

Prostate Stem Cell Antigen Is Overexpressed in Prostate Cancer Metastases

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Abstract Purpose: Prostate stem cell antigen (PSCA) is expressed by a majority of prostate cancers and is a promising therapeutic target. PSCA protein and mRNA expression was examined in prostate cancer bone, lymph node, and visceral metastases to assess the potential of PSCA as an immunotherapeutic target in advanced prostate cancer.

Experimental Design: Immunohistochemical analysis of PSCA protein expression and quantitative mRNA expression analysis of PSCA was done on clinical specimens of prostate cancer bone, lymph node, and visceral metastases. PSCA protein and mRNA expression levels were quantified and compared between available matched pairs of bone and lymph node or visceral metastases.

Results: Bone metastases stained with higher intensity of PSCA compared with lymph node or liver metastases in seven of eight (87.5%) matched pairs ($P = 0.035$). PSCA mRNA expression was equal or greater than that of LAPC-9, a PSCA expressing xenograft, in 12 of 24 (50%) cases of prostate cancer metastases and was significantly correlated with PSCA protein expression ($\sigma = 0.84$, $P = 0.0019$). Overall, PSCA protein expression was detected in 41 of 47 (87.2%), four of six (66.7%), and two of three (66.7%) cases of bone, lymph node, and liver metastases, respectively. Mean PSCA staining intensity was significantly higher in prostate cancer bone metastases compared with lymph node metastases (2.0 ± 0.02 versus 0.83 ± 0.31 , $P = 0.014$).

Conclusions: Prostate cancer metastases express PSCA. However, greater PSCA staining intensity and level of PSCA mRNA expression was associated with bone metastases compared with lymph node metastases. This study suggests that PSCA is a promising tumor marker and potential therapeutic target for patients with metastatic prostate cancer.

An estimated 232,090 men in the United States will be newly diagnosed with prostate cancer and ~30,350 deaths will have resulted from this disease in 2005, making it the most commonly diagnosed cancer and the second leading cause of cancer death among American men (1). Although great progress has been made in the diagnosis and management of localized disease, significant challenges remain in the management of this disease. Few successful therapeutic options exist for men who present with metastatic disease or for the 30% who have recurrence following local treatment. Median survival for

patients with metastatic, hormone-refractory disease is 12 to 18 months (2). Clearly, there is a need to develop improved systemic therapies that, ideally, are applicable to the full spectrum of disease ranging from micrometastatic disease at early diagnosis to overt metastatic disease.

A large number of novel therapeutic strategies are currently being pursued, including the development of cancer vaccines, antisense oligonucleotides, and monoclonal antibodies (mAb; ref. 3). Prostate stem cell antigen (PSCA) is a 123-amino-acid glycoprotein first identified in the LAPC-4 prostate xenograft mode of human prostate cancer (4). PSCA is a glycosyl phosphatidylinositol-anchored cell surface protein related to the Ly-6/Thy-1 family of cell surface antigens that bears 30% homology to stem cell antigen type 2 (SCA-2), a cell surface marker of immature thymic lymphocytes (5, 6). PSCA expression in normal tissues is largely prostate-specific, but PSCA transcripts and protein have been found in the transitional epithelium of the bladder and the stomach (7, 8). PSCA has also recently been shown to be expressed by a majority of bladder and pancreatic cancers (9). *In situ* hybridization and immunohistochemical analyses have shown that PSCA expression is detected in 94% and overexpressed in about 40% of clinically localized prostate cancer specimens (7). Elevated PSCA expression has been shown to correlate with increased tumor grade and stage (7). A tissue microarray analysis constructed from 246 radical prostatectomy specimens

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has also shown that high PSCA intensity is associated with adverse prognostic factors such as high Gleason score, seminal vesicle invasion, and capsular involvement (10). PSCA maps to chromosome 8q24.2, a region of genetic gain/amplification in a large percentage of advanced prostate cancers (4, 11).

These characteristics make PSCA a potentially attractive target for high-risk and metastatic prostate cancer. Supporting this hypothesis, we recently showed that mAbs against PSCA can inhibit tumor growth and metastasis formation and can prolong survival in mice bearing human prostate cancer xenografts (12). Furthermore, a PSCA-derived peptide has been shown to be capable of eliciting a PSCA-specific T-cell response in a patient with metastatic prostate cancer (13). PSCA continues to be evaluated as a diagnostic and therapeutic target for prostate cancer. Although studies have examined the utility of PSCA in localized prostate cancer, little work has been done in metastatic prostate cancer. In this study, we show that PSCA is expressed by a majority of prostate cancer metastases. Moreover, PSCA is overexpressed at the protein and mRNA levels in the majority of bone metastases compared with lymph node or visceral metastases.

Materials and Methods

Tissue samples. All of the tissue specimens were obtained with Institutional Review Board approval from the Tissue Procurement Core Laboratory of the Department of Pathology at the University of California at Los Angeles Medical Center. Tissues were obtained from the University of Washington and Mayo Clinic through approved protocols. Formalin-fixed, paraffin-embedded tissue blocks were cut into 4- μ m sections and mounted on charged slides in the usual fashion. H&E-stained sections of the neoplasms were graded by an experienced urological pathologist (J.W.S.) according to the criteria set forth in the WHO Classification of Tumors (14). The tissue samples consisted of 47 bone metastases and nine lymph node or visceral (liver and peritoneal) metastases.

Immunohistochemistry. The mouse mAb 1G8 was derived from BALB/c mice immunized with a glutathione *S*-transferase-PSCA fusion protein, as previously described (7, 12). PSCA mAb 1G8 was generated in the CellPharm System 100 as described previously (7). Immunohistochemical staining was done with anti-PSCA 1G8 antibody (1:20 dilution) using modifications of an immunoperoxidase technique previously described (15). Briefly, after deparaffinization of histologic sections, the slides were incubated with 70% hydrogen peroxide in methanol for 10 minutes and washed with PBST (0.01 mol/L sodium phosphate, 0.15 mol/L NaCl, and 0.05% Tween 20) for 5 minutes. The slides were then incubated for 2 hours at room temperature with the primary antibody diluted in 2% bovine serum albumin (1:20) and washed with PBST. The slides were then incubated with Envision horseradish peroxidase polymer (DAKO Co., Santa Barbara, CA), washed with PBST, and incubated with diaminobenzidine for 10 minutes and washed in tap water. The slides were counterstained with Harris hematoxylin and dehydrated in 95% ethanol, 100% ethanol, and xylene. Positive and negative controls were done on tissues obtained from mouse xenograft tumors that were derived from the human prostate cancer cell lines, LAPC-9 and PC3, respectively. Negative controls for each stained section consisted of substitution of the primary antibody by a noncross-reacting isotype-matched mAb.

Histopathologic slides of the clinical specimens were read and scored by a single pathologist (J.W.S.) in a blinded fashion. Immunohistochemical staining intensity was graded on a scale of 0 to 3 (0, no staining; 1+, weak staining; 2+, moderately intense staining; 3+, severely intense staining). Staining density was quantified as the percentage of cells staining positive with the primary antibody.

Quantitative reverse transcription-PCR. Fresh human tissues were obtained at the time of autopsy in men who died from metastatic prostate cancer at the University of Washington in accordance with an Institutional Review Board–approved clinical protocol. Total cellular RNA was extracted using Ultraspec RNA isolation systems (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. Total RNA (3 μ g) was reverse-transcribed to generate first strand cDNA using random hexamers and oligo-(dT)₁₅ primers and Superscript II according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Real-time PCR was used to quantify PSCA expression. Primers were designed using Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA). The primer sequences used were 5'-PSCA, 5'-ATCAGGAGGCCAGTAAAG-3' and 3'-PSCA, 5'-TCCCAGGAACCTCAGTCAAC-3'. Real-time PCRs were carried out using iCycler iQ Real-time PCR Detection System and iQ SYBR Green Supermix following the manufacturer's instructions (Bio-Rad, Hercules, CA). The PSCA transcripts were normalized to the content of the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase. The human prostate cancer cell lines, LAPC-9 and LNCaP, were used as positive and negative controls, respectively. PSCA mRNA expression levels were compared with the positive control LAPC-9. All PCR samples were done in triplicate for each sample and mean values were used in subsequent analysis.

Statistical analysis. Comparison of immunohistochemical staining across two groups was done using the Wilcoxon rank sum test. $P < 0.05$ was considered significant. Correlation of two ordinal variables was done using the Spearman correlation coefficient and its corresponding P . Comparison of immunohistochemical staining intensities between tissues of the same patient (matched data) was done using the Sign test. All data was statistically analyzed using the freely available R statistical software package (<http://www.cran.r-project.org>).

Results

Prostate stem cell antigen protein expression in matched pairs of prostate cancer metastases. The expression pattern of PSCA between eight available matched pairs of prostate cancer bone and lymph node or liver metastases is summarized in Table 1 and illustrated in Fig. 1A-D. Nine of nine (100%) bone metastasis specimens stained positively for PSCA. PSCA expression for bone metastases was weak (i.e., 1+) in two of nine (22.2%) cases, intermediate (i.e., 2+) in three of nine (33.3%) cases, and strong (i.e., 3+) in four of nine (44.4%) cases. For lymph node and liver metastasis specimens, no tumor was identified in a lymph node metastasis specimen from one of the matched pairs. Six of nine (66.7%) lymph node or visceral metastasis specimens stained positively for PSCA. PSCA expression for lymph node or liver metastases was negative in three of nine (33.3%) cases, weak in three of nine (33.3%) cases, intermediate in one of nine (11.1%) cases, and strong in one of nine (11.1%) cases. Mean PSCA staining intensity was higher in bone metastases compared with lymph node or liver metastases (2.2 ± 0.28 versus 1.0 ± 0.38 , $P = 0.028$). Furthermore, bone metastases stained with higher intensity compared with lymph node or liver metastases in seven of eight (87.5%) matched pairs ($P = 0.035$). PSCA expression was not detected in three specimens (2 lymph node and 1 liver), whereas the matched bone metastasis specimens stained strongly.

Prostate stem cell antigen mRNA expression in prostate cancer metastases. PSCA mRNA expression was compared between prostate cancer bone and lymph node or visceral metastases using quantitative reverse transcription-PCR. PSCA mRNA expression was compared with LAPC-9, a prostate cancer

Table 1. Comparison of immunohistochemistry staining of PSCA between matched pairs of prostate cancer bone and lymph node or liver metastases

Patient	Tissue	Immunohistochemical staining intensity
1	Bone	3+
	Lymph node	0
2	Bone	3+
	Lymph node	1+
3	Bone	2+
	Lymph node	1+
4	Bone	2+
	Lymph node	1+
5	Bone	1+
	Lymph node	0
6	Bone	3+
	Liver	0
7	Bone	3+
	Liver	2+
8	Bone	2+
	Liver	3+
9	Bone	1+
	Lymph node	No tumor
10	Bone	NA
	Lymph node	2+

Abbreviation: NA, not available.

xenograft that expresses high levels of PSCA and responds to PSCA mAb in preclinical models, and LNCaP, a prostate cancer cell that does not express endogenous PSCA. Consistent with the immunohistochemistry findings, PSCA mRNA was detected in the majority of prostate cancer metastases. The level of PSCA expression was equal or greater than that of LAPC-9 in 12 of 24 (50%) cases (Fig. 2). Furthermore, PSCA expression was profoundly higher in 11 of 12 (91.7%) cases, with 2- to 15-fold higher expression levels noted. PSCA mRNA expression was also compared between seven available matched pairs of prostate cancer bone metastases and lymph node or visceral metastases. Bone metastases expressed higher levels of PSCA mRNA compared with lymph node or visceral metastases in five of eight (62.5%) matched pairs.

Comparison of real-time PCR and immunohistochemistry for prostate stem cell antigen expression analysis. In 9 cases, both mRNA and paraffin slides were available. In all cases, the mRNA result agreed with the immunohistochemistry result. Furthermore, the level of PSCA mRNA expression significantly correlated with the level of PSCA protein expression ($\sigma = 0.84$, $P = 0.0019$). In 2 specimens (one lymph node metastasis and one liver metastasis), both real-time PCR and immunohistochemistry detected an absence of PSCA expression. Conversely, PSCA mRNA and protein were detected in the remaining 7 cases. Importantly, the level of mRNA expression was consistent with the subjective immunohistochemistry score in all cases (Fig. 2).

Prostate stem cell antigen protein expression in prostate cancer metastases. A total of 47 bone metastases, six lymph node metastases, and three liver metastases from prostate cancer were

analyzed for PSCA protein expression via immunohistochemistry (Table 2). PSCA expression was present in 41 of 47 (87.2%) cases, four of six (66.7%) cases, and two of three (66.7%) cases for bone, lymph node, and liver metastases, respectively. PSCA expression for bone metastases was absent in 6 (12.8%), weak in 8 (17%), intermediate in 13 (27.7%), and strong in 20 (42.6%) of 47 cases. PSCA expression for lymph node metastases was undetectable in two (33.3%), weak in three (50%), intermediate in one (16.7%), and strong in zero (0%) of six cases. PSCA expression for liver metastases was absent in one (0%), weak in zero (0%), intermediate in one (33.3%), and strong in one (33.3%) of three cases. Mean PSCA staining intensity was significantly higher in prostate cancer bone metastases compared with lymph node metastases (2.0 ± 0.02 versus 0.83 ± 0.31 , $P = 0.014$). A statistically valid analysis comparing the liver metastases group was not possible due the small sample size.

Discussion

A large number of novel therapeutic strategies are currently being pursued for patients with advanced prostate cancer, which include the development of cancer vaccines, antisense nucleotides, and mAbs (3). Recent success with Herceptin and Rituxan have generated particular excitement about the potential of mAb therapy (16, 17). The cell surface expression of PSCA in localized and advanced prostate cancer, along with its restricted pattern of expression, makes PSCA an exciting potential therapeutic target. In the present study, we showed that PSCA is highly expressed in metastatic prostate cancer and in particular bone metastases, validating PSCA as a target in advanced disease. We were able to quantitate both PSCA protein and mRNA expression in prostate cancer metastases with relative accuracy. Quantitative PCR analysis showed that PSCA expression in prostate cancer metastases was equal or greater than LAPC-9, a xenograft established from a bony metastasis that stains intensely for PSCA, in at least 50% of cases. Moreover, PSCA expression was higher in bony metastases compared with either lymph node or visceral metastases. Thus far, preclinical studies have been done in prostate cancer xenograft models using anti-PSCA mAbs against LAPC-9. Saffran et al. (12) reported on the use of anti-PSCA mAbs in the androgen-dependent LAPC-9 and androgen-independent PC3-PSCA (i.e., PC3 cells expressing PSCA) prostate cancer xenograft models. 1G8, a mAb against the middle portion of PSCA inhibited the formation of LAPC-9 and PC3-PSCA xenografts, and the formation of s.c. and orthotopic xenograft tumors were inhibited by 1G8 in a dose-dependent manner. The administration of 1G8 led to decreased tumor growth in an established orthotopic model, inhibited metastases to distant sites, and prolonged the survival of treated mice. These results have been corroborated by Ross et al. (8), who reported on the regression of established tumors treated with a PSCA-maytensinoid immunotoxin conjugate. These encouraging preclinical results show the therapeutic potential for anti-PSCA mAb immunotherapy in treating advanced and metastatic prostate cancer. Insight into the preclinical efficacy of PSCA mAbs may lead to combination strategies to enhance their activity. The toxicity of PSCA mAbs will also need to be carefully assessed, most likely in phase I clinical trials.

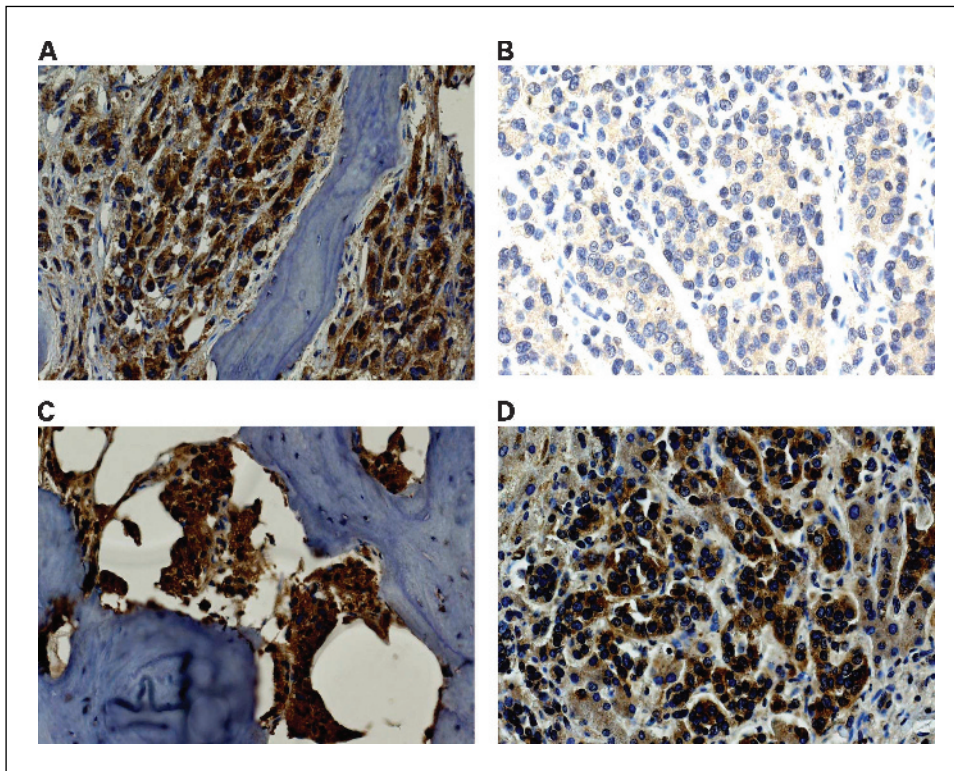


Fig. 1. PSCA immunohistochemical staining in matched sets of prostate cancer metastases. PSCA staining of a metastatic bony lesion (A) and a matched lymph node (B) metastasis from one patient. PSCA staining of a metastatic bony lesion (C) and a matched liver (D) metastasis from a second patient. H&E. Reduced from $\times 40$.

Recent advances in tumor immunology have enabled the identification of many genes encoding tumor antigens and their peptides that are recognized by CTLs (18, 19). Dannull et al. (13) has reported generating a PSCA-specific T-cell response in human lymphocytes cultured from a patient with metastatic prostate cancer. These CTLs were specific for an HLA-A0201-restricted PSCA epitope and recognized peptide-pulsed targets as well as three prostate cancer cell lines in cytolytic assays. Matsueda et al. (20, 21) has also identified PSCA-derived

peptides immunogenic in HLA-A2* and HLA-A24* prostate cancer patients. These findings indicate that PSCA, as well as this peptide epitope, may be a potential target for antigen-specific, T cell-based immunotherapy.

We have also previously reported that PSCA mRNA is overexpressed in a subset of prostate cancers (4, 7). Furthermore, the level of PSCA protein expression in prostate tumors was associated with higher Gleason score, higher tumor stage, and progression to androgen independence (7). Han et al. (10) corroborated these results and showed that PSCA overexpression was correlated with an increased risk of biochemical recurrence. Ross et al. (8) did not confirm a positive correlation between PSCA mRNA expression and high Gleason grade. A possible explanation may be that they used *in situ* hybridization technology to measure PSCA mRNA levels, whereas in the above studies, PSCA protein expression analysis with immunohistochemistry was performed. PSCA may be in part regulated post-transcriptionally, potentially explaining the

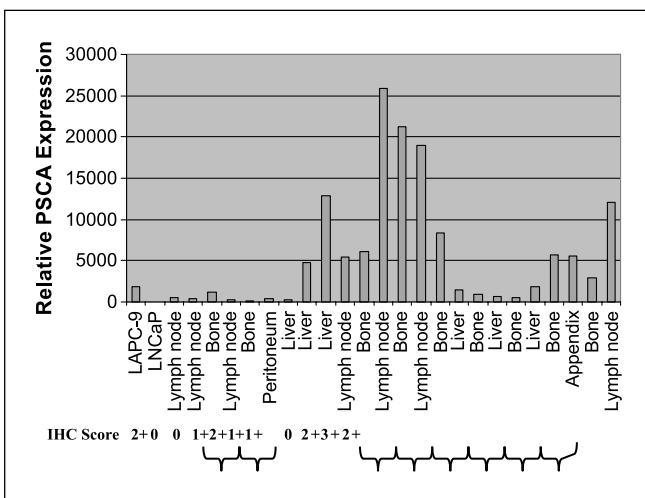


Fig. 2. Quantitative reverse transcriptase-PCR of PSCA expression in prostate cancer metastases. Level of PSCA expression detected in prostate cancer metastases relative to LAPC-9. Matched pairs of bone and lymph node or visceral metastases are grouped together with brackets. Available PSCA immunohistochemical staining intensity scores matched to samples analyzed for PSCA mRNA expression.

Table 2. Summary of immunohistochemical staining of PSCA among prostate cancer bone, lymph node, and liver metastases

Immunohistochemical staining intensity	frequency (%)		
	Bone	Lymph node	Liver
0	6 (12.8)	2 (33.3)	1 (33.3)
1+	8 (17)	3 (50)	0 (0)
2+	13 (27.7)	1 (16.7)	1 (33.3)
3+	20 (42.6)	0 (0)	1 (33.3)
Overall	41 (87.2)	4 (66.7)	2 (66.7)

overall difference in positivity as well as the lack of correlation with tumor grade. Hara et al. (22) recently reported the outcomes of men who had circulating PSCA-positive cells at the time of prostate cancer diagnosis. They reported that patients with detectable PSCA positive circulating cells had a higher mean Gleason score than those who were PSCA negative (5.71 versus 4.14, $P < 0.05$). Detectable PSCA positive circulating cells also correlated with extraprostatic extension. Whereas all cases of organ confined prostate cancer were PSCA negative, 47% of extraorgan disease cases were PSCA positive. In addition, patients who were PSCA reverse transcription-PCR positive had a significantly worse disease-specific survival compared with their counterparts who were PSCA PCR negative. Additional studies of PSCA expression in tumors that progress clinically and in micrometastases are necessary to draw conclusions regarding the prognostic significance of PSCA expression. It would also be interesting to compare PSCA reverse transcription-PCR expression between patients with localized versus metastatic prostate cancer.

Our results are in agreement with previous reports on PSCA expression in prostate cancer metastases. Gu et al. (7) previously showed intense and homogeneous immunohistochemical staining of PSCA on nine of nine (100%) bone metastasis specimens. Dannull et al. (13) also reported that PSCA mRNA expression was present in all metastatic prostate cancer tissues examined. Furthermore, a bone metastasis specimen had exceptionally high levels of PSCA mRNA expression compared with the matched primary tumor and metastatic liver specimen from the same patient. Ross et al. (8) conducted expression analysis of PSCA in normal urogenital tissue, benign prostatic hyperplasia, prostatic intraepithelial neoplasia, and primary and metastatic prostate cancer using isotopic *in situ* hybridization on tissue microarrays. The incidence and intensity of PSCA expression in the metastatic lesions, which included only lymph node or visceral metastases, was similar to our study. Ross et al. (8) also showed the percentage of metastatic prostate cancer cases positive for PSCA mRNA expression was higher (64%) than that of benign prostate disease and organ-confined prostate cancer (48%). Although we did not compare this directly in our present study, our previous results are consistent with that, in that the proportion of metastatic specimens with intense (i.e., 3+) staining was higher than that of the primary tumor specimens (7).

In our study, we noted that bone metastases expressed higher levels of PSCA mRNA compared with lymph node or visceral metastases in 62.5% of the matched pairs, which was lower than that seen with the group that underwent PSCA protein expression analysis. There may be several reasons why this

occurred. This may be secondary to RNA degradation because it is often difficult to extract good quality RNA from bone at autopsy, due to heating from the saw used to cut the bone, as well as decalcification. In addition, the bone metastasis specimens were from bone marrow, and this may be contaminated with normal osteoblasts. Thus, the percentage of tumor in any one specimen is not known; thus, this may cause a sampling error and could explain why in some cases PSCA expression in bone metastases was less than or equal to nonbony metastases at the RNA level. In addition, differences have often been reported to exist between mRNA and protein levels for many genes. Therefore, it is possible that post-transcriptional mechanisms may play a role and explain why mRNA and protein may not always agree (23). Furthermore, the protein epitope may also be altered secondary to glycosylation, which may prevent recognition by the antibody and leading to lower levels of protein detected compared with that of mRNA.

It is not known why bone metastases seem to express higher levels of PSCA than lymph node metastases. One possibility is that cells expressing high levels of PSCA may localize preferentially to bone. PSCA may also be regulated directly by factors expressed in bone. Comparable PSCA expression levels were also observed between liver and bone metastases. One explanation may be that hematogeneous metastases, such as those to the liver, may express higher levels of PSCA than lymphatic metastases. Unfortunately, due to the small sample size of the liver metastases group, statistically valid comparisons could not be generated. Therefore, it will be important for future studies to validate our observations. The functional role of PSCA and the mechanisms governing elevated PSCA expression in prostate carcinogenesis remain poorly understood. Further elucidation of PSCA function and regulation will be required to understand the apparent specific up-regulation of PSCA in bone.

The prostate cancer specimens in this study were derived from patients who had received antiandrogen therapy and/or chemotherapy for the treatment of metastatic disease. It is unclear whether an association exists between these treatments and the level of PSCA expression. However, PSCA expression is maintained in androgen independent tumors (7, 12). LAPC-9, a xenograft established from a bony metastasis, has previously been shown to stain intensely for PSCA and has been used as a positive control.

In summary, we have shown in this study that PSCA is expressed by a majority of metastatic prostate cancers. Furthermore, PSCA seems overexpressed in a majority of metastatic bony lesions. These results suggest that PSCA may be a valuable target for metastatic prostate cancer diagnosis and therapy.

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