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14. ABSTRACT

Overall, we have made exceptional progress in the first year funding period of this project. Tasks 1-3 are on schedule and although, as is often the case, we have made some unanticipated and necessary adjustments (replacement of 178-2BMAK cells with RM-9 cells for Task 3) we are moving toward our stated goals. Importantly, our data thus far, are yielding important mechanistic insight into the angiogenic activities of prostate cancer cell derived, secreted cav-1. In our view, after we further define these mechanisms we will be able to take the next important step, exploiting this new knowledge for prostate cancer diagnosis and therapy.

15. SUBJECT TERMS
Prostate cancer, angiogenesis, caveolin-1

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Introduction

Background Caveolin-1 (cav-1) is an important structural/regulatory molecule involved in many aspects of molecular transport and cell signaling. Cav-1 activities are dependent on protein level and cell context, yet an understanding of the biological consequences of inappropriate cav-1 expression in malignant cells has been elusive. We have shown previously that cav-1 up-regulation is associated with metastatic, androgen-insensitive prostate cancer. In studies funded by this grant we identified an underlying mechanism for the selection of cav-1 overexpression in prostate cancer cells during progression. We found that cav-1 binds to and inhibits the activities of PP1/PP2A serine / threonine phosphatases, preventing inactivation of Akt through dephosphorylation and thus sustaining levels of phospho-Akt and its oncogenic activities. Recently we have shown that cav-1 overexpression leads to increased levels of c-myc protein, and our preliminary data and the results of published studies lead us to speculate that the mechanisms for cav-1 mediated c-myc stabilization also involve PP1/PP2A inhibition and phospho-Akt stabilization. We have also shown that cav-1 overexpression leads to up-regulation and secretion of VEGF, FGF2 and TGF-β1, indicating that cav-1 stimulated molecular pathways can regulate these growth factors (GF) /angiogenic cytokines (AC). Importantly, we have also discovered that cav-1 itself is specifically secreted by prostate cancer cells and is taken up by prostate cancer cells and endothelial cells (EC).

Hypothesis These results suggest that cells expressing cav-1 can function as "feeder cells" for local and potentially distant prostate cancer cells and tumor-associated EC through secretion of cav-1 and cav-1 stimulated GF/AC. In support of this concept we have shown that experimentally induced metastasis is potentiated in host transgenic mice that overexpress cav-1 and is suppressed in host $cav-1^{-/-}$ mice. We propose that prostate tumor cells secrete cav-1 which induces specific changes in EC that potentiate angiogenesis and metastatic activities.

Specific Aims Using in vitro and in vivo models that involve novel genetically mutant mice we propose to resolve the molecular and cellular pathways that support these oncogenic activities through specific aims to: (1) to analyze the effects of cav-1 conditioned media on $cav-1^{-/-}$ endothelial cell gene expression and biological activity; (2) map the molecular pathways involved in EC angiogenesis stimulated by cav-1 protein alone or together with specific GF/AC; and (3) to correlate the effects of prostate cancer cell derived cav-1 uptake by TAEC with tumor growth activities.

Study Design We have generated and characterized mice that have a deletion of the $cav-1$ gene. These will be a source for isolation of EC that will be treated with conditioned media derived from prostate cancer cells with controlled levels of cav-1 protein (Aim 1) or with purified cav-1 protein (Aim 2). We will determine the molecular events that are induced by cav-1 in EC by analyzing expression of signalling molecules such as phosphorylated Akt, myc and relevant GF/AC (VEGF, FGF-2, and TGF-β1) at the transcription level by quantitative RT-PCR and at the protein level by western blotting. Additionally we will evaluate events further downstream of signalling such as nitric oxide (NO) production. Biological activities of the EC in response to cav-1 that we will examine will include proliferation, chemoattraction, motility and vasculogenesis. The validation of these activities will be accomplished by using specific molecular inhibitors of the signalling molecules. The final aim of the grant will focus on in vivo studies using a novel mouse metastatic prostate cancer cell line that overexpresses cav-1 and has a high level of metastasis to lung and to bone. We will inject this cell line orthotopically into $cav-1^{-/-}$ mice and then analyze TAEC responses locally and at distant sites of metastasis by immunohistochemistry. Treatment of the mice with cav-1 specific antibody will directly test the effects of blocking cav-1 uptake in vivo on the growth and progression of experimental prostate cancer in this model.

Relevance These studies could lead to the use of cav-1 positive TAEC as a potential prognostic/ predictive biomarker for prostate cancer in man. They will further serve to test the therapeutic potential of cav-1 antibody approaches for the treatment of prostate cancer.

Body

Task 1 *To analyze the effects of cav-1 conditioned media(CM) on cav1$^{-/-}$ endothelial cell gene expression and biological activity*

1. Prepare EC from aorta of 40 *cav-1$^{-/-}$* and 20 *cav-1$^{+/+}$* mice and CM from cav-1 (500ml) or pcDNA (500ml) transfected LP-LNCaP cells.
   (Months 1-3)
2. Perform western blotting and QRT-PCR on EC lysates treated with cav-1 CM. (Months 3-18)
3. Develop biological assays for the EC activity (Motility/invasion, migration, tubule formation, NO and PGI2 determination) and analyze the effect of soluble cav-1 on these activities.
   (Months 18-36)

We have prepared EC from the aorta of 10 *cav-1$^{-/-}$* and 5 *cav-1$^{+/+}$* mice and the cells were subcultured using endothelial cell growth medium (EGM) and confluent monolayer exhibited the typical cobblestone pattern and positive staining for uptake of Di-l-Ac-LDL. The cells were allowed to grow and passaged twice to generate sufficient numbers for analysis. Passage 4 will be used for all assays. CM from phcav-1 and pcDNA transfected LNCaP was prepared and stored in –80 ^{o}C. Mutagenesis experiments have identified the cav-1 scaffolding domain (CSD) residues 82-101 as the region of cav-1 responsible for mediating the interaction with a number of signaling proteins including eNOS, platelet activating factor (PDGF) receptors, epidermal growth factor (EGF), the kinases Src and Fyn, heterotrimeric G-protein and cholesterol binding. Therefore we constructed cav-1 plasmid with deleted CSD termed phΔcav-1-V5-His, and prepared CM from phΔcav-1-V5-His transfected LNCaP cells. The use of recombinant protein with deleted CSD (rΔcav-1) as well as CM will be important to investigate the role of CSD in the secretion, uptake and angiogenic activities of ECs.
The study of protein expression using western blotting and gene expression using QRT-PCR in EC lysates treated with cav-1 CM is underway.

Task 2 *Map the molecular pathways involved in EC angiogenesis stimulated by cav-1 protein aione or together with specific GF/AC.*
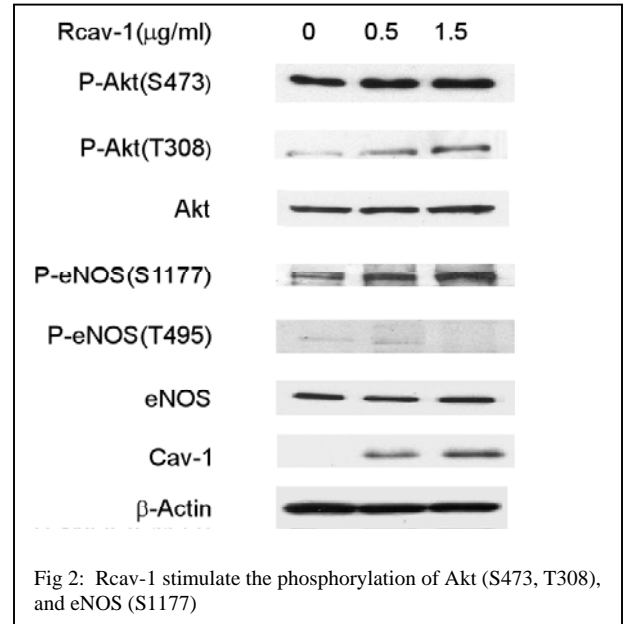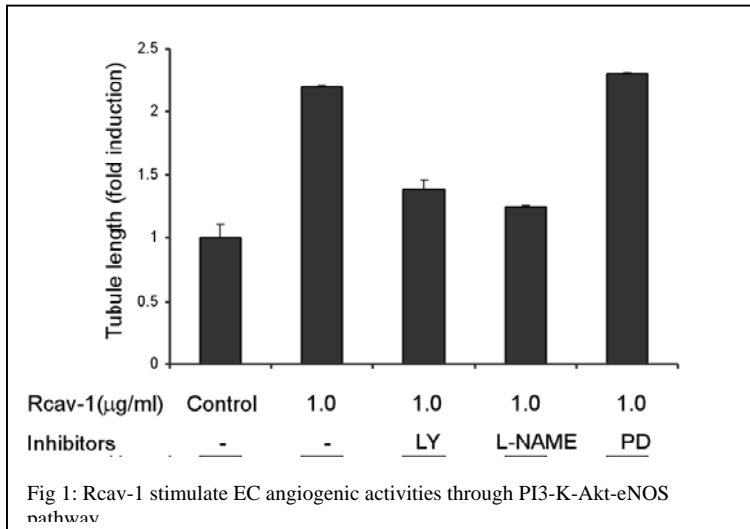
1. Preparation/purchase of reagents, including recombinant cav-1, VEGF, FGF-2, TGFβ1, SiRNA for VEGFR2, PI3-K, Akt, ERK1/2 and eNOS, chemical inhibitors for VEGFR2, PI3-K, ERK1/2 and eNOS.
   (Months 1-3)
2. Optimize conditions for SiRNA transfection and for chemical inhibitions.
   (Months 1-6)
3. Analysis of gene knock down, including QRT-PCR analysis for mRNA and western analysis for protein. Analysis range covers target genes and their downstream components.
   (Months 7-24)
4. Biological function end points.
   (Months 25-36)

We have purchased and tested all of the growth factors and siRNAs for VEGFR2, PI3-K, Akt, ERK1/2 and eNOS as well as chemical inhibitors such as LY294002, PD98059 and L-NAME . Recombinant cav-1 (rcav-1) and rΔcav-1 were purified from the 293PE cell lysates by transiently transfecting cav-1-V5-His or Δcav-1-V5-His and using NI-NTA agarose beads. The purified proteins were divided into small aliquots and stored at –80 ^{o}C. We are in the process of optimizing the conditions for SiRNA transfection and the chemical inhibitors. It is important to note that these processes take along time to perform as they require a large number of cells and the primary ECs are known for their slow rate of growth and multiplication.
We investigated the molecular pathways that mediate rcav-1 stimulated angiogenic activities and we tested the effects of inhibitors for PI3-K (LY294002), eNOS (L-NAME) and erk1/2 (PD98059) on tubule formation in rcav-1 treated *cav-1$^{-/-}$* EC. The results demonstrated that LY294002 and L-NAME but not PD98059 significantly suppressed these rcav-1 stimulated activities, suggesting the involvement of PI3-K-Akt–eNOS pathway (Fig. 1).We also analyzed the effects of rcav-1 on the phosphorylation status of Akt and its downstream target protein eNOS in *cav-1$^{-/-}$* EC. Rcav-1 treatment resulted in a dose dependent increase in Akt phosphorylation on S473 and T308 with no change in total Akt. Rcav-1 treatment also led to increased eNOS

phosphorylation on S1177 but not T495 which demonstrated low and potentially reduced phosphorylation following treatment (Fig 2).

The study of gene expression using QRT-PCR and proteins expression using western blotting in EC lysates treated with rcav-1 and/or GFs is underway.

Fig 1: Rcav-1 stimulate EC angiogenic activities through PI3-K-Akt-eNOS pathway

Fig 2: Rcav-1 stimulate the phosphorylation of Akt (S473, T308), and eNOS (S1177)

Task 3 *To correlate the effects of prostate cancer cell derived cav-1 uptake by TAEC with tumor growth activities.*

1. Inject 178-2 BMAK cells into dorsolateral prostate of twenty *cav-1$^{-/-}$* mice and analyze tumor weight and metastasis at 21 day, fixed time point. Analyze tissues by immunohistochemistry
    (Months 1-12)
2. Inject 178-2 BMAK cells into dorsolateral prostate of thirtynine *cav-1$^{-/-}$* mice then treat with HBSS, rabbit IgG, or rabbit anti-cav-1 serum (thirteen per group) and analyze tumor weight and metastasis at 21 day, fixed time point (12-18 months). Analyze tissues by immunohistochemistry
    (Months 12-36)
3. Inject 178-2 BMAK cells into dorsolateral prostate of thirty *cav-1$^{-/-}$* mice then treat with HBSS, rabbit IgG, or rabbit anti-cav-1 serum (ten per group) and analyze tumor weight and metastasis at survival time point (12-18 months). Analyze tissues by immunohistochemistry
    (Months 12-36)

In the first funding period (months 1-12) we made several attempts to use the 178-2BMAK cells in orthotopic *cav1$^{-/-}$* mouse model. The 178-2BMAK cells were originally isolated from 129sv mice and form orthotopic tumor and metastasis in the syngeniec mice. However, due to histo-compatibility issues *cav1$^{-/-}$* mice injected with 5000 or 10000 of 178-2BMAK cells did not form measurable tumors at the injection site within the 21 day period. Despite the fact that ES cells containing *cav1$^{-/-}$* allele were isolated from the mice of 129sv background, the founder *cav1$^{-/-}$* mouse was bred for several generations with C57/BL6 mice. The currently available *cav1$^{-/-}$* mice are mainly of C57/BL6 background that may explain poor "take" of 178-2BMAK (129SV) cells in this model.

To study the effect of prostate cancer cell derived cav-1 uptake by TAEC we replaced 178-2BMAK with RM-9 prostate cancer cells that were originally isolated from C57/BL6 mice. We have previously demonstrated that RM-9 cells secrete cav-1 into conditioned media in vitro and can be used in the orthotopic mouse prostate cancer model in vivo. In this new experimental setting 5000 RM-9 cells were injected into dorsolateral prostate of *cav1$^{-/-}$* or *cav1$^{+/+}$* mice and the tumor size was analyzed 21 days after injection. By day 21 *cav1$^{-/-}$* mice

developed significantly smaller prostate tumors (Fig 3a) that demonstrated reduced number of CD31 positive blood vessels compared to $cav1^{+/+}$ (Fig 3c). Analysis of pelvic lymph nodes also demonstrated that $cav1^{-/-}$ mice had less lymph node metastases compared to $cav1^{+/+}$ mice (Fig 3b). We will continue to analyze tissue collected in this experiment to demonstrate cav-1 uptake by TAEC.
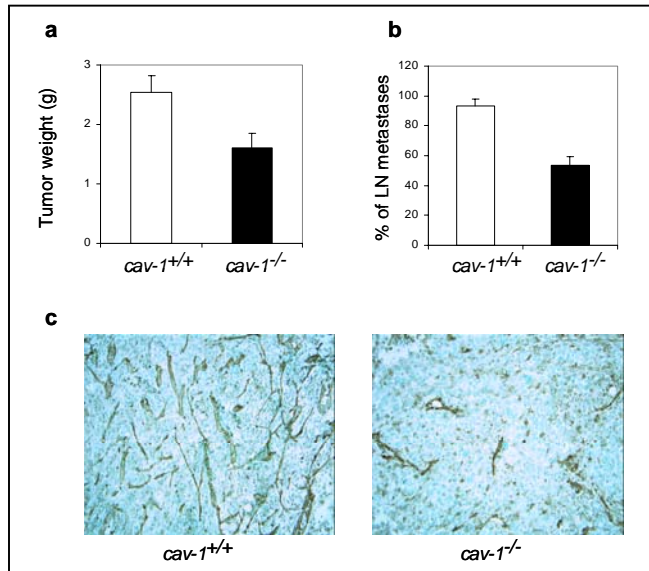

Fig 3. Secreted cav-1 promotes tumor progression and angiogenesis in orthotopic RM-9 mouse prostate cancer model. (**a**) Higher tumor wet weight in cav-1$^{+/+}$ than cav-1$^{-/-}$ mice. (**b**) Increased percentage of macroscopic LN metastases in cav-1$^{+/+}$ mice compared to cav-1$^{-/-}$. (**c**) Immunofluorescence staining of CD31 in tumor sections shows that $cav-1^{+/+}$ have higher number of microvessels than $cav-1^{-/-}$.

Key Research Accomplishments

1- Cav-1 stimulates angiogenic activities in $cav-1^{-/-}$ mouse EC.
2- Akt (S473, T308) and eNOS (S1177) are significantly phosphorylated in rcav-1 treated $cav-1^{-/-}$ mouse EC.

Reportable Outcomes

Presentations:
1.   Tahir SA, Yang G, Goltsov A, Watanabe M, Tabata K, and Thompson TC. Caveolin-1 uptake by endothelial cells promotes angiogenic activities and is associated with prostate cancer progression. American Association for Cancer Research, 97th Annual Meeting, Washington DC, April 1-5, 2006.
2.   Tahir SA, Yang G, Goltsov A, Watanabe M, Tabata K and Thompson TC. Caveolin-1 uptake by endothelial cells promotes angiogenic activities and is associated with prostate cancer progression. 4th Annual Cancer Center Symposium, Houston, TX, November 3, 2006.
3.   Miles BJ, Yang G, Addai J, Wheeler TM, Frolov A, Kadmon D and Thompson TC. Correlative evidence that prostate cancer cell-derived caveolin-1 mediates angiogenesis. American Society of Clinical Oncology annual meeting, June 1 – 5, 2007, Chicago, IL.
Manuscripts:
1.   Yang G, Addai J, Wheeler TM, Frolov A, Miles BJ, Kadmon D and Thompson TC. Correlative evidence that prostate cancer cell-derived caveolin-1 mediates angiogenesis. Human Path, submitted, 2006.
2.   Tahir ST, Frolov A, Hayes TG, Mims, MP, Miles BJ, Lerner SP, Wheeler TM, Ayala G, Wheeler TM, Thompson TC and Kadmon D. Preoperative serum caveolin-1 as a prognostic marker for recurrence in a radical prostatectomy cohort. Clin Can Res, 12(16):4872-4875, 2006.
3.   Tahir SA, Yang G, Goltsov AA, Watanabe M, Tabata K, Wheeler TM, Ayala G, Kadmon D and Thompson TC. Secreted caveolin-1 stimulates angiogenesis in prostate cancer. In preparation, 2006.

Conclusion:

Overall, we have made exceptional progress in the first year funding period of this project. Tasks 1-3 are on schedule and although, as is often the case, we have made some unanticipated and necessary adjustments (replacement of 178-2BMAK cells with RM-9 cells for Task 3) we are moving toward our stated goals.

Importantly, our data thus far, are yielding important mechanistic insight into the angiogenic activities of prostate cancer cell derived, secreted cav-1. In our view, after we further define these mechanisms we will be able to take the next important step, exploiting this new knowledge for prostate cancer diagnosis and therapy.

References:
None cited in text

Appendices:

Tahir ST, Frolov A, Hayes TG, Mims, MP, Miles BJ, Lerner SP, Wheeler TM, Ayala G, Wheeler TM, Thompson TC and Kadmon D. Preoperative serum caveolin-1 as a prognostic marker for recurrence in a radical prostatectomy cohort. Clin Can Res, 12(16):4872-4875, 2006.

Preoperative Serum Caveolin-1 as a Prognostic Marker for Recurrence in a Radical Prostatectomy Cohort

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Abstract

Purpose: Up-regulation of caveolin-1 (cav-1) is associated with virulent prostate cancer, and serum cav-1 levels are elevated in prostate cancer patients but not in benign prostatic hyperplasia. In this study, we evaluated the potential of high preoperative serum cav-1 levels to predict biochemical progression of prostate cancer. The value of the combined preoperative markers, prostate-specific antigen (PSA), biopsy Gleason score, and serum cav-1 for predicting biochemical recurrence was also investigated.

Experimental Design: Serum samples taken from 419 prostate cancer patients before radical prostatectomy were selected from our Specialized Programs of Research Excellence prostate cancer serum and tissue bank. Serum samples were obtained 0 to 180 days before surgery and all patients had complete data on age, sex, race, stage at enrollment, and follow-up for biochemical recurrence. Serum cav-1 levels were measured according to our previously reported ELISA protocol.

Results: Cav-1 levels were measured in the sera of 419 prostate cancer patients; the mean serum level was 4.52 ng/mL (median 1.01 ng/mL). Patients with high serum cav-1 levels had a 2.7-fold ($P = 0.0493$) greater risk of developing biochemical recurrence compared with those with low serum cav-1 levels. Importantly, patients with serum PSA ≥ 10 ng/mL and elevated levels of serum cav-1 had 2.44 times higher risk ($P = 0.0256$) of developing biochemical recurrence compared with patients with low levels of cav-1. In addition, high serum cav-1 levels combined with increasing biopsy Gleason score predicted much shorter recurrence-free survival in the group of patients with PSA ≥ 10 ng/mL ($P = 0.0353$). Cav-1 was also able to distinguish between high- and low- risk patients with biopsy Gleason score of seven, after adjusting, for patients PSA levels ($P = 0.0429$).

Conclusions: Overall, elevated preoperative levels of serum cav-1 predict decreased time to cancer recurrence. In the subset of patients with serum PSA of ≥ 10 ng/mL, the combination of serum cav-1 and biopsy Gleason score has the capacity to predict time to biochemical recurrence.

In 2005, ~90% of newly diagnosed prostate cancer patients had clinically localized disease (1). Consequently, the majority of patients are treated with curative intent by either radical prostatectomy or radiation therapy. It is well established, however, that 10% to 50% of patients who undergo radical prostatectomy will show biochemical evidence of disease recurrence [prostate-specific antigen (PSA) recurrence] within 5 years of surgery (2, 3). Various clinical variables have been used, singly and in combination (nomograms, tables, etc.), to predict, preoperatively, which patients are likely to fail definitive therapy (4). However, the predictive value of these variables has been thwarted by the vexing biological diversity of clinical prostate cancer. New markers are needed, preferably serum markers that have been mechanistically implicated in the progression of virulent disease. We believe that serum caveolin-1 (cav-1) may be such a marker.

Cav-1 is an important structural/regulatory molecule involved in many aspects of molecular transport and cell signaling (5, 6). Tissue cav-1 is overexpressed in metastatic and in hormone-resistant prostate cancer (7). Overexpression correlates with a shortened interval to disease recurrence following therapy for localized disease and tends to be associated with a high Gleason score pathologically (8–10). Interestingly, cav-1 is secreted by prostate cancer cells (11) and we have developed a sensitive ELISA immunoassay for the detection of cav-1 in the serum (12). In a preliminary study, we documented that prostate cancer patients have a higher serum cav-1 level when compared with age-matched controls with benign prostatic hyperplasia (12).

We report here the utility of a single preoperative measurement of serum cav-1 for predicting disease recurrence in a cohort of 419 prostate cancer patients undergoing radical prostatectomy at our institution.

Materials and Methods

Study population. The sera of 419 prostate cancer patients were obtained from the Specialized Programs of Research Excellence prostate cancer blood and tissue bank at Baylor College of Medicine. Entry into the study required availability of preoperative serum samples obtained within 6 months of surgery and complete data on age, race, stage at enrollment and follow-up, as well as availability of postoperative serum samples, as this is a part of a larger ongoing investigation. In addition, patients could have had no preoperative therapy. After completion of cav-1 measurements in the serum, it was discovered that seven patients were missing reliable data on their preoperative PSA and/or biopsy Gleason score and/or follow-up information. These patients were included in all analyses that did not require missing data. The preoperative serum collected from 355 patients was at a time period between prostate biopsy and surgery. No information was available in our database on the exact preoperative serum collection timing with respect to biopsy for 64 patients. The mean age of this patient group was 62.6 years (range 42.6-78.9 years); 91.4% were White males, with Hispanics, African-Americans, and Asians comprising 6.0%, 2.4%, and 0.2%, respectively. Mean follow-up time among this group of patients was 52 months, with a median follow-up time of 48 months. Biochemical recurrence is defined throughout this study as serum PSA level of ≥ 0.2 ng/mL on two consecutive measurements, using the first-generation postresection PSA assay (Hybritech, Beckman Coulter, Inc., Fullerton, CA). Patient data were gathered from the Informatics Core using the Specialized Programs of Research Excellence in Prostate Cancer Information System.

Determination of serum cav-1. Cav-1 was determined in the serum samples by the sandwich ELISA protocol developed in our laboratory (12). Briefly, Costar microplate wells were coated with 0.5 μg cav-1 polyclonal antibody (Transduction Laboratories, San Diego, CA) and blocked with TBS buffer containing 1.5% bovine serum albumin and 0.05% v/v Tween 20. Serum samples, calibrators, and controls (50 μL) were added to the well, and 50 μL TBS containing 0.5% v/v Tween 20 was added to each well. The plate was incubated at room temperature for 2 hours with shaking and after extensive washing, 100 μL horseradish peroxidase–conjugated cav-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in blocking buffer was added to each well. The microplate was incubated for 90 minutes at room temperature with shaking, the wells were then washed extensively, and 100 μL 3,3′,5,5′-tetramethylbenzidine substrate solution (Sigma-Aldrich, St. Louis, MO) was added and the blue color was allowed to develop for 20 minutes in the dark. The reaction was stopped by adding 50 μL of 2 N H_2SO_4, and the absorbance was read at

Table 2. Preoperative serum cav-1 is a univariate and multivariate predictor of decreased biochemical recurrence-free survival

	HR (95% CI)	*P*
Univariate model		
Preoperative cav-1	2.78 (1.003-7.70)	0.0493
Multivariate model		
Preoperative cav-1	2.57 (0.92-7.12)	<0.0704
Ln(PSA)	2.31 (1.60-3.33)	<0.0001
Biopsy Gleason score	1.74 (1.32-2.30)	0.0001

450 nm using a microplate reader (Sunrise Microplate Reader, Tecan US, Inc., Charlotte, NC).

Statistical analysis. Correlations of preoperative serum cav-1 levels with clinical and pathologic variables were evaluated using the Spearman correlation. The predictive value of cav-1 univariately and multivariately with other preoperative clinical and pathologic variables, such as preoperative PSA and biopsy Gleason score, as well as of the interactive terms, were analyzed using the Cox proportional hazards regression model. The minimum *P* value method was used to group patients into "low-level" and "high-level" cav-1 categories (13). The hazard ratio (HR) and 95% confidence intervals (95% CI) were computed for each marker. Kaplan-Meier survival curves were plotted for each risk category. $P < 0.05$ was considered statistically significant. All analyses were done using the SPSS 12.0 software package (SPSS, Inc., Chicago, IL)

Results

Serum cav-1 levels were measured in 419 prostate cancer patients. The mean cav-1 value was 4.52 ng/mL and the median level was 1.01 ng/mL (range 0.0-156.7 ng/mL). Serum cav-1 levels seemed to have a bimodal distribution, with positive values distributed log normally. The serum cav-1 levels were analyzed for correlation with other pathologic and clinical variables using the Spearman correlation. No statistically significant correlations with clinicopathologic variables were found (Table 1).
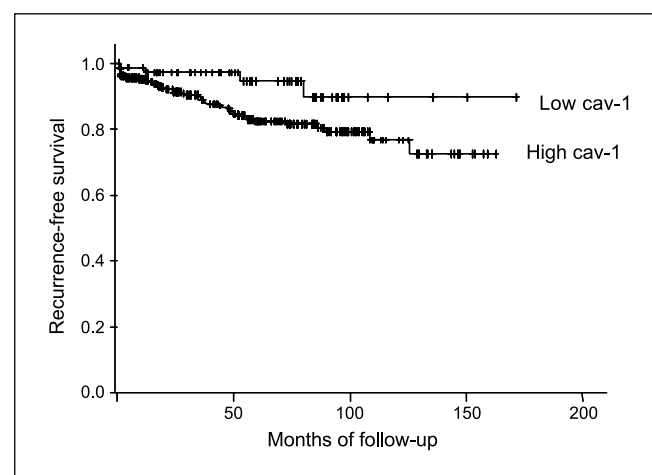
Table 1. Preoperative serum cav-1 level correlation with clinical and pathologic variables

	n	Mean (range), %	r^2	*P*
Preoperative cav-1	419	4.5 (0.0-156.7)	—	—
Preoperative PSA (ng/mL)	415	8.6 (0.4-53.2)	0.01	0.9013
Age	419	62.6 (42.6-78.9)	0.02	0.6268
Biopsy Gleason score	412	6.1 (3-9)	−0.06	0.2051
Seminal vesicle invasion	419	7.6%	−0.01	0.8543
Lymph node involvement	419	3.8%	0.08	0.1024
Extraprostatic extension	419	32.7%	0.07	0.1378
Margin positive	419	11.9%	0.06	0.2425
Gleason score	419	6.5 (3-9)	−0.01	0.8342

Fig. 1. High expression of cav-1 predicts decreased biochemical recurrence-free survival. This Kaplan-Meier plot illustrates the differences in recurrence-free survival between the low and high groups when separated by cav-1 cut point of 0.13 ng/mL. Patients with high level of cav-1 experienced significantly higher risk of recurrence than those with low levels ($P = 0.0493$).

There were 414 patients with complete follow-up information were included in the analysis of recurrence-free survival (mean follow-up 52.3 months, maximum 171.3 months); 54 patients had PSA recurrence during follow-up. Although it was clear that patients with no or very low levels of cav-1 had a better prognosis, the optimal cutoff was selected using the minimum P value method (13). This defined the low cav-1 group as patients with levels of <0.13 ng/mL and the high cav-1 group as those with >0.13 ng/mL. In univariate analysis, the risk of experiencing biochemical recurrence, estimated by HR, was 2.8 times higher (P = 0.0493) for the high cav-1 group compared with the low cav-1 group (Table 2). Kaplan-Meier plots illustrate the shorter time to biochemical recurrence following radical prostatectomy in the high cav-1 group compared with low cav-1 group. The 5- and 10-year recurrence-free survival rates were 94.4% and 90.5% for the low cav-1 group compared with 82.0% and 71.8% for the high cav-1 group. This corresponds to a consistent 12% to 21% increased progression-free survival for the low cav-1 group (Fig. 1). When the preoperative serum PSA level and the biopsy Gleason score were incorporated into the multivariate Cox proportional hazard model, the recurrence risk was 2.6 times higher for the high cav-1 group, but this effect was just below the level of significance (P = 0.0704; Table 2).

The effect of the serum cav-1 level on biochemical recurrence was further analyzed in patients with more advanced cancers, characterized by PSA of ≥10 ng/mL. The distribution shape remained the same and patients with low cav-1 levels continued to have a better prognosis. A new optimal cutoff of 2.86 ng/mL was identified for this subgroup of patients. Univariately, the estimated risk of recurrence was 2.44 times higher (P = 0.0256) in the high cav-1 group (serum cav-1 >2.86 ng/mL) than in its low cav-1 counterpart (serum cav-1 ≤ 2.86 ng/mL; Table 3). Kaplan-Meier plots illustrate that patients in the high cav-1 group had a much shorter time to recurrence than those in the low cav-1 group (Fig. 2). This figure also indicates a 10-year recurrence-free survival rate of 70.3% in the low cav-1 group compared with 47.4% in the high cav-1 group corresponding to a >20% decrease in progression-free survival in the low cav-1 group.

Incorporating the biopsy Gleason score into the Cox proportional hazard model (Table 3), we found that the interaction term between Gleason score and the cav-1 was the most predictive (P = 0.0353). This indicates that the biopsy Gleason score was an additional risk factor only in the high cav-1 group. The Kaplan-Meier plot (Fig. 3) illustrates this result by showing the highest recurrence risk in patients with high cav-1 and high biopsy Gleason score (7–9); and lower
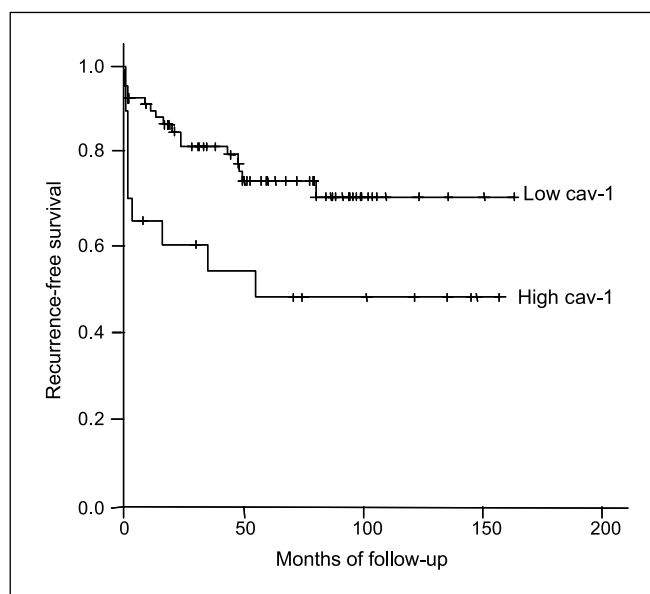
Table 3. Preoperative serum cav-1 is a univariate and multivariate predictor of decreased biochemical recurrence-free survival among patients with preoperative PSA of ≥10

	HR (95% CI)	*P*
Univariate model		
Preoperative cav-1	2.44 (1.12-5.34)	0.0256
Multivariate model		
Preoperative Cav-1 × biopsy Gleason score	1.13 (1.01-1.27)	0.0353

Fig. 2. For patients with PSA of ≥10, high expression of cav-1 is a strong predictor of decreased biochemical recurrence-free survival. For this high-risk patient subgroup, optimal cutoff was determined to be at 2.86 ng/mL. The Kaplan-Meier plot here shows the difference observed in the data.

recurrence risk in the high cav-1 and low biopsy Gleason score (<7) group, and those patients with low cav-1 regardless of the biopsy Gleason score. Recurrence-free survival curve for patients with low PSA (<10) was plotted for reference as well.

For biopsy Gleason 7 patients, the trend was the same: Higher cav-1 was observed in higher-risk patients. The difference in risk of recurrence, estimated by HR, between low and high cav-1 patients with cutoff defined at upper quartile of cav-1 (and confirmed by minimum P value method), was not statistically significant (P = 0.0953). However, after including preoperative PSA in the model, the
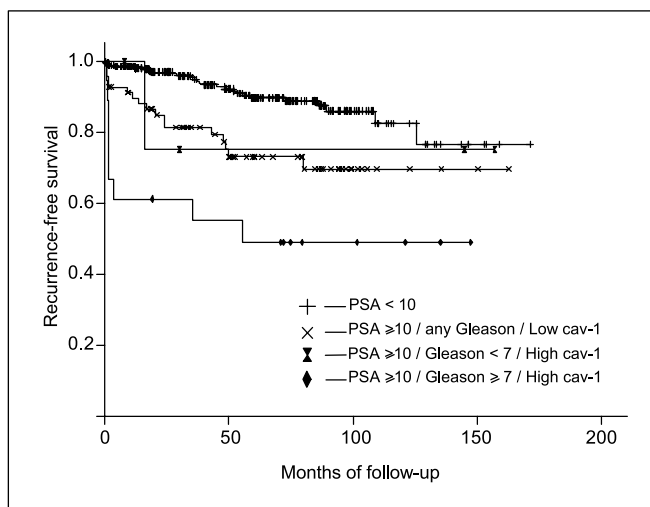

Fig. 3. Cav-1 works with biopsy Gleason score and preoperative PSA to predict biochemical recurrence-free survival. The interaction term between biopsy Gleason score and the cav-1, incorporated into the Cox proportional hazard, was the most predictive of recurrence-free survival among patients with PSA of ≥10 (P = 0.0353). This Kaplan-Meier plot illustrates how patients with both high cav-1 (>2.86 ng/mL) and biopsy Gleason score of ≥7 have the poorest prognosis. Curve for patients with PSA < 10 was plotted for reference.

difference became statistically significant (HR, 2.29; P = 0.0429). A patient with cav-1 in the upper quartile had over twice the risk of recurrence of one with cav-1 in the lower three quartiles if their preoperative PSA levels were the same.

Discussion

This study is part of our ongoing efforts to elucidate the biology and to define the clinical usefulness of serum cav-1 in prostate cancer. Although the factors modulating the serum levels of this biomarker remain largely unknown, the current study points out that a single preoperative serum cav-1 determination has prognostic value in a radical prostatectomy cohort. We observed the increase of the risk of biochemical recurrence with high levels of serum cav-1, and so we used the minimum P value method to segregate the patients into low-level and high-level groups. Remarkably, the risk of experiencing a PSA recurrence, estimated by HR, was 2.78 (95% CI, 1.003-7.70) times higher for the high-level cav-1 group (P = 0.0493), (see Fig. 1; Table 2). Incorporating the preoperative serum PSA level and the biopsy Gleason score into the model dropped the effect of cav-1 to just below the level of significance (HR, 2.57; P = 0.0704).

Interestingly, we found that the serum cav-1 levels are particularly important in predicting recurrence-free survival in patients with more advanced disease as defined by the preoperative serum PSA. When only patients with preoperative serum PSA levels of 10 ng/mL or higher were analyzed, cav-1 remained a significant predictor of recurrence-free survival (HR, 2.44; P = 0.0256). Additionally, the cav-1/biopsy Gleason score interaction term was a significant predictor (P = 0.0353). This implies that patients with both a high biopsy Gleason score and a high serum cav-1 level have a higher risk of biochemical recurrence than the remaining patients. Also, a subgroup of biopsy Gleason 7 prostate cancer patients, defined by the upper 25% of serum cav-1 levels, seems to harbor a biologically more aggressive prostate cancer after correction for individual PSA levels. All of these findings are consistent with our previous reports based on tissue up-regulation of cav-1 expression (8).

Notably, the distribution of the serum cav-1 values in the study population was not a normal distribution. About 10% of patients had undetectable serum levels by our sensitive ELISA essay. We can only speculate at this point as to the possible reason for this phenomenon. It is possible that the presence of any cav-1 in the serum is dictated by the genetic background of the individual and that, physiologically, there may be "secretors" and "nonsecretors." Within the secretor population, the specific makeup of the cancer may be contributing to the absolute serum level.

Surprisingly, we could not correlate the serum cav-1 levels with any of a large number of clinical and/or pathologic variables using the Spearman correlation (Table 1). We suggest that the reason is that cav-1 is an independent biomarker causally implicated in disease progression and not simply an epiphenomenon.

Many questions remain. For instance, we do not know the incidence of false-positive and/or false-negative elevated serum cav-1 values vis-à-vis the tumor tissue cav-1 expression. Only a correlative study of tissue and serum levels of cav-1 can answer this question. Likewise, the kinetics of the serum cav-1 has not been worked out, nor do we know what the stability of serum cav-1 is over extended periods of time. Clearly, we are at the beginning of the road leading to the establishment of serum cav-1 as a prognostic marker for prostate cancer. The data presented here suggest that this road is worth pursuing.

References

1. Cooperberg MR, Moul JW, Carroll PR. The changing face of prostate cancer. J Clin Oncol 2005;23: 8146–51.
2. Shipley WU, Thames HD, Sandler HM, et al. Radiation therapy for clinically localized prostate cancer: a multi-institutional pooled analysis. JAMA 1999; 281:1598–604.
3. Bracarda S, de Cobelli O, Greco C, et al. Cancer of the prostate. Crit Rev Oncol Hematol 2005;56:379–96.
4. Chin JL, Reiter RE. Molecular markers and prostate cancer prognosis. Clin Prostate Cancer 2004;3: 157–64.
5. Shaul PW, Anderson RG. Role of plasmalemmal caveolae in signal transduction. Am J Physiol 1998; 275:L843–51.

6. Ikonen E, Parton RG. Caveolins and cellular cholesterol balance. Traffic 2000;1:212–7.
7. Nasu Y, Timme TL, Yang G, et al. Suppression of caveolin expression induces androgen sensitivity in metastatic androgen-insensitive mouse prostate cancer cells. Nat Med 1998;4:1062–4.
8. Yang G, Truong LD, Wheeler TM, Thompson TC. Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. Cancer Res 1999;59:5719–23.
9. Satoh T, Yang G, Egawa S, et al. Caveolin-1 expression is a predictor of recurrence-free survival in pT₂N₀ prostate carcinoma diagnosed in Japanese patients. Cancer 2003;97:1225–33.
10. Yang G, Addai J, Ittmann M, Wheeler TM, Thompson

TC. Elevated caveolin-1 levels in African-American versus White-American prostate cancer. Clin Cancer Res 2000;6:3430–3.
11. Tahir SA, Yang G, Ebara S, et al. Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. Cancer Res 2001;61:3882–5.
12. Tahir SA, Ren C, Timme TL, et al. Development of an immunoassay for serum caveolin-1: a novel biomarker for prostate cancer. Clin Cancer Res 2003;9:3653–9.
13. Mazumdar M, Glassman JR. Categorizing a prognostic variable: review of methods, code for easy implementation and applications to decision-making about cancer treatments. Stat Med 2000; 19:113–32.