

## Original Article

# Expression of CD44 and prostate stem cell antigen and their prognostic significance in human pancreatic ductal adenocarcinoma

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**Abstract:** CD44 and prostate stem cell antigen (PSCA) have been investigated as two biomarkers for various cancers. However, either CD44 or PSCA expression alone has limited clinical value. In the present study, the expression of CD44 and PSCA in human pancreatic ductal adenocarcinoma (PDAC) and their values in prognosis were investigated by immunohistochemistry (IHC) and Kaplan-Meier method. The results suggested that CD44 and PSCA were significantly upregulated in pancreatic tumor tissue. No significant association were observed between CD44 expression and PSCA expression ( $r=0.15$ ,  $P=0.03$ ). High expression of CD44 was significantly correlated with poor tumor differentiation ( $P=0.021$ ), and indicated a poor outcome ( $P=0.003$ ). Expression of PSCA was positively related to tumor size ( $P=0.027$ ) and nodal metastasis ( $P=0.030$ ). Co-expression of CD44 and PSCA significantly indicated a poor outcome ( $P=0.005$ ). Moreover, multivariate survival analysis demonstrated that positive co-expression of CD44 and PSCA was an independent indicator for PDCA prognosis ( $P=0.040$ ). Therefore, our data indicated that co-staining of CD44 and PSCA should be recommended in PDCA patients for predicting prognosis.

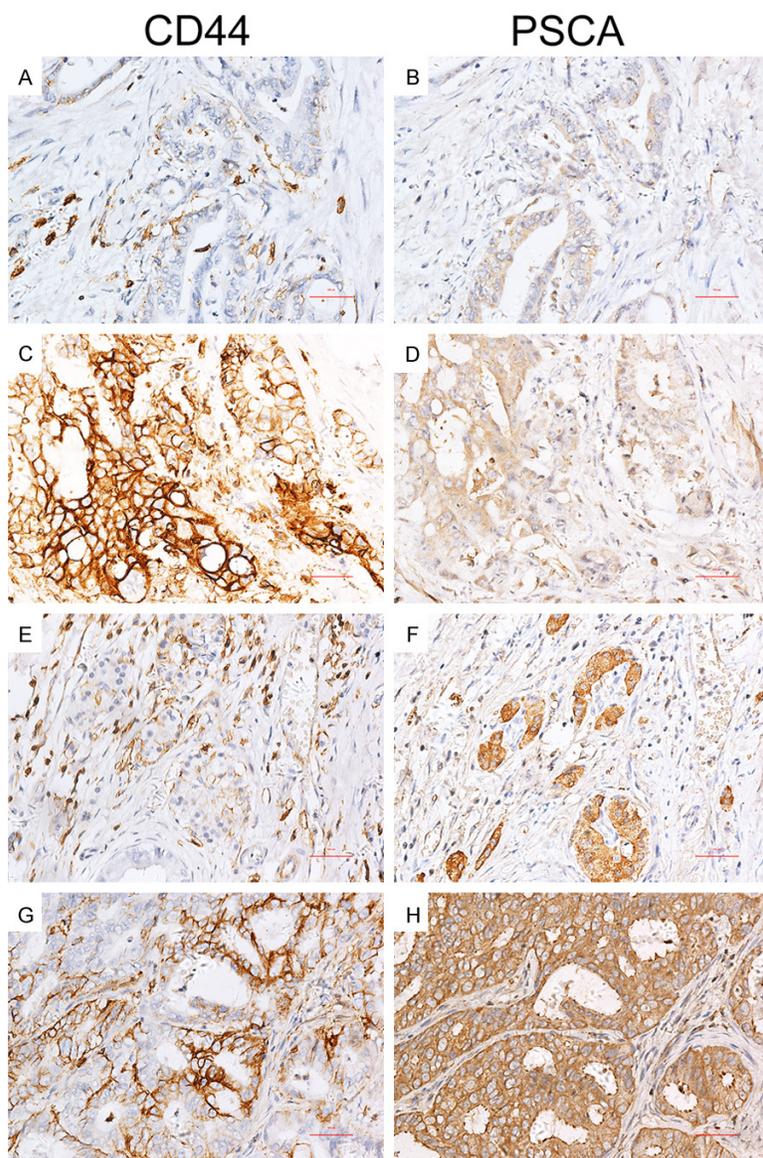
**Keywords:** Pancreatic ductal adenocarcinoma, CD44, PSCA, prognosis

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant cancers. Global statistical data in 2015 ranked PDAC as the fourth leading cause of cancer deaths. More than 90% of PDAC patients are diagnosed at an advanced stage, when it is too late to carry out surgery. The overall 5-year survival rate is less than 10% [1]. It is widely accepted that cancer stem cells (CSCs) are responsible for the poor prognosis of incurable solid tumors. CSCs are implicated in many biological processes in pancreatic cancers, including proliferation, differentiation, migration, invasion and resistance to therapy. Numerous studies have focused on the identification of CSC biomarkers to elucidate their significance for predicting patient outcomes [2-6]. CD44 is a putative CSC marker and involved in various biological processes, such as extracellular matrix binding, embryonic development, cell proliferation, survival, invasion, migration and the epithelial-mesenchymal transition [7-11]. CD44 expression is associated with overall survival (OS), tumor recurrence,

metastasis and resistance to chemo/radiation therapy in various cancers, including PDAC [12-15].

Prostate stem cell antigen (PSCA) is a cell surface antigen, which is initially identified as a prostate stem cell surface-specific marker [16]. Previous studies have suggested that PSCA can be expressed in various cancer cells of human solid cancers, including PDAC [17-24]. PSCA is implicated in the progress of carcinogenesis, such as cell proliferation, survival, adhesion and migration [25, 26]. The previous clinical trial demonstrated that gemcitabine plus AGS-1C4D4, a fully human monoclonal antibody to prostate stem cell antigen (PSCA), obviously improved 6-month survival rate than gemcitabine alone of PDCA patients [27]. However, as two putative biomarkers of cancer cells, there is still no research on the correlation between CD44/PSCA co-expression and clinical prognosis in PDCA. Here, we examined the expression of CD44 and PSCA proteins in PDAC using immunohistochemistry (IHC) with a tissue microarray (TMA) strategy, respectively, and first



**Figure 1.** Representative PDAC cases with different CD44/PSCA expression profiles were detected by IHC in the same field. (A and B) CD44-/PSCA- (A: CD44, B: PSCA). (C and D) CD44+/PSCA- (C: CD44, D: PSCA). (E and F) CD44-/PSCA+ (E: CD44, F: PSCA). (G and H) CD44+/PSCA+ (G: CD44, H: PSCA). Magnification 200 $\times$ .

assessed the clinical and prognostic significance of CD44/PSCA co-expression in PDAC.

### Materials and methods

#### *Patients and tissue specimens*

The following patient inclusion criteria were applied: (1) PDCA patients who had undergone surgery at Peking Union Medical College Hospital between January 2009 and December 2013; (2) the presence of complete clinicopathological and follow-up data; and (3) the avail-

ability of formalin fixed paraffin embedded (FFPE) tissue samples not subjected to chemotherapy, radiotherapy or targeted therapy. After their histopathological diagnoses were reconfirmed, 94 PDAC patients were finally included in our study. Tissue specimens were obtained from the Department of Pathology, Peking Union Medical College Hospital, China. Medical records were thoroughly reviewed for sex, age at the diagnosis, pathological diagnosis, tumor size, involved sites, resection margins, differentiation, lymph node involvement, TNM stage, tumor relapse and date of last follow-up. Experienced pathologists verified the pathological confirmations built on the 2010 WHO classification [28].

The ethics committee of Peking Union Medical College Hospital approved the study, and informed consent was obtained from all participants.

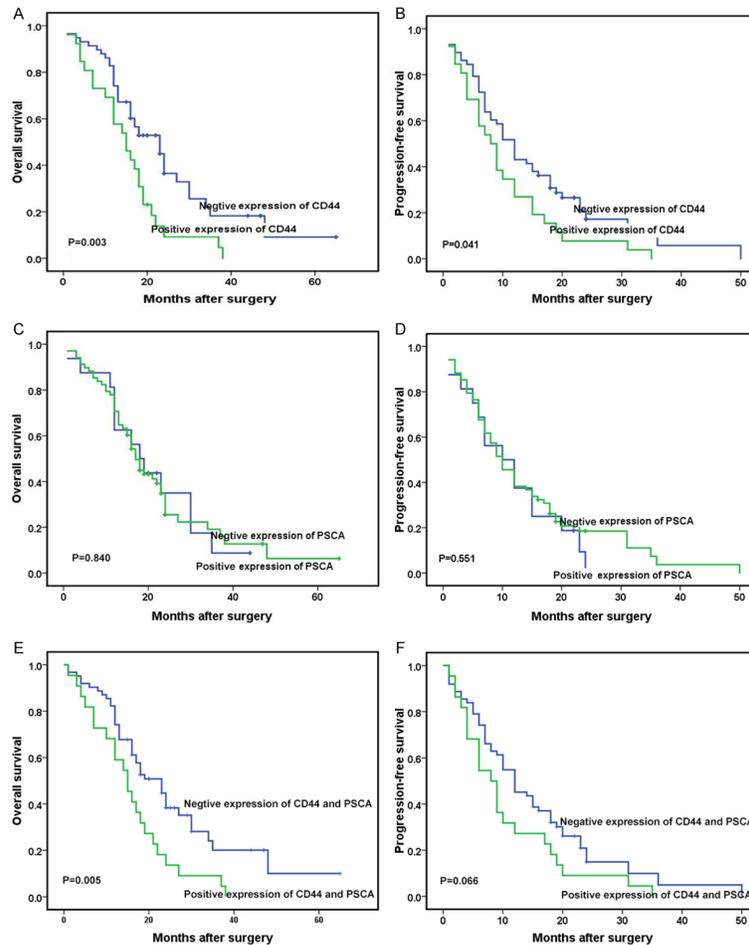
#### *TMA construction*

After reviewing all the tissue specimens included in our study, two experienced pathologists selected representative regions of PDAC tumors. TMAs were then constructed using 2-mm-diameter cores punched from representative regions of each FFPE tumor and normal block. The recipient blocks were cut into consecutive 4- $\mu$ m-thick sections. Each punched region to be sampled was reviewed prior to be use.

#### *IHC staining*

IHC staining was conducted on TMA slides to detect the expression of CD44 and PSCA proteins. IHC was performed using a previously described method [29]. TMA slides were baked for 1 h at 60 $^{\circ}$ C, deparaffinized in xylene, and

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**Figure 2.** Kaplan-Meier survival analysis showing OS and PFS of PDCA patients according to CD44 and PSCA expression. A and B: Correlation of CD44 protein expression and OS as well as PFS: negative expression, n=65; positive expression, n=29. C and D: Correlation of PSCA protein expression and OS as well as PFS: negative expression, n=17; positive expression, n=77. E and F: Correlation of CD44 and PSCA protein co-expression and OS as well as PFS: negative expression, n=69; positive expression, n=25.

dehydrated through graded ethanol. Antigen retrieval was performed using a high-pressure method of 10 mM citrate buffer (pH 6) for 4 min. One to three drops of 10% normal goat serum were added to the slides when the citrate buffer became cold at room temperature, and the slides were incubated at room temperature for 15 min. Slides were drained and incubated with anti-CD44 and PSCA mouse monoclonal antibodies at 37°C, for 2 h in a moist chamber. All the antibodies used in the present study were obtained from R&D Systems Inc., Minneapolis, USA). The slides were rinsed three times with 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) for 5 min and incubated with a secondary antibody (ZSGB-BIO, China) for 1 h at room temperature. The

slides were rinsed three times with 0.01 mol/L PBS (pH 7.4) for 5 min and stained with DAB (3,3-diaminobenzidine). Finally, the slides were stained with hematoxylin, dehydrated and mounted.

### IHC assessment

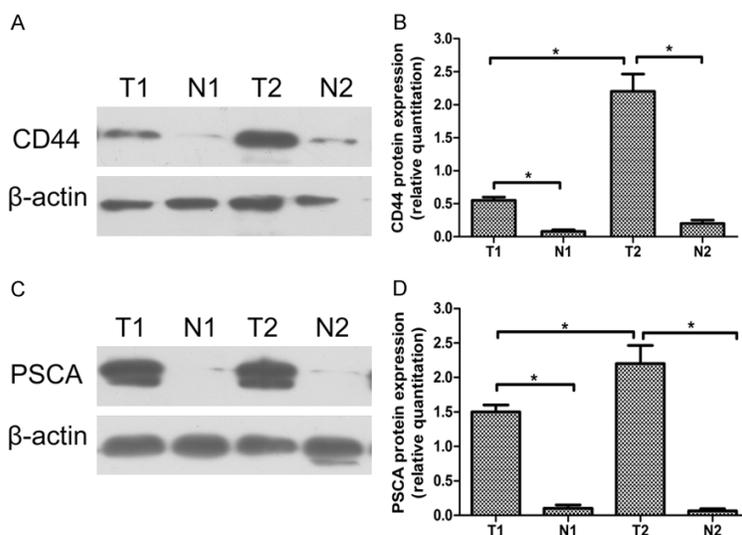
Two experienced pathologists independently evaluated the IHC staining using a light microscope at magnifications of 200×. We used semi-quantitative methods to score CD44 and PSCA expression. In detail, we simultaneously scored the percentage of positive-staining cells and the staining intensity of glandular cells in tumor and normal tissues. A final score was subsequently assigned to the tumor tissue by multiplying the two scores. The percentage of positive glandular cells was scored on a scale from 0 to 3: 0, less than 10% of cells stained; 1, 10%-25% cells stained; 2, 25%-50% cells stained; and 3, >50% cells stained. The staining intensities of the glandular cells were also scored using a scale from 0 to 3: 0, absolutely no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Based on clinical experience

and relevant references [30, 31], positive expression was defined as a final score greater than 4, and negative expression was defined as a final score less than or equal to 4 for both CD44 and PSCA proteins. Each of pathologists assessed the same tissue sample more than twice to confirm the reliability of the final scores. Discordant cases were examined by another pathologist, and a final consensus was reached.

### Western blot analysis

Two paired fresh PDCA tissues and normal pancreatic tissues were obtained from two PDCA patients, including one paired well differentiation specimens without nodal metastasis and one paired poor differentiation specimens with nodal metastasis. Tissue proteins were obtain-

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**Figure 3.** Western blot analysis of CD44 and PSCA proteins expression in two-paired tumor and normal pancreatic tissues. A and C: Immunoblot showing CD44 and PSCA proteins expression. B and D: Western blot data were quantified and normalized to an internal standard  $\beta$ -actin. Bars indicate mean  $\pm$  SD. \* $P < 0.05$ .

ed by liquid nitrogen grounding method. Western blot was performed according to the standard protocol. Briefly, added 30  $\mu$ g proteins of each sample per lane for gel electrophoresis, transferred onto polypropylene difluoride membranes and incubated overnight at 4°C with primary polyclonal antibody. Then, the second day, incubated with the secondary peroxidase-conjugated antibody (ZSGB-BIO, China) and detected by an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ), according to the manufacturer's instructions.

### Statistical analysis

SPSS 16.0 (SPSS Inc., Chicago, IL) was used to analyze the results. Correlations between CD44 and PSCA expression and clinicopathological features of the PDAC patients were tested using the  $\chi^2$ -test. Survival curves were calculated using the Kaplan-Meier method, and the results were compared using the log-rank test. Differences were considered significant at a  $P$  value  $\leq 0.05$  (two-tailed). A multivariate analysis was performed using a Cox model.

## Results

### Expression of CD44 and PSCA proteins in PDAC

CD44 and PSCA were primarily present in the cytoplasm and membrane of cancer cells, and

lowly expressed in normal pancreatic tissues (Figures 1 and 3). Positive expression of CD44, PSCA and CD44/PSCA accounted for 30.85%, 81.91% and 26.60% of the 94 PDAC patients, respectively (Table 1). Representative PDAC tissues with different CD44/PSCA expression profiles are shown in Figure 1.

### Correlations between CD44/PSCA expression and clinicopathological characteristics of PDAC patients

Table 1 summarizes the correlation between clinical data and CD44/PSCA expression levels of the 94 PDAC patients. The clinical data included sex, age at diagnosis, tumor size, tumor primary

sites, resection margins, tumor differentiation, nodal metastasis and TNM stage. SPSS 16.0 analysis revealed that positive expression of CD44 was significantly higher in poor differentiation cases (13/27, 48.1%) than in well and moderated cases (16/67, 23.9%) ( $P = 0.021$ ), while positive expression of PSCA was significantly associated with tumor size ( $P = 0.027$ ) and nodal metastasis ( $P = 0.030$ ) of PDAC (Table 1). Western blot analysis also indicated that expression of CD44 and PSCA was higher in poor differentiation specimen (T2) with nodal metastasis than in well differentiation specimen (T1) without nodal metastasis (Figure 3). In addition, positive co-expression of CD44 and PSCA was significantly associated with tumor differentiation ( $P = 0.003$ ) (Table 1). However, there was no significant correlation between CD44 and PSCA expression ( $r = 0.15$ ,  $P = 0.03$ ) (Supplementary Figure 1).

### Prognostic factors for PDAC: univariate and multivariate survival analyses

The present study investigated all of the well-established prognostic factors of PDAC patient survival using a Kaplan-Meier analysis. The median durations of follow-ups for overall survival (OS) and progression-free survival (PFS) of the 94 PDAC patients were 18.00 months and 10.00 months, respectively (Figure 2). We analyzed the influences of the following indi-

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**Table 1.** Correlation between CD44/PSCA expression with clinicopathology characteristics in pancreatic carcinoma

	All cases	CD44 protein			PSCA protein			CD44/PSCA protein		
		Negative expression	Positive expression	P-value <sup>a</sup>	Negative expression	Positive expression	P-value <sup>a</sup>	Negative expression	Positive expression	P-value <sup>a</sup>
Sex				0.667			0.449			0.583
Female	42	30 (71.4%)	12 (28.6%)		9 (21.4%)	33 (78.6%)		32 (76.2%)	10 (23.8%)	
Male	52	35 (67.3%)	17 (32.7%)		8 (15.4%)	44 (84.6%)		37 (71.2%)	15 (28.8%)	
Age at diagnosis				0.419			0.864			0.913
≤60	46	30 (65.2%)	16 (34.8%)		8 (17.4%)	38 (82.6%)		34 (73.9%)	12 (26.1%)	
>60	48	35 (72.9%)	13 (27.1%)		9 (18.8%)	39 (81.2%)		35 (72.9%)	13 (27.1%)	
Size (diameter), cm				0.992			0.027			0.571
≤2	26	18 (69.2%)	8 (30.8%)		1 (3.8%)	25 (96.2%)		18 (69.2%)	8 (30.8%)	
>2	68	47 (69.1%)	21 (30.9%)		16 (23.5%)	52 (76.5%)		51 (75.0%)	17 (25.0%)	
Tumor sites				0.210			0.214			0.410
Head	48	36 (75.0%)	12 (25.0%)		11 (22.9%)	37 (77.1%)		37 (77.1%)	11 (22.9%)	
Body/Tail	46	29 (63.0%)	17 (37.0%)		6 (13.0%)	40 (87.0%)		32 (69.6%)	14 (30.4%)	
Resection margins				0.820			0.210			0.519
Negative	79	55 (69.6%)	24 (30.4%)		16 (20.3%)	63 (79.7%)		59 (74.7%)	20 (25.3%)	
Positive	15	10 (66.7%)	5 (33.3%)		1 (6.7%)	14 (93.3%)		10 (66.7%)	5 (33.3%)	
Differentiation				0.021			0.601			0.003
Well/moderate	67	51 (76.1%)	16 (23.9%)		13 (19.4%)	54 (80.6%)		55 (82.1%)	12 (17.9%)	
Poor	27	14 (51.9%)	13 (48.1%)		4 (14.8%)	23 (85.2%)		14 (51.9%)	13 (48.1%)	
Nodal metastasis				0.249			0.030			0.206
No	44	33 (75%)	11 (25%)		12 (27.3%)	32 (72.7%)		35 (79.5%)	9 (20.5%)	
Yes	50	32 (64%)	18 (36%)		5 (10%)	45 (90%)		34 (68.0%)	16 (32.0%)	
TNM stage				0.674			0.399			0.435
I/II	83	58 (69.9%)	25 (30.1%)		14 (16.9%)	69 (83.1%)		62 (74.7%)	21 (25.3%)	
III/IV	11	7 (63.6%)	4 (36.4%)		3 (27.3%)	8 (72.7%)		7 (63.6%)	4 (36.4%)	

<sup>a</sup>Chi-square test.

vidual factors on survival: sex, age, tumor size, tumor sites, resection margins, differentiation, nodal metastasis, TNM stage, expression of CD44, expression of PSCA and co-expression of CD44 and PSCA. The univariate analysis revealed that positive expression of CD44 (P=0.003), positive co-expression of CD44 and PSCA (P=0.005), tumor resection margins (P=0.009), differentiation degree (P<0.001), nodal metastasis (P=0.002) and TNM stage (P=0.003) were adverse prognostic predictors for PDAC patients (Table 2). PDAC patients who had positive expression of CD44 had a median OS time of 15.0 months, whereas those patients with negative expression of CD44 had a median OS time of 23 months (P=0.003) (Figure 2). Similar to positive expression of CD44, positive co-expression of CD44 and PSCA also indicated a poor outcome, the median OS time of positive co-expression of CD44 and PSCA group (positive co-expression group) versus negative expression of CD44 or PSCA group (negative expression group) was 15.0 months versus 23.0 months (P=0.005) (Table

2). We used a Cox proportional hazards model to analyze variables, including tumor resection margins, differentiation degree, nodal metastasis, TNM stage and CD44 expression. The results demonstrated that positive co-expression of CD44 and PSCA (HR: 1.764, 95% CI: 1.026-3.031, P=0.040), tumor differentiation degree (HR: 2.730, 95% CI: 1.533-4.863, P=0.001), nodal metastasis (HR: 1.766, 95% CI: 1.005-3.095, P=0.048) and TNM stage (HR: 2.632, 95% CI: 1.23-5.61, P=0.013) were independent risk factors for poor clinical outcome, while CD44 positive expression cannot be served as an independent prognostic factor (HR: 1.582, CI: 0.921-2.717, P=0.097) (Table 2).

### Discussion

Numerous tumor biomarkers have been identified, including CD44 and PSCA. However, only a few of them are applied in clinic as an independent diagnostic or prognostic indicator. Sometimes, more than one biomarker were

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**Table 2.** Univariate and multivariate analyses of different prognostic factors in 94 patients with Pancreatic ductal carcinoma

Variable	All cases	Univariate analysis <sup>a</sup>		Multivariate analysis <sup>b</sup>	
		Median survival (months)	P-value	HR (95% CI)	P-value
Sex			0.968		
Female	42	23.00			
Male	52	18.00			
Age at diagnosis			0.763		
≤60	46	17.00			
>60	48	22.00			
Size (diameter), cm			0.416		
≤2	26	21.00			
>2	68	17.00			
Tumor sites			0.703		
Head	48	18.00			
Body/Tail	46	18.00			
Resection margins			0.009	1.626 (0.823-3.20)	0.161
Negative	79	21.00			
Positive	15	13.00			
Differentiation			0.000	2.730 (1.533-4.863)	0.001
Well/moderate	67	24.00			
Poor	27	13.00			
Nodal metastasis			0.002	1.766 (1.005-3.095)	0.048
No	44	24.00			
Yes	50	15.00			
TNM stage			0.003	2.632 (1.23-5.61)	0.013
I/II	83	19.00			
III/IV	11	11.00			
CD44 expression			0.003	1.582 (0.921-2.717)	0.097
Negative expression	65	23.00			
Positive expression	29	15.00			
PSCA expression			0.840		
Negative expression	17	19.00			
Positive expression	77	17.00			
CD44 and PSCA			0.005	1.764 (1.026-3.031)	0.040
Negative expression	69	23.00			
Positive expression	25	15.00			

<sup>a</sup>Log-rank test. <sup>b</sup>Cox regression model. HR indicates hazards ratio; CI indicates confidence interval.

needed in diagnosis and prognosis. In the present study, we found that positive co-expression of CD44 and PSCA was an independent prognostic indicator for PDCA patients.

The CSC theory suggests that a subpopulation of cancer cells with stem cell properties of self-renewal, differentiation and infinite proliferation drive cancer development [32]. CD44 is one of the most promising markers for CSCs. Emerging evidences suggest that positive ex-

pression of CD44 is associated with poor prognosis in PDCA [33, 34]. However, no consensus opinion on cancer progression has been reached. The present study demonstrated that CD44 was overexpressed in PDAC, and high CD44 expression was significantly associated with poor differentiation. CD44 is a widely accepted biomarker for stem cells, and it is reasonable that CD44 is overexpressed in poorly differentiated tumors. Thus, we suspect that CD44 overexpression may be associated with

tumor de-differentiation and aggressive biological behavior, and it may play a pivotal role in PDAC carcinogenesis and development. We also found that high CD44 expression may predict a poor patient prognosis. However, significant associations were not found between CD44 expression and metastasis or TNM stage, which differs from the results of several previous studies [33, 34]. Notably, we also found that high CD44 expression was significantly correlated with poor prognosis in PDAC patients, but it was not an independent prognostic factor for PDAC. These results are likely due to the strong association between CD44 and tumor differentiation.

PSCA is another stem cell-specific marker and proposed as a specific biomarker for PDCA early diagnosis. No significant correlation was found between PSCA and PDCA prognosis [35]. However, anti-PSCA target therapy showed inhibitory effect on tumor growth and progression of PDCA [36]. The present study demonstrated that PSCA was highly expressed in PDAC, and high PSCA expression was significantly correlated with tumor size and nodal metastasis, results that are consistent with previous studies [27, 37]. The present results indicated that PSCA may also be involved in PDAC development. However, PDAC expression was not significantly correlated with tumor differentiation and might be of little prognostic value. Since CD44 was not an independent prognostic factor for PDAC as mentioned above, we investigated the positive co-expression of CD44 and PSCA. Strikingly, positive co-expression of CD44 and PSCA was an independent indicator for PDCA prognosis.

In sum, our results demonstrated that CD44 and PSCA may play an important role in PDAC carcinogenesis and development. Positive co-expression of CD44 and PSCA was an independent prognosis indicator of PDCA which would provide a new perspective to the diagnosis and treatment of PDCA. Because the present study was not a multicenter research, the main limitation was its small sample size. Thus, exploration in a larger cohort and multicenter research may be needed to further verify our conclusion. In conclusion, we demonstrated that positive co-expression of CD44 and PSCA identified a subgroup of PDCA patients with more aggressive biological behavior and worse prognosis.

Our results suggested that CD44 and PSCA co-staining would be meaningful as an independent prognostic factor of PDCA in clinic.

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### Disclosure of conflict of interest

None.

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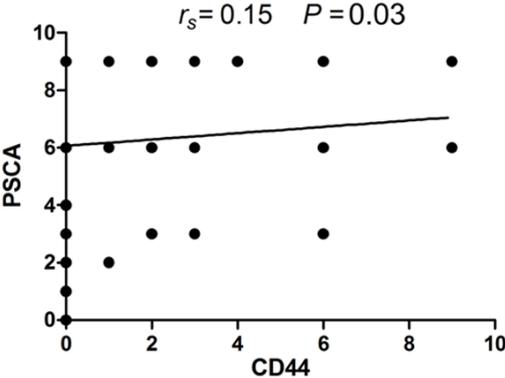
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Supplementary Figure 1. Correlation between CD44 and PSCA expression level.