Decreased expression of USP9X is associated with poor prognosis in Chinese pancreatic ductal adenocarcinoma patients

WEI LIU*, YANMIAO HUO*, JIANYU YANG, XUELIANG FU, MINWEI YANG, LINGYE TAO, DEJUN LIU, JUNFENG ZHANG, RONG HUA and YONGWEI SUN

Department of Biliary-Pancreatic Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, P.R. China

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Abstract. The present study aimed to investigate the expression level of ubiquitin specific peptidase 9X (USP9X) and its clinical significance in Chinese patients with pancreatic ductal adenocarcinoma (PDAC). The mRNA expression levels of USP9X in 30 paired PDAC tissue samples were examined by reverse transcription-quantitative polymerase chain reaction. The protein expression of USP9X was analyzed by immunohistochemistry (IHC) in a tissue microarray containing 205 PDAC specimens. All analyses were performed by SPSS 20.0 and GraphPad Prism 5.0 software. The USP9X mRNA level was significantly decreased in 18/30 (60.0%) PDAC tissue samples compared with matched surrounding non-tumor tissue samples. The results of IHC revealed that decreased expression of USP9X was inversely associated with liver metastasis (P=0.032). Kaplan-Meier survival curves indicated that patients with high expression of USP9X presented a longer clinical overall survival time (P<0.001). Univariate and multivariate COX regression analysis revealed that USP9X protein expression level was a significant, and independent prognostic factor for the overall survival rate of patients with PDAC. The results of the present study indicate that USP9X may serve as a candidate tumor suppressor and prognostic biomarker in PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and fatal types of malignancy, ranking the fourth highest cause of disease-associated mortality worldwide (1). Statistical studies have revealed that the 5-year survival of PDAC is <5%, and ~50% of patients are diagnosed at the advanced stage (2,3). Only <20% of patients are eligible for curative resection, and among these patients, the majority experience recurrence within a year. The identification of reliable clinical markers to predict the prognosis is required for the management of patients with PDAC. It is necessary to identify novel molecular markers for early diagnosis and prediction of prognosis.

Ubiquitin specific peptidase 9X (USP9X), a member of the deubiquitinating protease family, is encoded on the X chromosome and is widely expressed in all tissues (4). Previous studies have demonstrated that the aberrant expression of USP9X is associated with multiple human cancer types, including lung cancer (5), breast cancer (6,7), esophageal carcinoma (8), colorectal carcinoma (9,10), prostate cancer (11), cervical cancer (12), chronic myelogenous leukemia (13), lymphoma and multiple myeloma (14), suggesting the role of USP9X in tumorigenesis, and progression. It has been revealed that USP9X primarily affects proteins that are meant to undergo proteasomal degradation by removing ubiquitin components. Consistently, in a xenograft model with the KRAS wild-type PDAC cell line, tumor volume decreased when USP9X was knockdown (14). However, a report noted that the knockout of USP9X enhances the transformation and protects cancer cells from anoikis in a murine model of PDAC, indicating that USP9X behaves as a tumor suppressor (15). The data of another research argued that USP9X promoted cell growth for established PDAC tumor cells and served a context-dependent role in PDAC (16).

In the present study, the expression level of USP9X was analyzed in Chinese patients with PDAC using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC). Then, the association between clinicopathological parameters and USP9X expression of PDAC was explored, and the prognostic value of USP9X expression for the overall survival of Chinese patients with PDAC was evaluated.

Materials and methods

Patients and tissue specimens. The clinicopathological data of 205 patients with PDAC with surgical resection were

Correspondence to: Dr Yongwei Sun or Dr Rong Hua, Department of Biliary-Pancreatic Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, 160 Pujian Road, Shanghai 200127, P.R. China
E-mail: syw0616@126.com
E-mail: lordhuarong@sohu.com

*Contributed equally

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retrospectively analyzed at the Biliary-Pancreatic Department of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China) between January 2002 and June 2014. In this cohort, there were 88 females and 117 males, age ranging from 38 to 90 years old, with a median age of 65 years. The diagnosis was confirmed by pathological examination. Patients who received preoperative chemotherapy, radiotherapy or other anticancer therapies were excluded from this study. An additional 30 fresh frozen cancerous and corresponding non-cancerous tissues of PDAC were also obtained from the same department. The present study was reviewed and approved by the Ethics Committee of the Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. Written informed consent was obtained from all participating patients.

RNA extraction and RT-qPCR. According to the manufacturers' protocols, total RNA from cancerous and corresponding non-cancerous tissue samples was extracted with TRIzol® reagent (Takara Bio, Inc., Otsu Japan), and reverse transcribed using a PrimeScript RT-PCR kit (Takara Bio, Inc.). 1 ug total RNAs were added to each reaction. qPCR reactions were then performed on a 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the SYBR Premix Ex Taq II (Takara, Japan) in a 10 ul system. The reactions were incubated at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, and 60˚C for 34 sec. All quantitative PCR reactions were performed in triplicate. The primer sequences used for USP9X detection were as follows: Forward, 5'-GTAATCCTGAGGAGGAAGAG-3' and reverse, 5'-ACCACAGGCAGCGAAACAT-3'. The relative levels of USP9X expression were normalized to GAPDH RNA (forward, 5'-GACATCAAGAAGGTGGTGAA-3' and reverse, 5'-TGTCATACCACGAAATGAGC-3') using the $2^{-\Delta\Delta Ct}$ method (17).

Tissue microarray (TMA) construction. TMAs were assembled using 1.5-mm diameter cores. In all 205 cases, corresponding non-cancerous tissue specimen cores were used as internal controls. After marking the representative area of each tissue, the samples were punched out and fitted into the paraffin array blocks.

IHC staining and scoring. The tissue microarray sections were rehydrated and treated with 3% hydrogen peroxide, followed by antigen retrieval. After being blocked with 10% normal goat serum (Shanghai Long Island Biotec, Co., Ltd., Shanghai, China) at room temperature for 30 min, the sections were incubated with primary antibodies at 4˚C overnight, followed by incubation, with a peroxidase-labeled secondary antibody for 30 min at room temperature. Finally, Diaminobenzidine tetrahydrochloride (DAB; Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) was used for the color-reaction followed by nucleus counterstaining with hematoxylin. The following antibodies were used: Rabbit anti-USP9X (1:100, Abcam, Cambridge, UK); Elivision plus Polyer HRP (Mouse/Rabbit) IHC Kit (Fuzhou Maixin Biotech Co., Ltd.). IHC staining was performed on the TMA containing 205 paired PDAC samples by two senior pathologists independently as previously described (18-20). The percentage of positively stained cells were scored using the following scale: 0, 0-5%; 1, 6-35%; 2, 36-70%; and 3, >70% of cells stained. The staining intensity was graded as following: Negative, 0; weakly graded, 1; moderately graded, 2; and strongly graded, 3. The final score was the product of the scores for the positive-staining rate and intensity as follows: '-' for a score of 0-1, '+' for a score of 2-3, '++' for a score of 4-6 and '+++' for a score of >6. A total score <4 in USP9X expression was considered to exhibit low expression and ≥4 as high expression.

Follow-up. The results of clinical and laboratory examinations were followed-up periodically until patients succumbed. Overall survival was defined as the duration between the date of surgery and the date of mortality or the last follow-up.

Statistical analysis. All statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Paired-samples t-tests were used to compare the mRNA levels of USP9X in the tissue samples. The chi-squared test for proportion was used to analyze the comparison of IHC grades in PDAC and normal pancreatic tissues, and the association between clinicopathological characteristics and USP9X expression. The Kaplan-Meier method and the log-rank test were applied in the comparison of the survival curves. Univariate and multivariate Cox proportional hazard analysis was used to investigate the association between clinicopathological variables and USP9X expression on survival. Two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

USP9X expression in PDAC. RT-qPCR assays were performed using 30 pairs of fresh specimens from patients with PDAC to determine the mRNA levels of USP9X. The USP9X mRNA levels were significantly decreased in PDAC tissue samples (18/30, 60.0%) compared with the matched surrounding non-tumor tissue samples (P=0.039; Fig. 1A). Based on the aforementioned scoring criteria mentioned, the IHC data from the 205 PDAC samples revealed that 73 (35.6%) exhibited high USP9X expression (USP9X ++ or USP9X +++), whereas the remaining 132 cases (64.4%) exhibited low USP9X expression (USP9X- or USP9X +) (Fig. 1B; Tables I and II).

Association between USP9X expression and PDAC clinicopathological parameters. The IHC staining scoring of USP9X level was statistically analyzed to explore the association between USP9X expression and clinicopathological parameters in PDAC. The clinicopathological parameters included age, sex, tumor location, tumor size, differentiation status, clinical stage, lymph node metastasis, liver metastasis and vascular invasion. Although the number of patients with PDAC with low USP9X expression was more than that with high expression, USP9X expression was only significantly associated with liver metastasis (P=0.032; Table I).

Prognostic significance of USP9X expression in PDAC. Survival analysis of patients was conducted using the Kaplan-Meier curve and log-rank test. As shown in Fig. 2A, patients with a high expression of USP9X exhibited a longer clinical overall survival time compared with those with low USP9X expression

Table I. Associations between USP9X expression and clinicopathological features in patients with PDAC.

Characteristics	Total	USP9X expression		P-value (χ^2 test)
		Low (n=132) (%)	High (n=73) (%)	
Age, years				0.153
<65	98	68 (69.4)	30 (30.6)	
≥65	107	64 (59.8)	43 (40.2)	
Sex				0.491
Male	117	73 (62.4)	44 (37.6)	
Female	88	59 (67.0)	29 (33.0)	
Tumor location				0.875
Head	139	89 (64.0)	50 (36.0)	
Body/tail	66	43 (66.2)	23 (34.8)	
Size, cm				0.662
≤2	28	17 (60.7)	11 (39.3)	
>2	177	115 (65.0)	62 (35.0)	
Tumor differentiation				0.244
Well	14	7 (50.0)	7 (50.0)	
Moderate/poor	191	125 (65.4)	66 (34.6)	
AJCC stage				0.238
Stage I-II	150	93 (62.0)	57 (38.0)	
Stage III-IV	55	39 (70.9)	16 (29.1)	
Lymph node metastasis				0.328
Absent	143	89 (62.2)	54 (37.8)	
Present	62	43 (69.4)	19 (30.6)	
Liver metastasis				0.032[a]
Absent	184	114 (62.0)	70 (38.0)	
Present	21	18 (85.7)	3 (14.3)	
Vascular invasion				0.055
Absent	176	107 (60.8)	69 (39.2)	
Present	29	23 (79.3)	6 (20.7)	

[a]Statistically significant (P<0.05). AJCC, American Joint Committee on Cancer; PDAC, pancreatic ductal adenocarcinoma; USP9X, ubiquitin specific peptidase 9X.

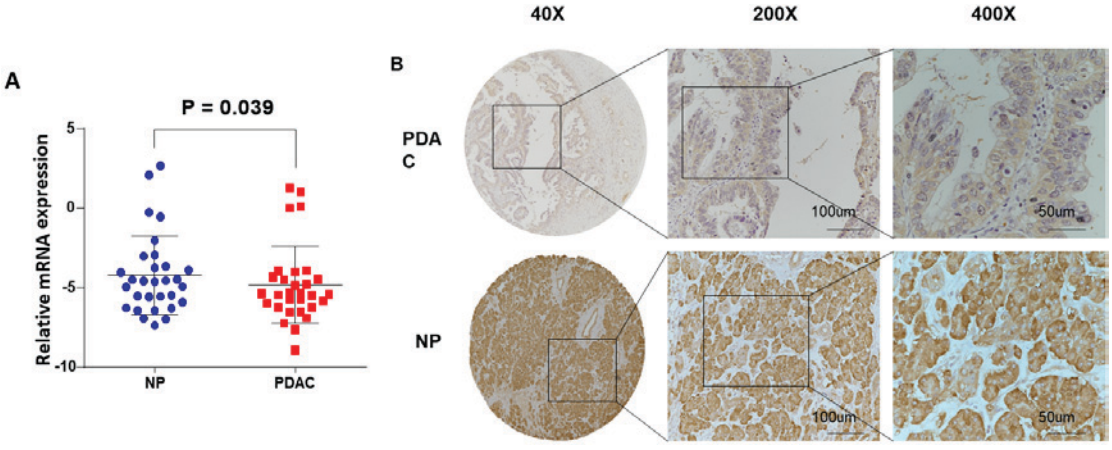

Figure 1. USP9X mRNA and protein expression in PDAC. (A) Paired-samples t-test comparison of USP9X mRNA expression in 30 matched PDAC tumor and surrounding non-tumor tissue was detected by reverse transcription-quantitative polymerase chain reaction. (B) Immunohistochemical detection of USP9X protein expression in PDAC and NP samples. PDAC, pancreatic ductal adenocarcinoma; NP, surrounding non-tumor tissue; USP9X, ubiquitin specific peptidase 9X.

Table II. Comparison of high and low USP9X immunohistochemical expression in PDAC and normal pancreatic tissues.

Immunohistochemical grade	Tissue		P-value (χ^2 test)
	PDAC (n=205,%)	NP (n=205,%)	
High USP9X expression	73 (35.6)	98 (47.8)	0.012[a]
Low USP9X expression	132 (64.4)	107 (52.2)	

[a]P<0.05. PDAC, pancreatic ductal adenocarcinoma; NP, surrounding non-tumor tissue; USP9X, ubiquitin specific peptidase 9X.

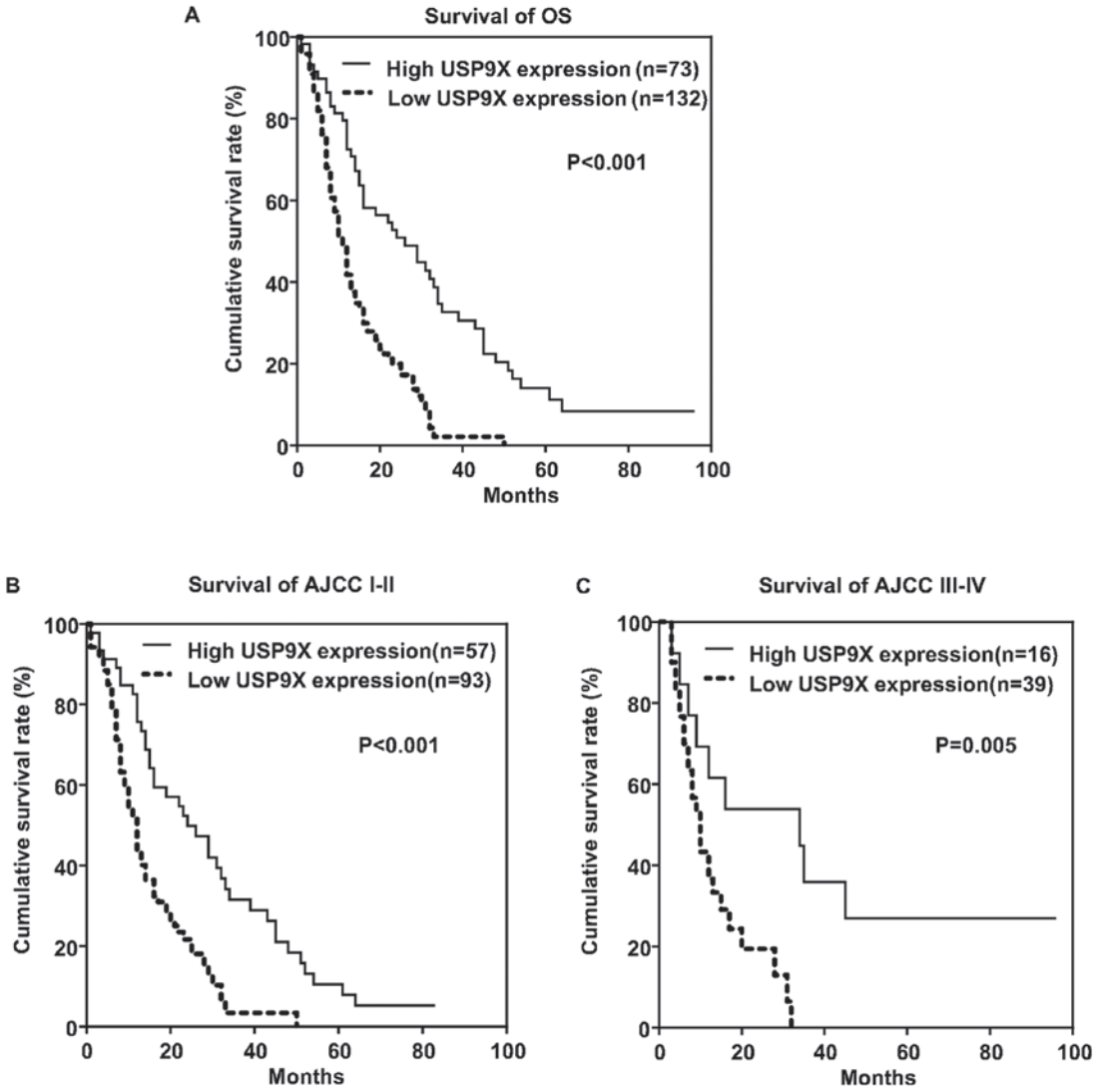

Figure 2. Kaplan-Meier survival curves for patients with PDAC (n=205) following surgical resection. (A) USP9X expression is associated with the overall survival rate in patients with PDAC. Low expression of USP9X was significantly associated with poor survival of PDAC (log-rank test, P<0.001). (B and C) Low expression of USP9X was significantly associated with poor survival of PDAC in early clinical stage (I-II) cohorts (log-rank test, P<0.001) and advanced clinical stage (III-IV) cohorts (log-rank test, P=0.005). PDAC, pancreatic ductal adenocarcinoma; OS, overall survival; USP9X, ubiquitin specific peptidase 9X; AJCC, American Joint Committee on Cancer.

(P<0.001). The association between overall survival and USP9X expression level was evaluated according to American Joint Committee on Cancer (AJCC) stage 7th edition (Fig. 2B and C) (21). Similarly patients with stage I-II and stage III-IV with high USP9X expression exhibited a longer overall survival time compared with their low USP9X expression counterparts.

Furthermore, univariate Cox regression analysis was performed in the 205 PDAC cases (Table III). Overall survival was significantly associated with USP9X expression, age, sex, tumor size, tumor differentiation and liver metastasis (P<0.05). Furthermore, following multivariate Cox regression analysis, all six parameters remained significantly associated with

Table III. Univariate and multivariate analyses of prognostic parameters for survival in patients with PDAC.

	Univariate analysis			Multivariate analysis		
Prognostic parameter	RR	95% CI	P-value	RR	95% CI	P-value
USP9X (low vs. high)	0.332	0.232-0.477	<0.001[a]	0.375	0.263-0.534	<0.001[a]
Age (<65 vs. ≥65 years)	1.486	1.111-1.988	0.008[a]	1.601	1.183-2.166	0.002[a]
Sex (male vs. female)	0.723	0.534-0.978	0.035[a]	0.708	0.515-0.974	0.034[a]
Tumor location (head vs. body/tail)	1.045	0.767-1.424	0.781			
Tumor size (≤2 vs. >2 cm)	1.589	1.038-2.430	0.033[a]	1.735	1.115-2.698	0.014[a]
Tumor differentiation (Well vs. moderate/poor)	3.907	1.950-7.826	<0.001[a]	3.470	1.715-7.020	0.001[a]
AJCC stage (I-II vs. III-IV)	1.042	0.739-1.470	0.815			
Lymph node metastasis (present vs. absent)	1.288	0.939-1.765	0.116			
Liver metastasis (present vs. absent)	3.474	1.887-6.394	<0.001[a]	4.457	2.373-8.371	<0.001[a]
Vascular invasion (present vs. absent)	1.300	0.843-2.004	0.235			

[a]Statistically significant (P<0.05). AJCC, American Joint Committee on Cancer; PDAC, pancreatic ductal adenocarcinoma; USP9X, ubiquitin specific peptidase 9X; CI, confidence interval; RR, risk ratio.

overall survival and were identified as independent prognostic factors for PDAC (Table III).

Discussion

Pancreatic cancer is one of the most aggressive and fatal types of malignant tumor in solid tumor oncology in the world. One of the reasons for the poor prognosis of pancreatic cancer is that it is difficult to diagnose at the early stage. In the present study, USP9X expression, and its association with clinicopathological features and clinical prognosis were investigated in Chinese patients with PDAC. It was demonstrated that USP9X may serve as a candidate tumor suppressor and prognostic biomarker in PDAC.

First, USP9X expression was demonstrated to be decreased at the protein level by IHC analysis in 205-paired PDAC sample TMA (132/205, 64.4%). USP9X expression was also significantly decreased in PDAC tissues compared with the matched surrounding non-tumor tissue samples at the mRNA level (P=0.039). Furthermore, liver metastasis is an important negative prognostic indicator of PDAC. In the current study, a potential association was identified between USP9X expression and liver metastasis by statistical analysis with various clinicopathological parameters, which indicated that low USP9X expression suppressed liver metastasis in PDAC (P=0.032). Nevertheless, no statistical significance was identified between USP9X expression and tumor differentiation (P=0.244) or AJCC stage (P=0.238) in PDAC, which may be attributed to the variability in the distribution of different stages across the cohort.

In the further study, it was demonstrated that longer clinical overall survival times occurred in patients with high expression of USP9X when compared with those with low USP9X expression, which verified the suppressor role of USP9X. Perez-Mancera *et al* (15) reported that knockdown the USP9X expression increased the colony of formation rate of pancreatic cancer cells and protected pancreatic cancer cells from anoikis. The present study revealed that the level of USP9X was significantly reduced in Chinese PDAC tissues compared with the matched surrounding non-tumor tissues. It may be possible that USP9X serves a tumor suppressor role in PDAC. Several previous reports presented that USP9X was a cancer promoter in other malignant solid tumors, including lung cancer (5), breast cancer (6,7), esophageal carcinoma (8), colorectal carcinoma (9,10), prostate cancer (11) and pancreatic cancer (14). Cox *et al* (16) downregulated the USP9X expression using short hairpin RNA, which resulted in a reduction in the growth of human PDAC cells, indicating that USP9X serves as an oncogene. Notably, it was also reported that the growth of PDAC cell lines was impaired by deubiquitinating protease inhibitor WP1130, which revealed that USP9X may promote PDAC cell growth (16). We hypothesized that USP9X contributes to PDAC by a relatively complicated mechanism involving other participants, including an association with a Kras gene mutation (15), which requires further studies to verify, although Cox *et al* (16) argued that there is a context-dependent function of USP9X in different stages of PDAC.

In conclusion, the present study demonstrated that USP9X may serve as a candidate tumor suppressor and prognostic biomarker in Chinese patients with PDAC. Further studies are necessary to explore the probable mechanism pathway regarding the regulation of USP9X, which may be a potential target for therapeutic intervention in PDAC.

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Competing interests

The authors declare that they have no competing interests.

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