig. 1). The standard recombinant purified cav-1 with a V5–6X-His tag migrated at a somewhat slower rate than the authentic cav-1.

Development of the Cav-1 Assay. The optimum cav-1 antibody pair was selected for the standard assay by testing a number of commercially available mouse and rabbit cav-1 antibodies using a direct ELISA and a known quantity of the recombinant cav-1. The antibody pair that gave the highest sensitivity with lowest background signal was then tested with different concentrations of both the capture and detection anti-

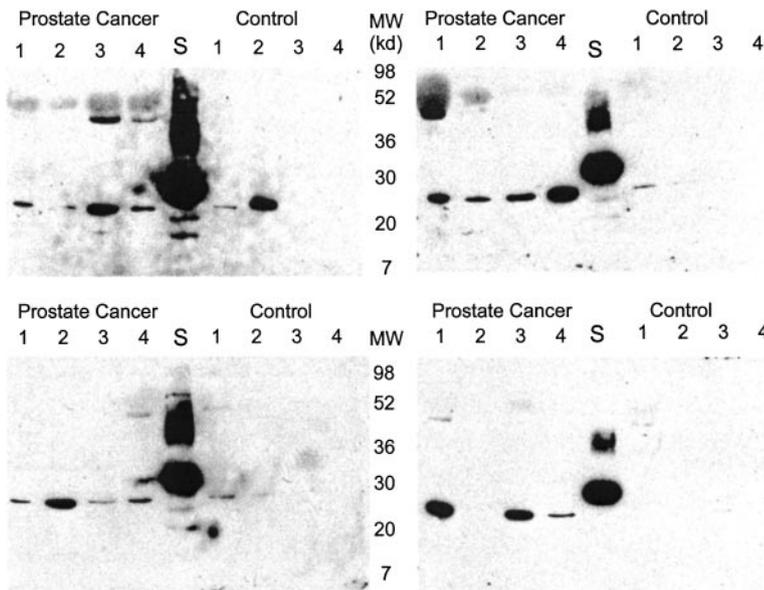


Fig. 1 Detection of cav-1 in HDL₃ fraction of serum. Serum samples from men with or without prostate cancer were screened by Western blot. The amount of the HDL₃ fraction equivalent to 50 μ l of serum was loaded in each lane. S: purified cav-1-V5-His protein standard (0.1 μ g).

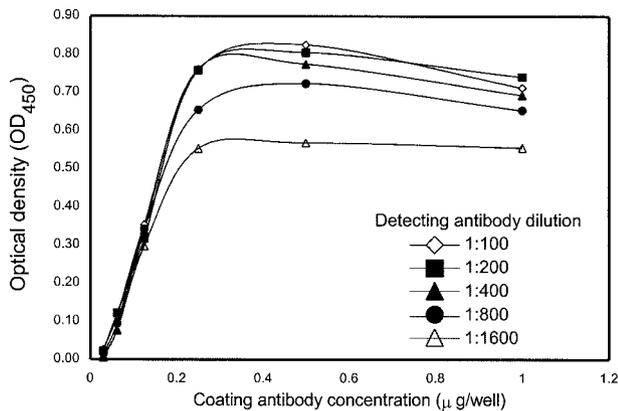


Fig. 2 Effect of coating antibody and detection antibody concentrations on sensitivity. The coating antibody was serially diluted in carbonate buffer, and the detection antibody was serially diluted in TBS blocking buffer. Each data point is the mean of three determinations. Coating antibody concentration of 0.5 μ g/well, and a 1:200 detection antibody dilution was selected for the standard assay protocol.

bodies (Fig. 2). A concentration of 0.5 μ g/well for coating (capture) cav-1 antibody and a dilution of 1/200 of the HRP-conjugated detection antibody (0.2 mg/ml of antibody) were selected for the standard assay. The incubation time, coating buffer, serum volume, and diluting buffer selected in this immunoassay were based on experiments that revealed optimum detection sensitivity with minimal background or nonspecific signal.

A linear standard curve was constructed using a concentration range (0.0–28.2 ng/ml) of recombinant cav-1-V5-His-tagged protein added to a control serum with undetectable cav-1. The reproducibility of the standard curve was demonstrated in five independent assays (Fig. 3).

The specificity of the cav-1 assay was tested in three cell

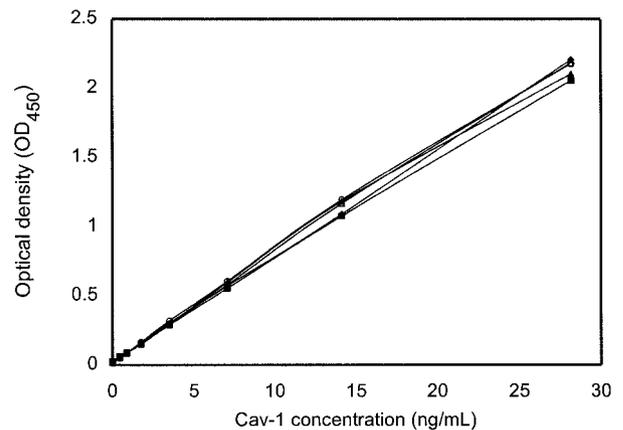


Fig. 3 Cav-1 calibration curves. Individual lines represent five independent calibration curves. Stock standard cav-1 concentration of 28.8 ng/ml in zero control serum was serially diluted using the same serum as diluent and then used in the cav-1 assay. Each data point is the mean of three determinations.

lysates, two with a high cav-1 content (human prostate cancer cell line, DU145, and mouse endothelial cell line, MS1) and one (human prostate cancer cell line, LNCaP) with undetectable cav-1 level. The absorbance for LNCaP lysate was equivalent to the background, and those for DU145 and MS1 were extremely high, 3.62 and 2.99, respectively (Table 1). The specificity of the assay was also tested by competitive inhibition using a synthetic peptide specific for the detection antibody. The peptide caused a dose-dependent reduction of the signal using recombinant cav-1 as a calibrator (data not shown).

Detection Limit, Precision, and Recovery. The minimum detection limit of the assay was 0.017 ng/ml when calculated using the response for the zero calibrator plus 3 SD, repeated in eight different independent assays. Interassay preci-

Table 1 Specificity of the cav-1 ELISA

Cell lysate	A _{450nm}	Cav-1 level ^a
Blank	0.064	
LNCaP ^b (low passage)	0.076	Undetectable
MS1 ^c	2.99	High
DU145 ^b	3.62	High

^a Western blot.^b Human prostate cancer cell line.^c Mouse endothelial cell line.

Table 2 Precision of the cav-1 ELISA

Sample	Cav-1 level	Interassay		Intra-assay	
		ng/ml	CV (%)	ng/ml	CV (%)
A	Very low	0.4–0.48	6.43	0.25–0.285	6.74
B	Low	1.84–2.1	5.6	2.6–2.8	3.1
C	Medium	6.3–7.8	4.26	13.2–14.6	3.07
D	High	28.56–30.76	2.81	30.29–31.89	2.29

sion was determined using four different pools of serum in eight independent assays over a period of 1 month using two different lots of capture antibody and one lot of HRP-conjugated antibody (CV < 6.43%). The intra-assay precision was determined using four different pools of serum assayed in eight replicates in one assay (CV < 6.74%; Table 2).

The recovery of cav-1 in a positive control serum sample was determined in three serial dilutions (1:2, 1:4, and 1:8), and the median recovery was 92.7% (range, 89.55–99.25%). Using the His-V5-cav-1 calibrator spiked into a serum with zero cav-1 concentration and serially diluted, the median recovery was 100.14% (range, 98.3–100.28%; Table 3). We additionally determined the recovery using a positive control serum with extremely high cav-1 (126.13 ng/ml), and when this sample was diluted 10-fold, the recovery was 104.95%.

Cav-1 in Plasma Samples. The cav-1 immunoassay for the measurement of cav-1 in plasma specimens showed similar sensitivity and reproducibility to that of serum using two positive control sera, as well as standard cav-1 calibrator spiked into two negative control sera and plasma samples (Table 4).

Cav-1 Levels in Prostate Cancer, BPH Patients, and Controls. The serum cav-1 concentration in 81 control subjects was measured, and the median was 0.324 ng/ml (range, 0.0–5.73 ng/ml). The median cav-1 concentration in the sera of the men with BPH was 0.172 ng/ml (range, 0.0–59.9 ng/ml). The serum levels in the control group were not significantly different from the BPH group ($P = 0.835$, Mann-Whitney test). Comparisons between levels of cav-1 were made with Mann-Whitney test, which uses the number of times a value in one group precedes a value in the other group when values are sorted in ascending order. The median cav-1 concentration in the sera of the prostate cancer patients was 0.461 ng/ml (range, 0.0–95.8 ng/ml), which was significantly higher than the control group ($P = 0.0446$, Mann-Whitney test) or the BPH group ($P = 0.0317$, Mann-Whitney test; Table 5). We also analyzed the prostate cancer samples for statistical correlation of serum cav-1 levels with serum PSA and specific clinical and pathological

Table 3 Analytical recovery of cav-1

Dilution	Serum-positive control		Standard recombinant cav-1	
	ng/ml	% recovery	ng/ml	% recovery
1:2	8.04	99.25	14.12	98.3
1:4	4.02	89.55	7.06	100.14
1:8	2.01	92.7	3.53	100.28

Table 4 Comparison of cav-1 detection in serum and plasma

	Serum cav-1 (ng/ml)	Plasma cav-1 (ng/ml)
Specimen A ^a (ng/ml)		
0	0.0	0.0
3.53	4.15	4.76
7.06	8.83	9.25
14.1	16.7	16.35
Specimen B ^a (ng/ml)		
0	0	0
3.53	4.25	4.84
7.06	9.06	9.19
14.1	16.2	16.29
Positive control 1 ^b	17.18	20.97
Positive control 2 ^b	4.88	4.46

^a Different concentrations of cav-1 calibrator spiked into the serum and plasma specimens of two independent control subjects with undetectable cav-1 levels.

^b Two independent prostate cancer patients with serum and plasma collected at the same time.

parameters associated with these prostate cancer specimens (Gleason score, positive margin status, extracapsular extension, and seminal vesicle invasion), but no significant correlations were observed (Spearman Rank correlation).

DISCUSSION

In this study, we report the development of a specific and sensitive immunoassay for the measurement of serum cav-1 and the preliminary evaluation of its potential to discriminate men with or without prostate cancer. We have developed insight into the progression of prostate cancer through investigations of cav-1 expression specifically in metastatic disease and have documented its potential as a biomarker for prostate cancer. Our previous clinical studies have included (a) the initial identification of cav-1 overexpression in metastatic prostate cancer (9); (b) the determination of cav-1 as an independent prognostic marker for prostate cancer progression in lymph node-negative patients who have biochemical recurrence of disease after radical prostatectomy (10); and (c) a significant association of increased cav-1 in prostate cancer in African-American men versus white-American men (11). Our basic studies have elucidated one aspect of the mechanism of action of cav-1 by showing that cav-1 has antiapoptotic properties under a variety of clinically relevant circumstances, including androgen and growth factor deprivation and oncogene overexpression (12, 17, 18). Overall, our research in this area has contributed to our understanding of androgen-insensitive metastatic prostate can-

Table 5 Statistical comparison of serum cav-1 in control, BPH, and prostate cancer patients

Group	n	Median (ng/ml)	Range (ng/ml)	Mean ranks ^a / <i>P</i> (versus) control	Mean ranks ^a / <i>P</i> (versus) cancer
Control	81	0.324	0.0–5.7		83 vs 99/0.0446
BPH	107	0.172	0.0–59.9	94 vs 95/0.8350	96 vs 114/0.0317
Prostate cancer	102	0.463	0.0–95.8	99 vs 83/0.0446	

^a Calculated using combined, sorted, and ranked two-group data.

cer. We recently documented that prostate cancer cells secrete cav-1 and that secreted cav-1 can stimulate viability and clonal growth in prostate cancer cells that do not express cav-1 (13). The concept of a secreted autocrine or paracrine factor that directly contributes to androgen resistance in prostate cancer is novel and represents an efficient mechanism for maximizing resistance to various proapoptotic stimuli that metastatic cells often encounter during the highly inefficient process of metastasis (19). Interestingly, although many cell types express cav-1, the secretion of cav-1 appears to be restricted. Thus far, secretion of cav-1 has been reported for normal pancreatic exocrine cells (20) and pituitary cells (8) in addition to prostate cancer cells (13). The biological activities of secreted cav-1 indicate that this molecule represents a potential therapeutic target for prostate cancer. Indeed, proof of principle for this concept has been established as we have shown that cav-1 antibody can have antimetastatic activities *in vivo* when administered systemically (13).

Importantly, the detection of secreted cav-1 in patient sera has a significant potential for clinical use. Unlike PSA, secreted cav-1 is associated with malignant properties of prostate cancer cells, and serum cav-1 may have unique diagnostic/prognostic capacity. Our preliminary observation that cav-1 is detected in the HDL₃ fraction of serum samples of patients with prostate cancer (13) is consistent with the view that HDL₃ is an acceptor of excess cholesterol in the plasma membrane. It is also consistent with previous studies showing that caveolae are a major site of cholesterol efflux from cells and that cav-1 mediates the intracellular movement of cholesterol (21). Our data further raise the possibility that cav-1 can be associated with cholesterol in the context of a serum lipoprotein particle. A previous study reported that normal human serum does not contain cav-1 based on Western blotting analysis of the HDL fraction of human serum (20). Our current results are in agreement with the results of this study in that the normal human serum level of cav-1 is very low to undetectable. However, based on Western blot analysis, we report here (Fig. 1) the levels of cav-1 in a number of prostate cancer patients are significantly higher than those of controls. This result was confirmed by analysis of 102 prostate cancer patient sera and 81 control sera using an immunoassay for the measurement of cav-1. Significantly higher levels of cav-1 were detected in the patient sera than the control sera ($P = 0.0446$, Mann-Whitney test).

Our direct sandwich immunoassay was developed using two polyclonal antibodies for cav-1. The HRP-conjugated detection antibody showed a remarkable specificity to detect serum, plasma, and tissue-derived cav-1, as well as a recombinant COOH-terminal His-tagged cav-1 with minimal nonspecific background. Monoclonal antibodies for cav-1 that are commercially available were tested, but a consistently low signal was

detected using cav-1-positive sera. Some monoclonal antibodies also failed to detect the recombinant His-tagged cav-1, potentially because of a conformational change in the modified protein. A common problem encountered using polyclonal antibodies as reagents in immunoassays is that different batches of antibodies may vary markedly in their specificity and affinity for the antigen. In our assay system, we overcame this problem by extensive pretesting and comparing any new batch or lot of antibody with the previous batch; only lots that differed by <5% from the original lot were used.

The use of pooled serum for the calibrator in our assay likely improves the accuracy of the assay as it guarantees that the binding affinity between the antibody and the calibrator is the same as that between the sample analyte and antibody. The use of inappropriate calibrators in many early PSA immunoassay protocols contributed to disagreement among assays of PSA values produced on the same specimens (22).

Among the possible future applications of the cav-1 serum assay is its use as a prognostic tool before or after prostatectomy or radiation therapy for clinically localized disease. Therefore, the sensitivity of the assay is a major consideration, and the capability to detect low levels of cav-1 (below 1.0 ng/ml) is highly desirable. In general, the sensitivity of an ELISA is largely determined by the affinity between the antibody and the analyte. However several other assay parameters can be optimized to improve assay sensitivity such as total reaction volume, sample concentration, diluent composition, and incubation time for the interaction between the coated antibody and the analyte and between the antibody-bound analyte and the secondary or detecting antibody. We found that the use of 0.5% v/v Tween in the incubation buffer improved the sensitivity of the assay considerably; this may be attributable to increased solubility of serum cav-1 without affecting the binding capacity or the dissociation of the coating antibody from the microplate. Tween concentrations > 0.5% v/v resulted in reduced assay sensitivity.

The results that show serum cav-1 levels in BPH patients were significantly lower than the prostate cancer patients and that the BPH serum levels were not significantly different from the controls clearly indicate that serum cav-1 could be a valuable tool to discriminate between BPH and prostate cancer patients. A close examination of the cav-1 serum levels of the BPH group showed a relatively large sample to sample variation with ~6.5% of the patients having cav-1 values > 10 ng/ml. We have no clear explanation for the observed high cav-1 levels. However, it is conceivable that some of these patients harbored prostate cancer that was not clinically detectable at the time of examination. Unfortunately, clinical follow-up information on these patients is not available.

Our correlation analysis of serum cav-1 levels with path-

ological parameters associated with prostate cancer did not reveal any significant associations. In our previous studies, we demonstrated that cav-1 expression was focally positive in primary tumors (9), yet the percentage of cav-1-positive cells increased to ~40% in androgen-insensitive disease (13). As these results indicate that an increase in cellular cav-1 and therefore potentially secreted cav-1 is associated with virulent disease, a failure to demonstrate positive correlations with pathological markers of progression was not unexpected for this specific cohort of prostate cancer specimens. The clinical and pathological data of this group of patients indicate that the majority of the patients are in an early phase of the disease with relatively low Gleason grades, seminal vascular invasion, or positive margins. The median PSA level of this group was relatively low, 5.55 ng/ml.

In conclusion, we describe here the initial development of a direct sandwich ELISA for the measurement of serum cav-1 using two polyclonal antibodies and a purified recombinant cav-1 protein as a standard. This assay is highly sensitive, specific, and reproducible for the detection of serum cav-1. The median serum cav-1 levels in the prostate cancer patient group were significantly higher than controls or BPH, suggesting that cav-1 in serum could be an important biomarker for prostate cancer diagnosis/prognosis and progression. We anticipate that additional studies using larger and more specific patient populations, including men with more advanced disease, will establish significant clinical utility for serum cav-1 analysis.

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