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Original Article

Elevated expression of PMEPA1 predicts poor prognosis in pancreatic ductal adenocarcinoma

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Abstract: Prostate transmembrane protein, androgen induced 1 (PMEPA1) has been reported to be upregulated and involved in the progression in multiple types of cancer. However, expression of PMEPA1 and its clinical significance in pancreatic ductal adenocarcinoma (PDAC) remain unclear. In this study, we measured the expression of PMEPA1 mRNA using qRT-PCR in 15 pairs of resected PDAC tissues and corresponding adjacent non-tumor tissues. In addition, we assessed the expression of PMEPA1 protein using immunohistochemistry in tissue microarray (TMA) which contains 74 pairs of PDAC tissues and corresponding adjacent non-tumor tissues, as well as another 18 PDAC tissues. Our analysis revealed that expression of PMEPA1 mRNA and protein were elevated in PDAC tissues compared with corresponding non-tumor pancreatic tissues ($P<0.001$). High PMEPA1 expression was positively correlated with histological grade ($P = 0.033$) and regional lymph node metastasis ($P = 0.015$). Survival analysis showed that overall survival of patients with high PMEPA1 expression is significantly shorter than that of patients with low PMEPA1 expression (median survival 7.7 vs. 23 months, $x^2 = 6.979$, $P = 0.008$). The multivariate analysis indicated that high PMEPA1 expression was an independent prognostic factors for poor overall survival in PDAC (hazard ratio = 1.956, $P = 0.015$). High T stage (hazard ratio = 2.491, $P = 0.001$) and regional lymph node metastasis (hazard ratio = 2.245, $P = 0.002$) were also independent prognostic factors for poor survival in PDAC. In conclusion, PMEPA1 might be a valuable prognostic biomarker in PDAC and helpful to selecting patients who need adjuvant treatment.

Keywords: PMEPA1, pancreatic ductal adenocarcinoma, immunohistochemistry, prognosis

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of all pancreatic cancers and is one of the most lethal cancers worldwide [1]. As PDAC is usually diagnosed at advanced stage, only around 20% PDAC patients are candidates for surgical resection and 5% survive to 5 years [2]. Therefore, there is a crucial and urgent need to explore the underlying mechanism and potential therapeutic target of PDAC.

Prostate transmembrane protein, androgen induced 1 (PMEPA1), also known as TMEPAI or STAG1, is a protein coding gene located on chromosome 20q13, which was initially identified in normal prostate tissue as an androgen-induced gene in 2000 and has been found to be overexpressed in many solid tumors including breast, lung, colon and ovarian cancer [3-10]. PMEPA1 encodes a single-pass transmembrane protein mainly located in lysosome, endosome and golgi apparatus, which contains a SIM motif interacting with R-Smad, and two PY motifs interacting with E3 ubiquitin ligases [11, 12]. It has been supposed to be involved in the growth and metastasis of certain cancers via multiple mechanisms [11-14].

Previous studies have demonstrated that PMEPA1 could be induced by transforming growth factor beta (TGF-β), which could activate signaling transduction via canonical Smad-dependent pathway and other Smad-independent pathways including PI3-K/Akt, RhoA/ROCK1 and DAXX pathway [15-18]. Through a negative feedback loop, PMEPA1 protein in turn functions as a negative regulator of TGF-β signaling [11]. Given that various studies have shown that TGF-β has important functions in the cell proliferation, metastasis and drug resistance of PDAC [19-22], PMEPA1, as a TGF-β-induced gene, is likely to play a role in the progression of PDAC.

In this study, the expression of PMEPA1 mRNA and protein was assessed in resected specimens of PDAC. To further evaluate the significance and prognostic value of PMEPA1 in PDAC, the correlation between PMEPA1 expression and clinicopathological characteristics as well as survival data was analyzed.

Materials and methods

Collection of clinical information and specimens

Patients in this study were diagnosed as PDAC and underwent pancreatectomy in Chinese PLA general hospital, Beijing, China, from May 2005 to September 2010 and none of the patients had received neoadjuvant chemotherapy or radiotherapy before operation. The resected tissue samples were pathologically diagnosed as PDAC and tumors were staged according to the 7th edition of the AJCC cancer staging manual [23]. The patients were followed up until the patients' death or at November 2015. 15 pairs of liquid-nitrogen-frozen fresh PDAC tissues and corresponding adjacent non-tumor pancreatic tissues were used to extract total RNA. A total of 166 formalin-fixed paraffin-embedded (FFPE) tissues from 92 PDAC patients were used to construct tissue microarray (TMA), which contained 74 PDAC tissues and 74 corresponding adjacent non-tumor pancreatic tissues as well as another 18 PDAC tissues. The specimen collection and study protocol have been approved by the Medical Ethics Committee of the Chinese PLA General Hospital.

Real-time quantitative reverse transcription PCR analysis

Total RNA was isolated from liquid-nitrogen-frozen fresh tissues using TRIzol Plus RNA Purification Kit (Ambion, ThermoFisher, USA). Subsequently, total RNA was reverse-transcribed to complementary DNA (cDNA) using PrimeScript RT reagent Kit (Takara, Daliang, China). Real-time quantitative PCR for the cDNA was implemented in triplicate using GoTaq qPCR Master Mix (A6001, Promega, Shanghai, China) and 7500 real time PCR system (Applied Biosystems, CA, USA). The PCR cycling program was 95°C for 2 minutes, 40 cycles with 95°C for 15 seconds and 60°C for 60 seconds. The UBC served as an internal reference gene. The primer sequences for PMEPA1 and UBC were as followed: PMEPA1, 5'-AGAACACTCCGCGCTTCTTA-3 (forward), 5'-GCTTGTGCATTCAGACCAGA-3 (reverse), UBC, 5'-ATTTGGGTCGCAGTTCTTG-3 (forward), 5'-TGCCTTGACATTCTCGATGGT-3 (reverse). Relative expression of PMEPA1 mRNA were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to UBC mRNA and to that of non-tumor tissue [24].

Tissue microarray construction

A total of 166 FFPE tissues containing 74 PDAC tissues and 74 corresponding adjacent non-tumor pancreatic tissues, as well as another 18 PDAC tissues were used to construct TMA with a tissue arrayer (Beecher Instruments, Silver Spring, MD). The tissue cores selected for TMA construction were 1.5 mm in diameter and were inserted into a recipient paraffin block and cut into 4 μm thick. Finally, the TMA selections were moved to the slide for immunohistochemistry (IHC) staining.

IHC staining and scoring

For IHC staining, the TMA slides were deparaffinized in xylene and rehydrate in different gradients of ethanol. The heat-induced epitope retrieval was performed in sodium citrate buffer (pH 6, 0.01 M) using a pressure cooker. The endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 minutes. Then the slides were incubated with 10% normal goat serum at 37°C for 1 hour and subsequently with the primary anti-PMEPA1 antibody (1:100, 16521-1-AP, Proteintech, Chicago) at 4°C overnight. For negative control, PBS was used instead of the primary antibody. In the following day, the slides were Incubated with horseradish peroxidase labeled anti-rabbit secondary antibody (GK500505A, Genetech, Shanghai, China) at room temperature for 30 minutes and then stained using DAB (GK347010, Genetech, Shanghai, China) and restained with hematoxylin (Solarbio, Beijing, China).

The results of IHC staining were evaluated by two independent pathologists. Expression of the PMEPA1 protein was quantified by a modified scoring system as previously described [25], according to the intensity of IHC staining (0, none; 1, weak staining; 2, moderate staining; 3, strong staining) and the percentage of positive cells (0-100). The expression score (0-300) was calculated by multiplying the inten-

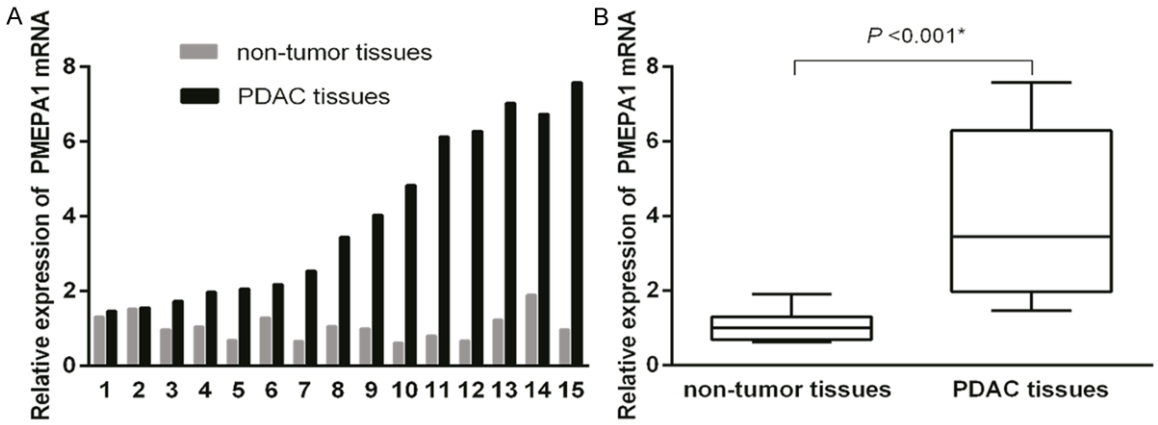

Figure 1. Expression of PMEPA1 mRNA in fresh surgical resected tissues by qRT-PCR. A: Relative expression of PMEPA1 mRNA in 15 pairs of PDAC tissues compared with corresponding adjacent non-tumor tissues. B: Mean level of relative PMEPA1 mRNA expression was elevated in PDAC tissues.

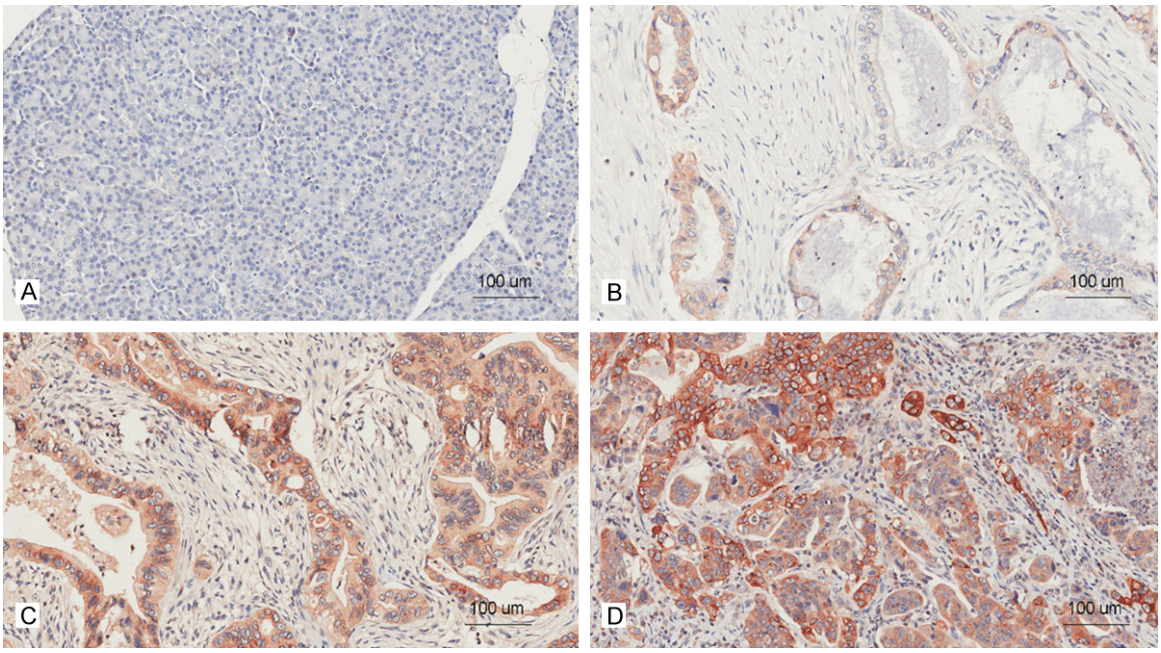

Figure 2. Representative pattern of PMEPA1 expression in PDAC tissues and adjacent non-tumor tissues. A. Negative staining for PMEPA1 in adjacent non-tumor tissue; B. Mild to moderate positive staining for PMEPA1 in well differentiated PDAC tissue; C. Strong positive staining for PMEPA1 in moderately differentiated PDAC tissue; D. Strong positive staining for PMEPA1 in poorly differentiated PDAC tissue.

sity and percentage of staining. The cutoff value for PMEPA1 expression score was obtained using X-tile software version 3.6.1 (Yale University, New Haven, CT, USA) [26].

Statistics analysis

Statistical analysis was performed using the IBM SPSS statistics software version 22 (SPSS, Chicago, IL, USA). The paired t-test was used to compare the expression of PMEPA1 mRNA and the McNemar test was used to compare the expression of PMEPA1 protein between paired PDAC tissues and adjacent non-tumor tissues, respectively. The chi-square test was performed to evaluate the correlation between the expression of PMEPA1 protein and clinicopathologic characteristics. The overall survival (OS) was estimated using the Kaplan-Meier method

Table 1. Correlation between PMEPA1 expression and clinicopathologic characteristics of patients with pancreatic ductal adenocarcinoma

Variables	PMEPA1		Pearson χ^2	*P*-value*
	Low n = 33	High n = 59		
AGE (years)			0.001	0.977
≤70	15	27		
>70	18	32		
Sex			0.131	0.717
Man	13	21		
Woman	20	38		
Histological grade			4.552	0.033*
G1+G2	29	40		
G3	4	19		
T stage			1.906	0.167
T1+T2	22	47		
T3+T4	11	12		
N stage			5.902	0.015*
0	21	22		
1	12	37		
Perineural invasion				
Yes	16	20	1.890	0.169
No	17	39		

*P<0.05.

Table 2. Univariate and multivariate Cox analysis of the overall survival

Variables	HR	*P*-value	95% CI
Univariate analysis			
PMEPA1 expression (High vs. low)	2.006	0.010*	1.178-3.415
Age (>70 vs. ≤70)	0.692	0.136	0.427-1.122
Sex (man vs. woman)	1.003	0.991	0.612-1.643
Histological grade (G3 vs. G1+G2)	2.087	0.007*	1.224-3.559
T stage (T3+T4 vs. T1+T2)	2.134	0.006*	1.245-3.659
N stage (N1 vs. N0)	2.350	0.001*	1.428-3.867
Perineural invasion (Yes vs. No)	1.306	0.279	0.806-2.118
Multivariate analysis			
PMEPA1 expression (High vs. low)	1.956	0.015*	1.139-3.360
T stage (T3+T4 vs. T1+T2)	2.491	0.001*	1.431-4.337
N stage (N1 vs. N0)	2.245	0.002*	1.350-3.731

HR, hazard ratio; CI, confidence interval; *P<0.05.

and the comparison of OS between subgroups was analyzed using the log-rank test. Univariate and multivariate analysis were performed using the Cox proportional hazards regression model. Results were expressed as mean ± standard deviation (SD). Difference was considered to be statistically significant when $P<0.05$.

Results

Expression of PMEPA1 in PDAC tissues and adjacent non-tumor pancreatic tissues

qRT-PCR was used to measure the level of PMEPA1 mRNA in 15 pairs of PDAC tissues and adjacent non-tumor tissues. The mean level of relative expression of PMEPA1 mRNA in PDAC tissues and adjacent non-tumor tissues were 3.972±2.255 and 1.053±0.357, respectively. The relative expression of PMEPA1 mRNA in pancreatic cancer tissues was significantly higher than that in corresponding adjacent non-tumor tissues ($t = 4.957$, $P<0.001$) (**Figure 1**).

To further evaluate the expression of PMEPA1 protein, IHC was performed on the TMA, which showed that PMEPA1 protein was primarily detected in the cytoplasm and no or weak positive signals were detected in the nucleus. According to the IHC results and survival data, we calculated the cutoff value using X-tile software and defined that score <100 as low expression and score ≥100 as high expression. Among the 74 pairs of matched PDAC tissues and corresponding adjacent non-tumor tissues, high expression of PMEPA1 was detected in 63.51% of PDAC tissues (47/74) and 10.81% of adjacent non-tumor tissues (8/74). The proportion of high PMEPA1 protein expression was significantly higher in PDAC tissues when compared with corresponding adjacent non-tumor tissues ($\chi^2 = 33.581$, $P<0.001$) (**Figure 2**).

Correlation between the expression of PMEPA1 and clinicopathological characteristics

The correlation between the expression of PMEPA1 protein and the clinicopathologic characteristics of 92 PDAC patients was summarized in **Table 1**. High expression of PMEPA1 was positively correlated with histological grade ($P = 0.033$) and N stage (regional lymph nodes) ($P = 0.015$). However, expression of PMEPA1

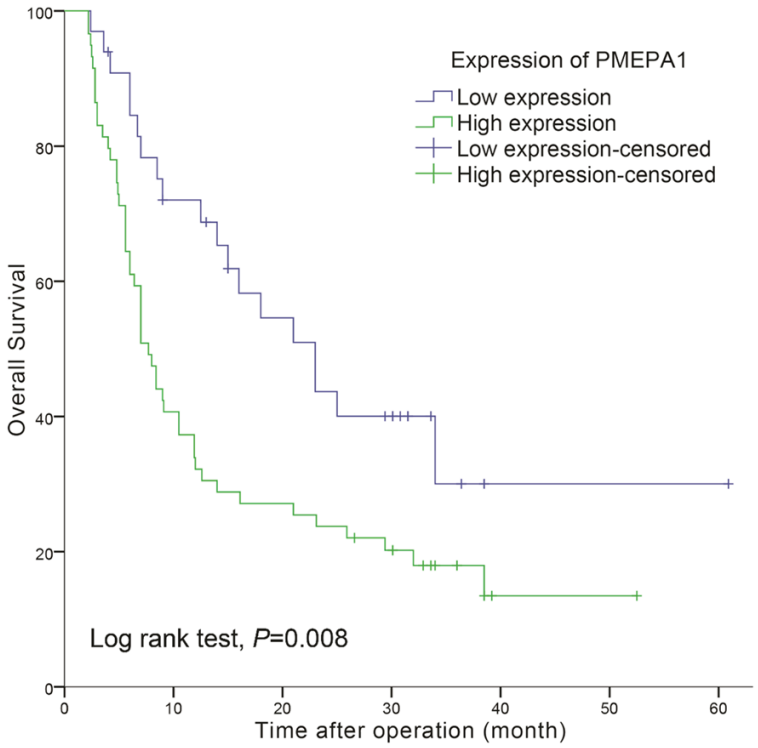

Figure 3. Survival analysis by the Kaplan-Meier method and log-rank test. Overall survival of patients with high PMEPA1 expression (green line) was significantly shorter than that of patients with low PMEPA1 expression (blue line).

protein was not statistically correlated with age (P = 0.977), sex (P = 0.717), T stage (P = 0.167) and perineural invasion (P = 0.169).

Prognostic value of PMEPA1 expression in PDAC

The univariate analysis showed that high PMEPA1 expression (HR = 2.006, P = 0.010), high histological grade (HR = 2.087, P = 0.007), high T stage (HR = 2.134, P = 0.006) and regional lymph node metastasis (HR = 2.350, P = 0.001) predicted poor overall survival (**Table 2**). The multivariate analysis showed that high PMEPA1 expression (HR = 1.956, P = 0.015), high T stage (HR = 2.491, P = 0.001) and regional lymph node metastasis (HR = 2.245, P = 0.002) were independent prognostic factors for poor overall survival. Kaplan-Meier survival curve and log-rank test showed that the overall survival of patients with high PMEPA1 expression was significantly shorter than that of patients with low PMEPA1 expression (median survival 7.7 vs. 23 months, χ^2 = 6.979, P = 0.008) (**Figure 3**).

Discussion

PDAC is the ninth most common cancer and the sixth leading cause of cancer death in China [27]. Although modern diagnostic and therapeutic approaches have been applied, the prognosis of PDAC remains dismal. Therefore, many studies have focused on the factors which may affect the prognosis of PDAC, and various genes have been identified as prognostic marker in PDAC [28, 29]. However, little known is about the prognostic value of PMEPA1 in PDAC.

In the present work, we demonstrated that PMEPA1 was predominantly expressed in the cytoplasm and the expression of PMEPA1 was significantly higher in PDAC tissues than that in adjacent non-tumor pancreatic tissues. In addition, high PMEPA1 expression was also positively correlated with histological grade, regional lymph node metastasis, as well as poor prognosis in PDAC.

PMEPA1 has been detected to be highly expressed in many types of cancer tissues [4-10]. Additionally, It has been considered to function as a negative regulator of TGF-β signaling and AR signaling [11, 13, 14]. In the canonical TGF-β pathway, PMEPA1 competes with Smad anchor for receptor activation (SARA) for Smad2 binding and inhibits the phosphorylation and nuclear translocation of Smad2/3, thereby suppressing the TGF-β/Smad signaling [30]. Evidence have shown that PMEPA1 could interact with the NEDD4 protein and mediate lysosome degradation of TGF-β receptor and AR [12, 14].

Previous studies have shown PMEPA1 is strongly induced by TGF-β which regulates proliferation, differentiation, migration and other processes in normal and cancer cells [9, 31-33]. In PDAC, TGF-β could induce epithelial-to-mesenchymal transition (EMT), which is considered as

a possible mechanism of early dissemination of cancer stem cell-like pancreatic cells and is important for invasion and metastasis in PDAC [34-36]. Hu et al [6] revealed that PMEPA1 contributes to TGF-β-induced EMT and cancer cell migration through ROS production and IRS-1 downregulation in lung cancer. Therefore, in PDAC, high PMEPA1 expression might be partially involved in the EMT-associated cancer cell dissemination to regional lymph nodes and remote metastatic sites. Besides, Nie et al [31] reported that PMEPA1 expression is increased in invasive breast ductal carcinomas (IDCs) and is positively associated with IDC histological grade. In this study, we also found that high PMEPA1 expression was positively correlated with histological grade.

Recent studies suggest that PMEPA1 expression participate in the formation and maintenance of breast cancer stem cells (CSCs) induced by TGF-β and promote PI3K/Akt signaling via suppressing PTEN in ER negative breast cancer [5, 31]. Singha et al [37] reported that ER negative breast cancer patients with high PMEPA1 expression has a significantly shorter recurrence-free survival than those with low PMEPA1 expression, which might be associated with the potential role of CSCs and PTEN/PI3K/Akt signaling in tumorigenesis and metastasis [38, 39]. Consistent with this, Fournier et al [9] reported high level of PMEPA1 predicts shorter overall survival and recurrence-free survival in lung cancer. However, high PMEPA1 expression was also reported to be correlated with longer survival in breast cancer [9]. The obscure results about the prognostic value of PMEPA1 in different types of cancer indicate that PMEPA1 might function differently in certain context of cancer. In our study, we evaluated the survival status and PMEPA1 expression of 92 cases of PDAC patients. The multivariate analysis using the Cox proportional hazards regression model suggested that together with high T stage and regional lymph nodes metastasis, elevated PMEPA1 expression is an independent prognostic factor for poor survival in PDAC. However, the histological grade was correlated with poor survival in univariate analysis but uncorrelated in multivariate analysis. Since the present study is a retrospective, non-randomized study with limited simple size, larger cohort and further research are needed to validate our data and explore the precise mechanism of PMEPA1 in PDAC.

In conclusion, our findings demonstrate PMEPA1 is elevated in PDAC tissues and correlated with regional lymph node metastasis and high histological grade. Moreover, elevated expression of PMEPA1 predicts poor prognosis for PDAC patients after curative resection. Altogether, PMEPA1 might be a valuable prognostic biomarker in PDAC and helpful to selecting patients who need adjuvant treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

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