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Clinical Significance of Prostate Stem Cell Antigen Expression in Non-small Cell Lung Cancer

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Objective: Prostate stem cell antigen was originally identified as an overexpressed gene in prostate cancer and its overexpression correlated with disease progression and prognosis. In this study, we investigated the clinical significance and therapeutic potential of prostate stem cell antigen expression in non-small cell lung cancer.
Methods: Prostate stem cell antigen expression was examined by immunohistochemistry in 97 primary tumors and 21 metastatic lymph nodes from non-small cell lung cancer patients who underwent curative resection from January 2001 through March 2003. Therapeutic potential of targeting prostate stem cell antigen was further examined by small interfering RNA method using human lung cancer cell line (A549).
Results: Prostate stem cell antigen protein expression was detected in 94 of 97 primary lesions (97%) and all metastatic lymph nodes. Prostate stem cell antigen expression intensity was positively correlated with advanced pathological T-factor and stage (T1 vs. T2–4, $P = 0.014$; Stage I vs. Stages II–IV, $P = 0.029$, respectively). The prognosis of patients with low prostate stem cell antigen expression was significantly better than those with high prostate stem cell antigen expression (5-year disease-free survival rate; 90% vs. 53%, $P = 0.001$). Finally, small interfering RNA-mediated knockdown of prostate stem cell antigen resulted in the inhibition of lung cancer cell growth.
Conclusions: Prostate stem cell antigen is highly expressed in non-small cell lung cancer and may be functionally important for this fatal disease.

Key words: non-small cell lung cancer – prostate stem cell antigen – immunohistochemistry – prognosis

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in Japan as well as in other countries. Despite the improvement of conventional antitumor therapies including chemotherapy, radiotherapy as well as surgical resection, the prognosis of lung cancer patients remains extremely poor (1). Most patients have advanced disease and few therapeutic options at presentation. Moreover, the treatment response, even if feasible, is usually very limited (1,2). Therefore, to improve patients' prognosis, there is an urgent need for developing novel therapeutic strategies. Recent advances in tumor biology have led to the development of specific molecular-targeted therapies (3). These alternatives to conventional treatment have been greatly expected to provide a new standard for cancer therapy. However, clinical response of the current molecular-targeted therapeutic agents including anti-epidermal growth factor receptor tyrosine kinase inhibitors and monoclonal antibodies is only limited

(4). Thus, further studies are clearly required to find more suitable and promising molecular targets.

Prostate stem cell antigen (PSCA), a 123 amino acid glycoprotein, is a member of the Thy-1/Ly-6 family of glycosylphosphatidylinositol-anchored cell surface antigens. It is predominantly prostate-specific and expressed in a subset of basal and secretory cells in normal prostate. It is overexpressed in both androgen-dependent and -independent prostate cancer (5). The expression has been demonstrated to increase tumor grade, stage, metastasis and recurrence in prostate cancer (5–7). In addition, Zhigang et al. (8) reported that the PSCA expression in prostatic intraepithelial neoplasia decreased after preventive androgen ablation treatment and the decline of PSCA expression after the treatment could be a predictor for the subsequent development of prostate cancer. Furthermore, anti-PSCA monoclonal antibody treatment has been demonstrated to exhibit tumor-specific cytotoxicity *in vitro* and inhibit tumor growth and metastasis *in vivo* (9–11). It has also been reported that several PSCA-derived peptide vaccine therapy could effectively induce PSCA-specific and long-lasting cellular and humoral immune responses both *in vitro* and *in vivo* (12–14). Thus, PSCA may hold a great promise as a useful predictor of tumor biology and clinical outcome, and also a potential therapeutic target for prostate cancer. Recent reports have shown that PSCA was also detected in other few malignancies including bladder, pancreatic and renal cancer (15–19). However, the precise role and function of PSCA in various human malignancies are largely unknown. In this study, we investigated the clinical significance and therapeutic potential of PSCA expression in human non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS

Patients and Tissue Samples

We examined 97 NSCLC patients who underwent curative resection at our institution from January 2001 through March 2003. The median age of the patients was 68 years with a range of 41–83 years. All patients were diagnosed as NSCLC: 64 in adenocarcinoma, 25 in squamous cell carcinoma and 8 in large cell carcinoma. Tumors were classified according to World Health Organization classification and TNM classification of the International Union Against Cancer (20,21). Pathological stages were as follows: 62 in Stage I, 13 in Stage II, 19 in Stage III and 3 in Stage IV. The median follow-up period for the 63 patients without recurrence was 53 months. Materials for immunohistochemical analysis, 20% formalin-fixed and paraffin-embedded blocks, were retrieved from surgical pathology files of pathology section at our institution. The blocks were cut into 4 μm sections and mounted on charged slides according to standard protocols (7). Documentation of informed consent was obtained from all patients. All experiments were conducted under a protocol approved by our institutional review board.

Immunohistochemistry

Ninety-seven primary tumors and 21 metastatic lymph nodes were examined. The immunohistochemical staining was performed with the anti-PSCA rabbit polyclonal antibody ab64919 (Abcam Inc., MA, USA) diluted at 1:25 as reported previously (22). Tissue sections on glass slides were dewaxed in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline. This buffer was used for all subsequent washes. Antigen retrieval was performed at 99°C for 40 min in target retrieval solution (0.01 M citrate buffer, pH 7). Detection steps were done using a commercially available kit (Dako EnVision™ System-HRP, DakoCytomation, Kyoto, Japan) according to the manufacturer's instructions. Hematoxylin was used as counter stain. Evaluation of immunostaining was performed by a consensus of two pathologists blinded to clinical and pathological data. The staining intensity was graded on a scale of 0–2 (0, no staining; 1, weak staining; and 2, strong staining) (22,23). Specimens in which one or more tumor areas with different staining intensities were present were scored for the most prevalent intensity. PSCA expression level was divided into two categories: low (score 0–1) and high (score 2) expression.

Human Lung Cancer Cell Line

A549 (human lung adenocarcinoma cell line) was used for mRNA analysis. It was obtained from the Cell Resource Center for Biochemical Research, Tohoku University (Sendai, Japan). A549 cells were grown in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated from A549 using RNAspin Mini (GE, Healthcare, Japan) and the first-strand cDNA was synthesized from 2 μg RNA by using a ReverTra Ace (TOYOBO, Tokyo, Japan) according to the instructions of the manufacturer. Reverse transcriptase–polymerase chain reaction (RT–PCR) was performed by using KOD FX (TOYOBO). PCR primers were designed to amplify a 202 bp cDNA PSCA fragment (5′-CCACCCTTAACCCTGTGTTC-3′, sense; 5′-AAACTCCCAGGAACTCACGTC-3′, antisense). PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosystems, CA, USA) using 1 μl of cDNA in a 20 μl final reaction volume. The PCR conditions were as follows: initial step at 95°C for 2 m; 30 cycles of amplification (98°C for 10 s, 60°C for 30 s and 72°C for 60 s); and a final extension step of 4 m at 72°C. The PCR products were resolved by electrophoresis in a 2% agarose gel and stained ethidium bromide. Loading was controlled by the simultaneous PCR of housekeeping gene β-actin. Real-time quantitative PCR analysis was done by using StepOnePlus Real Time PCR System (Applied Biosystems). All primer/

probe sets were purchased from Applied Biosystems (GAPDH, Hs99999905_m1; PSCA, Hs00194665_m1). The PCR thermal cycle conditions were as follows: initial step at 95°C for 10 m, followed by 40 cycles of 95°C for 15 s and 60°C for 1 m. The expression level of the housekeeping gene GAPDH was measured as an internal reference with a standard curve to determine the integrity of template RNA for all of the specimens. The ratio of mRNA level of each gene was calculated as follows: (absolute copy number of PSCA)/(absolute copy number of GAPDH).

RNA Interference

A549 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Stable knockdown of PSCA gene in A549 cells was done using the short hairpin RNA (shRNA) method (24). shRNA coding for PSCA was introduced into the pSINsi-hU6 DNA vector (Takara Bio Inc., Shiga, Japan) at the BamH1 and Cla1 ligation sites according to the manufacturer's instruction. shRNA oligonucleotides were synthesized corresponding to the published sequence of PSCA mRNA (NM005672). The oligonucleotide sequences used in the construction of the RNA interference (RNAi) vector were as follows: RNAi, 5′-GATCCGCTAACGCAAG TCTGACCATTAGTGCTCCTGGTTGATGGTCAGACTTG CGTTAGCTTTTTTAT-3′ and 5′-CGATAAAAAAGCTAA CGCAAGTCTGACCATCAACCAGGAGCACTAATGGTCA GACTTGCGTTAGCG-3′; RNAi scramble, 5′-GATCCGTC TTAATCGCGTATAAGGCTAGTGCTCCTGGTTGGCCT TATACGCGATTAAGACTTTTTTAT-3′ and 5′-CGATAAA AAAGTCTTAATCGCGTATAAGGCCAACCAGGAGCAC TAGCCTTATACGCGATTAAGACG-3′. These plasmids that formed into linear shape by restriction enzyme SpeI (Takara Bio Inc.) were, respectively, transfected into A549 by electroporation using Nucleofector™ II and the Cell Line Nucleofector Kit T (Amaxa Biosystems, MD, USA); 48 h after electroporation, the cells were harvested by trypsin–EDTA (Wako, Osaka, Japan), diluted to six times and reseeded in 10 cm culture dishes. For stable transfection, the transfected cells were selected with geneticin (G418; 600 mg/ml). After 10 days, colonies were picked with a cloning ring and reseeded in the culture plate. For the evaluation of PSCA knockdown, PSCA expression was determined by RT–PCR.

Cell Proliferation Assay

MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) assay was used for evaluation of cell proliferation. Aliquots of 2×10^3 cells/well were cultured in 96-well plates. After 72 h, MTS assay was performed using CellTiter 96 Aqueous One Solution (Promega, WI, USA) following the manufacturer's instructions.

Statistical Analysis

Disease-free survival (DFS) was analyzed by the Kaplan and Meier method, and the survival curves were compared using the log-rank test. The endpoint of DFS was defined as relapse of the lung cancer and the length was measured from the day of the operation. The observation was censored at the last follow-up when the patient was alive without recurrence or at the date of death when the patient died from causes other than lung cancer. Comparison of the clinicopathological variables according to the PSCA expression was determined by χ^2 test. The results of MTS assay were compared by Student's *t*-test. Statistical analysis was considered to be significant when the probability value was <0.05.

RESULTS

PSCA Protein Expression in Surgical Specimens of NSCLC

First, we evaluated the PSCA protein expression in clinical human lung cancer tissues. Ninety-seven surgical specimens of NSCLC were examined by immunohistochemistry. The expression of PSCA was demonstrated mainly in the cytoplasm of tumor cells (Figs 1 and 2). On the other hand, PSCA was not expressed in normal lung epithelium. Ninety-four out of the 97 specimens (97%) were stained positively for PSCA. They consisted of 63 lesions (24 in weakly positive and 39 in strongly positive) in adenocarcinoma, 23 (4 in weakly positive and 19 in strongly positive) in squamous cell carcinoma and 8 (2 in weakly positive and 6 in strongly positive) in large cell carcinoma. Most specimens showed a uniform staining in different tumor areas of each specimen. Only three specimens (one in adenocarcinoma and two in squamous cell carcinoma) showed negative staining.

Correlations Between PSCA Expression and Clinicopathological Characteristics

According to the immunohistochemical staining intensity, PSCA expression level of 97 specimens was divided into two categories: low ($n = 33$) and high ($n = 64$) expression. The correlations between PSCA expression and various clinicopathological characteristics are shown in Table 1. PSCA expression had significant correlations with several factors. Patients with high PSCA expression had more advanced disease than those with low expression (T1 vs. T2–4, $P = 0.014$; Stage I vs. Stages II–IV, $P = 0.029$, respectively).

Correlations Between PSCA Expression and Post-operative Prognosis

The 5-year DFS rate of patients with low PSCA expression was significantly better than that of those with high PSCA expression (90% vs. 53%, $P = 0.001$) (Fig. 3). When the

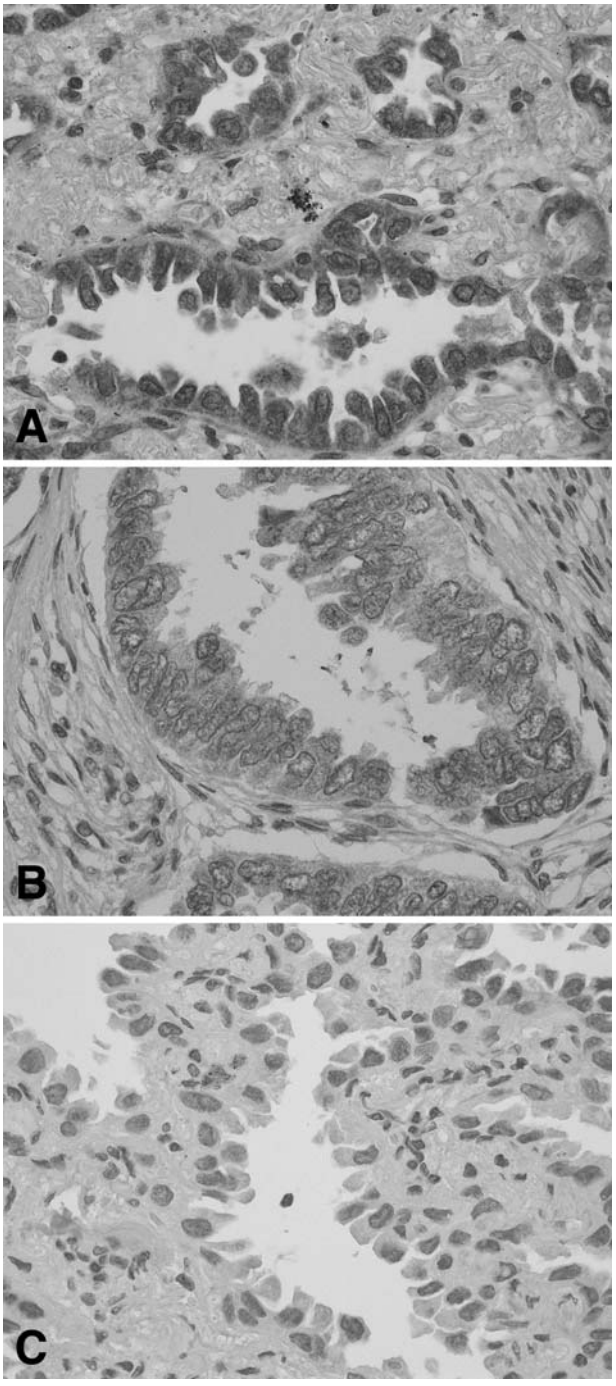

Figure 1. Representative immunohistochemical staining of prostate stem cell antigen (PSCA) in adenocarcinoma. (A) Strongly positive, (B) weakly positive and (C) no staining. Original magnification, ×400. A color version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org.

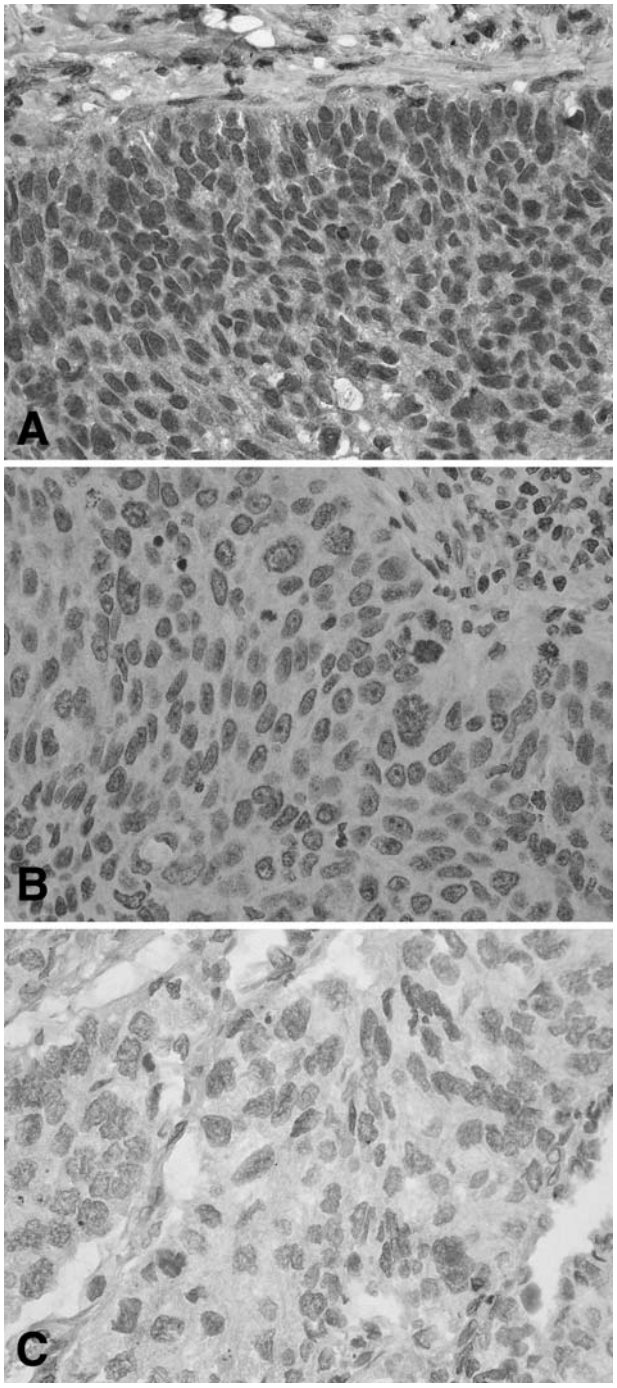

Figure 2. Representative immunohistochemical staining of PSCA in squamous cell carcinoma. (A) Strongly positive, (B) weakly positive and (C) no staining. Original magnification, ×400. A color version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org.

survival analysis was performed in 64 patients with adenocarcinoma, the prognostic difference was also significant (5-year DFS rate: 88% vs. 52%, $P = 0.005$) (Fig. 4). Furthermore, patients with non-adenocarcinoma of low PSCA expression had a tendency to have a better postoperative prognosis compared with those with the high expression (5-year DFS rate: 100% vs. 61%, $P = 0.236$), although the difference did not reach statistical significance possibly due to the small number of patients (Fig. 5).

PSCA Expression in Metastatic Lymph Nodes of NSCLC

Furthermore, the PSCA expression in available 21 specimens with metastatic lymph nodes was examined by immunohistochemistry (16 specimens in adenocarcinoma, 3 in squamous

Table 1. Correlations between PSCA expression and clinicopathological characteristics

Clinicopathological features	PSCA expression		*P* value
	High ($n = 64$)	Low ($n = 33$)	
Gender			
Male	47	20	0.177
Female	17	13	
Smoking			
Yes (male/female)	50 (45/5)	20 (19/1)	0.068
No (male/female)	14 (2/12)	13 (1/12)	
Histology			
Ad	39	25	0.144
Non-Ad (Sq/La)	25 (19/6)	8 (6/2)	
Pathological T classification			
T1	22	20	0.014
T2–4	42	13	
Pathological N classification			
N0	42	27	0.095
N1–2	22	6	
Pathological stage			
I	36	26	0.029
II–IV	28	7	

PSCA, prostate stem cell antigen; Ad, adenocarcinoma; non-Ad, non-adenocarcinoma including squamous cell carcinoma (Sq) and large cell carcinoma (La).

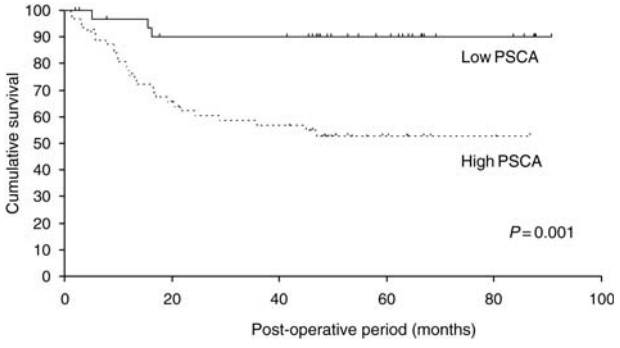

Figure 3. Disease-free survival (DFS) curves of all patients according to the PSCA expression. Patients with high PSCA expression ($n = 64$, solid line) had a worse prognosis than those with low PSCA expression ($n = 33$, dotted line).

cell carcinoma and 2 in large cell carcinoma). PSCA was positively expressed in all metastatic lymph nodes (3 in weakly positive and 18 in strongly positive) (Fig. 6). Correlations of PSCA expression in metastatic lymph nodes and matched primary lesions are shown in Table 2. In four out of six patients having primary lesion with low PSCA expression, the PSCA expression level in metastatic lymph node was higher than the primary lesion.

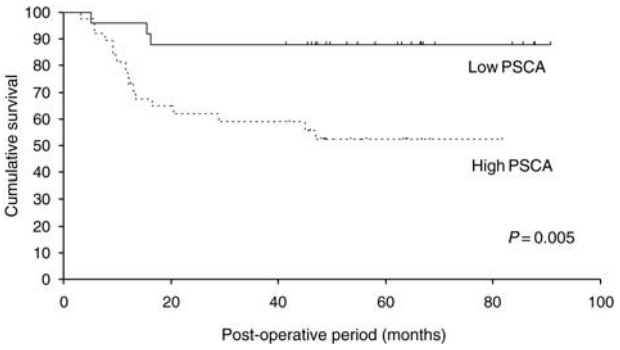

Figure 4. DFS curves of patients with adenocarcinoma according to the PSCA expression. Patients with high PSCA expression ($n = 39$, solid line) had a worse prognosis than those with low PSCA expression ($n = 25$, dotted line).

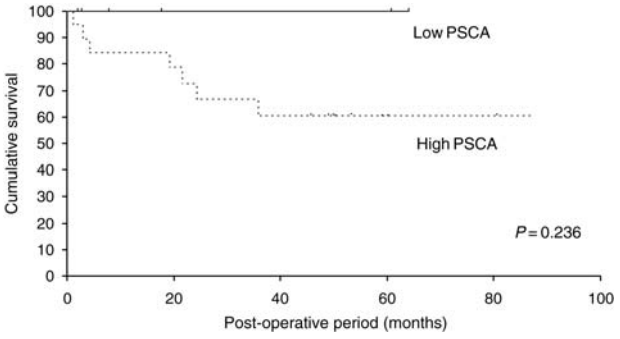

Figure 5. DFS curves of patients with non-adenocarcinoma according to the PSCA expression. Patients with high PSCA expression ($n = 25$, solid line) had a tendency to have a worse prognosis than those with low PSCA expression ($n = 8$, dotted line).

Small interfering RNA Knockdown of PSCA Inhibits Lung Cancer Cell Growth

Finally, we investigated the therapeutic efficacy of targeting PSCA in a human lung adenocarcinoma cell line (A549). To this end, we employed conventional small interfering RNA (siRNA) knockdown method. We first confirmed the PSCA gene expression in A549, and it was successfully depleted by designed siRNA (Fig. 7A). By MTS assay, we found that depletion of PSCA by siRNA significantly inhibited the growth of A549 compared with control (Fig. 7B, $P < 0.001$). There was ~40% inhibition in A549 cell growth.

DISCUSSION

PSCA is a cell surface antigen and predominantly prostate-specific in normal tissues. It has been reported to be overexpressed in more than 80% of prostate cancer (5,6). In addition, PSCA expression was also identified in some other malignancies including bladder, pancreatic and renal cancer (15,16,18,19). They have suggested that PSCA might have a potential in the diagnosis and treatment of human cancer (5–7,9–11).

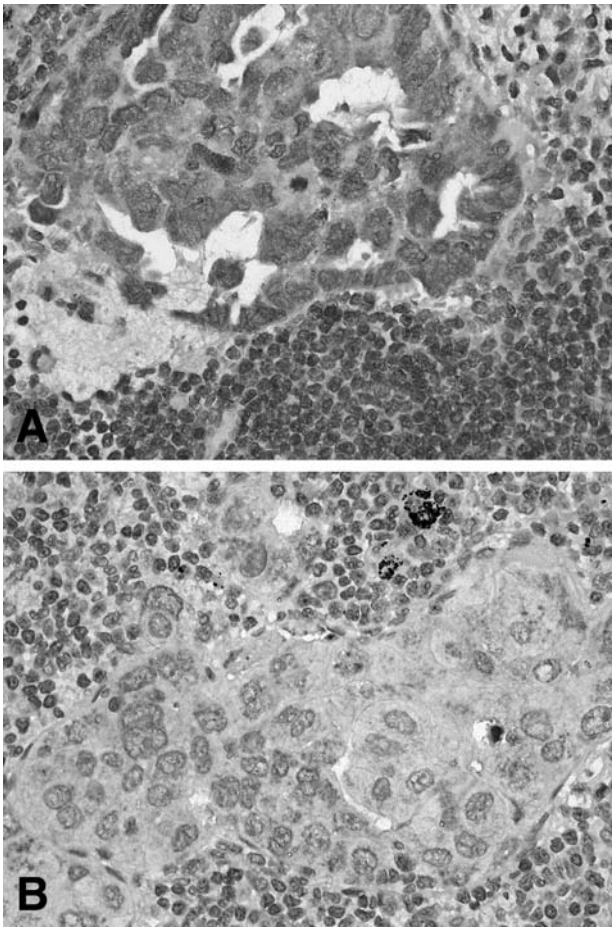

Figure 6. Representative immunohistochemical PSCA staining in metastatic lymph nodes. (A) Strongly positive and (B) weakly positive. Original magnification, ×400. A color version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org.

Table 2. PSCA expression in metastatic lymph nodes and matched primary lesion

Metastatic LN	Primary tumor		Total
	High	Low	
High	14	4	18
Low	1	2	3
Total	15	6	21

LN, lymph node.

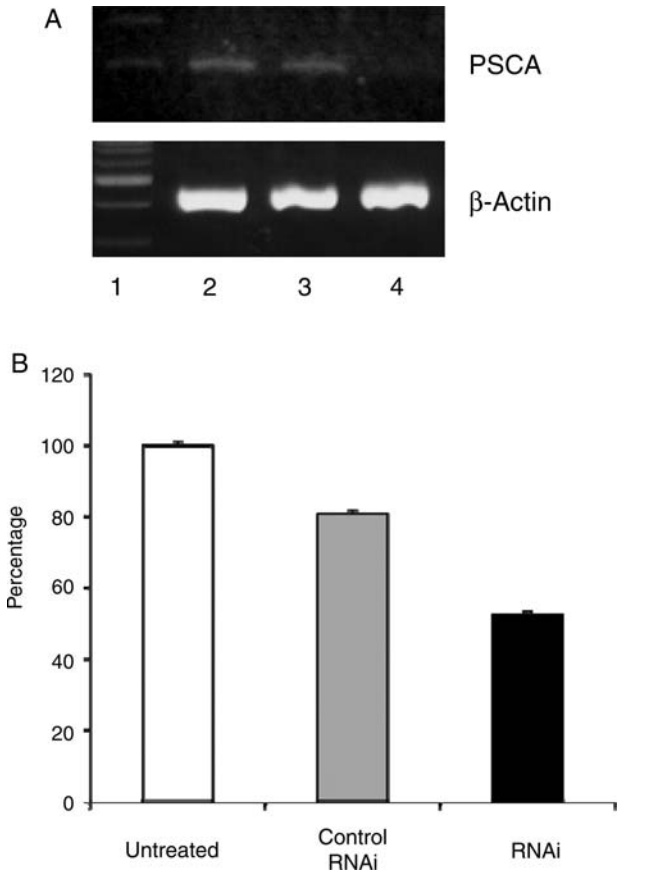

Figure 7. Small interfering RNA (siRNA) knockdown of PSCA. (A) Reverse transcriptase–polymerase chain reaction analysis of PSCA mRNA on A549, adenocarcinoma lung cancer (lane 1, size marker; lane 2, untreated A549; lane 3, control non-specific siRNA treated; and lane 4, RNA1 treated). (B) Cell growth inhibition was examined by MTS assay. The results were expressed mean ± SEM obtained from three to five wells in each group. Experiments were repeated at least three times with similar results. There was ~40% inhibition in cell growth of A549 treated with RNAi in comparison with control RNAi ($P < 0.001$). Open bar, untreated A549; gray bar, A549 treated with control non-specific RNAi; closed bar, A549 treated with RNAi.

In this study, we examined the PSCA expression in surgically resected NSCLC tissues. First, we confirmed that a large percentage of clinical lung cancer tissues had PSCA protein expression, whereas normal lung epithelium did not express PSCA. The result suggested that PSCA might play some roles in NSCLC. To determine the clinical significance of PSCA expression, we analyzed the correlations of PSCA expression level with several clinicopathological factors. We found that the intensity of PSCA expression was significantly correlated with pathological T-factor and stage. In addition, PSCA expression was also found in metastatic lymph nodes. Thus, PSCA might play a critical role in tumor progression as well as metastasis in human lung cancer. Our data may corroborate with the previous reports on other malignancies including prostate and renal cancer (6,7,16,18,19). Furthermore, this interpretation was further strengthened by the prognostic data. The patients with high PSCA expression had significantly poorer post-operative prognosis than low PSCA expression. The difference was also statistically significant in the patients with adenocarcinoma. Furthermore, the patients with non-adenocarcinoma had a similar trend in prognosis. Taken together, tumor-related PSCA expression may be functionally important in lung cancer, irrespective of the histological type.

The precise physiological and pathological function of PSCA in tumor biology has not been fully elucidated yet.

Previous studies have revealed the activities of some homologues of PSCA including CD177, CD59 and uPAR belonging to Ly-6 gene superfamily (25–28). Their activities comprise cell adhesion, cell migration and the inhibition of complement-mediated hemolysis (25,26). Moreover, Ly-6A (Sca-1) negatively regulates lymphocyte activation via influencing T cell signaling and T cell receptor expression (27,28). In tumors, the high level of Ly-6E expression was associated with high malignancy and metastatic phenotype (29). Furthermore, the regulatory mechanisms of PSCA expression were also remain unknown. Gu et al. (6) speculated that c-myc amplification might associate with PSCA overexpression in prostate cancer. Their speculation was based on the following data: (i) PSCA mapped distal to c-myc on chromosome 8q24.2 and (ii) PSCA was co-amplified with c-myc in prostate cancer (30). In addition, c-myc is also known to be one of the important oncogenic proteins of lung cancer, and its tumorigenesis is contributed by cigarette smoke (31,32). Taken together, c-myc might be involved in PSCA expression in lung cancer. Further studies are required to define these unknown mechanisms relating to PSCA in cancer.

PSCA has been primarily studied in prostate cancer. Based on those various experimental and clinical research data, it has been considered as a promising therapeutic target for metastatic and hormone-refractory prostate cancer (9–14,33). Proposed strategies include peptide vaccine as well as monoclonal antibody therapy. Several animal studies have demonstrated that each treatment effectively induced PSCA-specific cellular and humoral immune responses *in vivo* (33). In addition, more importantly, we found that siRNA-mediated knockdown of PSCA resulted in the significant reduction of lung cancer cell growth. Although further studies are required to confirm antitumor effect of targeting PSCA using other different lung cancer cell lines, our data support that PSCA could be a therapeutic target for human malignancies including lung cancer. Furthermore, a Phase I/II clinical trial with PSCA peptide vaccination against hormone and chemotherapy refractory prostate cancer has already proven a certain clinical benefit (34). Therefore, these novel therapies can be easily applied to lung cancer and may hold a promise to improve patient survival.

In conclusion, we have demonstrated for the first time that PSCA is highly expressed in human lung cancer and its knockdown significantly inhibited human lung cancer cell growth. Both our clinical and experimental data suggest that PSCA may be a functionally important and novel therapeutic molecular target for lung cancer.

Conflict of interest statement

None declared.

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