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Original Article

Expression of CD44 in pancreatic cancer and its significance

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Abstract: Background: CD44 is a potentially interesting prognostic marker and therapeutic target in pancreatic cancer. The expression of CD44 has been reported to correlate with poor prognosis of pancreatic cancer in most literatures. The purpose of this study is to investigate the roles of CD44 in pancreatic caner, and their correlation with the prognosis of pancreatic cancer patients. Methods: 67 pancreatic cancer samples were collected in Xinhua hospital affiliated to Shanghai Jiaotong University dating from Jan 2010 to Dec 2012. Immunohistochemistry was applied to test the expression of CD44 in pancreatic cancer. The clinical data of the patients were collected including their gender, age, the histology and location, lymph node metastasis and so on. The correlation between the CD44 expression and the clinicopathological factors of patients with pancreatic cancer was analyzed by the software SPSS 13.0. We devise and synthesis of effectively interference of shRNA sequence of CD44, which was transefected to the pancreatic cancer cells PANC-1. Colony formation assay, cell migration assays and western blot were performed. Results: The positive rates of CD44 expression in pancreatic samples were 73.1% (49/67). Univariate analysis showed that there were a significant differences between the CD44 expression and the pancreatic cancer' T staging, TNM staging, lymph node metastasis, the differentiation degree, tumor location ($P < 0.05$). The Cox proportional hazards model showed that differentiation, CD44 expression and nerve invasion were independent prognostic factors. Knockdown of CD44 expression in pancreatic cancer cells led to decreased cellular proliferation and migration ability, accompanied by downregulation of p-ERK and p-AKT. Conclusion:CD44 were related to the distant metastasis and aggressive malignant behaviors of pancreatic cancer. CD44 may regulate tumorigenesis and cancer metastasis partially via PI3K/AKT or MAPK/ERK regulatory pathway.

Keywords: CD44, pancreatic carcinoma, molecular mechanism, prognosis, RNAi

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth common cause of cancer-related death in the world [1, 2]. At the time of diagnosis, most patients are not eligible for curative surgery resection. The 5 year survival rate of patients with locally advanced disease is less than 10% due to both local disease progression and metastasis [3]. At present, many researchers are devoted to find biologic markers for early diagnosis and prediction of metastasis and recurrence of pancreatic cancer, but have not yet found a satisfactory index [4].

Research showed that the fundamental cause of cancer may be the intracellular changes in gene structure and abnormal gene expression [5]. Tumor invasion and metastasis includes the fracture of linkage between cells, extracellular matrix degradation, invasion of the basement membrane, was mediated by cell adhesion molecules and matrix degrading enzymes. A large number of adhesion molecules expressed abnormally in pancreatic cancer and associated with pancreatic cancer' survival and prognosis [6]. CD44 is involved in many cellular mechanisms such as cell adhesion, proliferation, migration, and chemoresistance. Up-regulation of CD44 is correlated with tumor progression and metastatic phenotype in many cancers, including pancreatic cancer [7, 8].

Here, we show that CD44 is overexpressed in pancreatic cancer cell lines and pancreatic cancer tissues, and its expression correlates with

patients' poor prognosis. The inhibition of CD44 by its shRNA attenuated tumor formation and growth, consistent with the down-regulation of p-AKT and p-ERK. Our observation suggests that CD44 could be targeted as novel therapies for pancreatic cancer.

Materials and methods

Clinical samples and immunohistochemical analyses

67 pancreatic cancer samples were collected in Xinhua hospital affiliated to Shanghai Jiaotong University dating from Jan 2010 to Dec 2012. 4-μm sections of tissue were transferred to an adhesive-coated slide and immunohistochemical staining to detect the expression of CD44, p-AKT and p-ERK in paraffin sections were performed as described [9]. The rat anti-mouse CD44 antibody KM114 from BD bioscience was used at a 1:1,000 dilution and p-AKT and p-ERK was detected by a mouse monoclonal antibody (Santa Cruz Biotech, CA). The number of positively stained cells and the intensity of positive staining was scored by 2 pathologists independently, and averaged to obtain a final score for the tissue. More than 10% of the cells were stained with moderate or strong staining intensity was considered positive. Otherwise, the sample was considered negative. The immunostaining of each tissue was assessed in 5 areas of the acquired images of each tissue section and the mean of these 5 scores was calculated.

The clinical data of the patients were collected including their gender, age, the histology and location, lymph node metastasis and so on. The clinicalpathologic variables were obtained from the medical records and the disease stages of the patients were classified according to the 2010 AJCC pancreatic cancer TNM staging system [10]. The correlation between the CD44 expression and the clinicopathological factors of patients with pancreatic cancer was analyzed. For the use of these clinical materials for research purposes, prior patients' consent and approval from the Institute Research Ethics Committee was obtained.

Cells, antibodies, and methods

Two human pancreatic cancer cell lines (Panc-1, BxPC-3) were purchased from Cell Bank of China Academy of Sciences (Shanghai) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. Control or target-specific shRNAs were purchased from GeneChem Inc. (Shanghai, China). The scrambled shRNA was used as the negative control (Ctrli) [11]. The transfected cells were used for either the sphere formation assay or western blot. The colony formation assay was conducted to assess clonogenic potential of the cells, as described previously [12]. Transwell chamber (Corning Costar, Cambridge, MA) migration assay was performed as described to detect cell migration ability [12]. Cells were lysed in RIPA buffer (Sigma-Aldrich; R0278) and protease inhibitor (Sigma-Aldrich; P8340). Western blot analysis was performed as previously described [12]. The antibodies against CD44, p-Akt, and p-ERK were obtained from Cell Signaling Technology. The antibody against β-actin was obtained from Sigma.

Statistical analysis

All statistical analyses were done by using the SPSS 13.0 software package (IBM, Endicott, NY). In the set of IHC assay of paraffin embedded tissue samples, the Pearson χ^2 test was used to estimate the correlations between CD44, p-AKT and p-ERK, and clinicopathologic characteristics. Data were expressed as the means ± standard errors of all experiments. Median overall survival (OS) was estimated using the Kaplan-Meier method, and differences were measured using the log-rank test, defined as the time from surgery until death (living patients were censored at the time of their last follow-up). A χ^2 test and multivariable Cox proportional hazards model were used to study associations of all variables with patient survival as well as independent associations between CD44 positivity, tumor stage, or lymph node metastasis. The results are presented as the median survival in months with 95% confidence interval (CI), the relative risk with 95% CI, and the number of patients at risk. A P value less than 0.05 was considered to be statistically significant as indicated.

Results

Clinicopathologic characteristics and outcomes

By IHC analysis, 49 (73.1%) paraffin-embedded archival pancreatic tumor tissues showed a

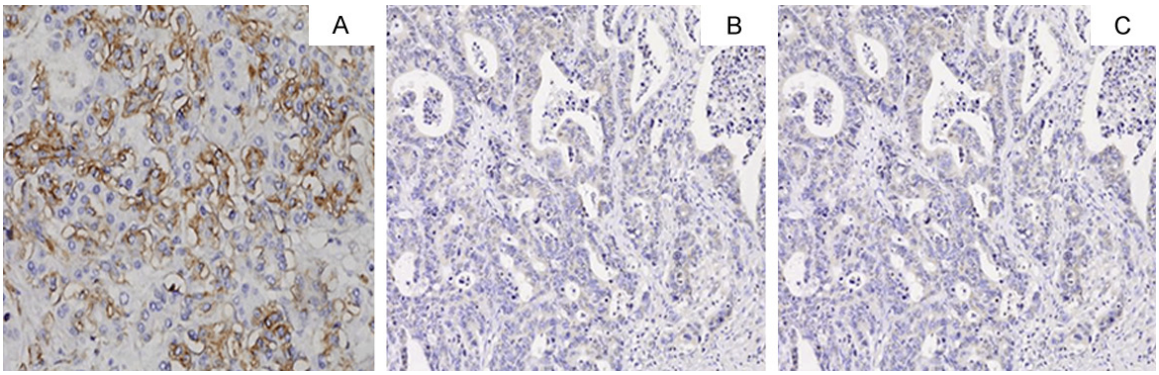

Figure 1. The expression of CD44, p-AKT and p-ERK in pancreatic tumor tissues A. CD44 B. p-AKT C. p-ERK.

Table 1. The correlation of CD44 expression with clinic-pathological parameters

	n	CD44 (+)	CD44 (-)	*P* value
Lymph node metastasis				0.004
negative	41	25	16	
positive	26	24	2	
T classification				0.005
T1-2	23	12	11	
T3-4	44	37	7	
TNM stage				
I	5	2	3	0.003
II	31	18	13	
III	18	16	2	
IV	13	13	0	
Differentiation				
Well differentiated	8	1	7	0.004
Moderately differentiated	8	6	2	
Poorly differentiated	51	36	15	
Vascular invasion				
positive	6	6	0	0.163
negative	61	53	18	
Nerve invasion				
positive	10	8	2	0.602
negative	57	41	16	
Age (years)				
≥ 60	26	19	7	0.993
< 60	41	30	11	
Location				
Head	48	31	17	0.012
Body and tail	19	18	1	
Sex				
Male	43	33	10	0.380
Female	24	16	8	

positive staining for CD44 (**Figure 1A**). Patients' clinical data between the CD44-positive and CD44-negative groups are listed in **Table 1**. Differences in age, sex, vascular invasion and nerve invasion between the two groups were not significant. Most tumors located in the head of the pancreas (71.6%). The majority of tumors were poorly differentiated (76.2%), and the remaining tumors were well differentiated (11.9%) and moderately differentiated (11.9%). Most patients had stage II disease (46.3%), 38.9% of patients had lymph node metastases. Fifty four patients received radical surgery, and forty nine followed by gemcitabine based post-operative adjuvant chemotherapy for patients with advanced stage (T3/4 or N1-3). Thirteen patients were found to have liver or peritoneal metastases during operation and received palliative operation, followed by gemcitabine based palliative chemotherapy.

In paraffin-embedded archival pancreatic tumor samples, there was a significant positive correlation between CD44 expression with clinical stage and lymph node metastasis (N classification), and a significant negative correlation between CD44 expression and the pancreatic cancer' T staging, TNM staging, lymph node metastasis, the differentiation degree, tumor location, which suggested that overexpression of CD44 correlated with a more aggressive phenotype in pancreatic cancer.

All the patients were followed up to get the survival data. The median follow-up time was 39 months, and forty four patients had died at the last follow-up time. We examined the correlation of CD44 expression with patients' survival of 67 pancreatic cancers that had survival data available, by Kaplan-Meier survival analysis. The median overall survival time of patients in the CD44-negative group were 28 months, whereas that in the CD44-positive group was

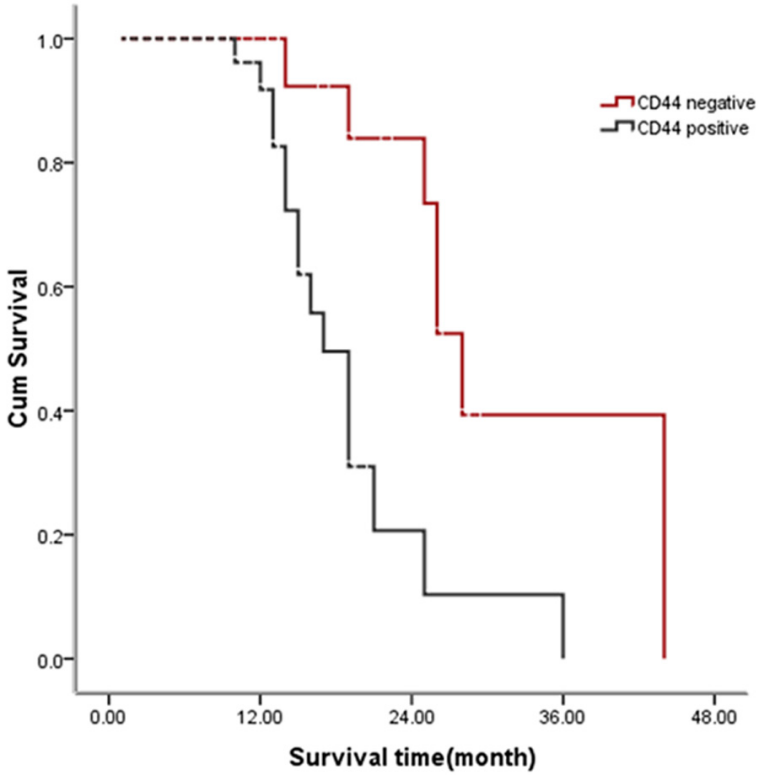

Figure 2. Effects of CD44 expression on pancreatic cancer survival.

Table 2. Multivariate analysis of prognostic factors of pancreatic cancer

	Hazard Ratio	95% CI	*P* Value
CD44	0.312	0.125-0.778	0.012
p-AKT	0.511	0.370-1.616	0.275
p-ERK	0.605	0.421-1.892	0.364
T classification	2.990	0.863-10.363	0.084
Lymph node metastasis	0.699	0.315-1.552	0.379
TNM stage (I + II/III+ IV)	0.439	0.124-1.558	0.203
Differentiation (Well + Moderately/Poorly)	0.335	0.114-0.985	0.047
Vascular invasion	1.102	0.317-3.828	0.879
Nerve invasion	0.119	0.043-0.327	0.000
Age	0.490	0.240-1.002	0.051
Sex	0.871	0.443-1.710	0.687
Location	0.745	0.452-1.487	0.582

only 17 months, and the difference between the two groups was significant (hazard ratio = 0.264; 95% confidence interval, 0.126-0.437; *P* = 0.005). The cumulative survival curve is shown in **Figure 2**. The results suggest that overexpression of CD44 correlates with poor prognosis in patients with pancreatic cancer. In a multivariable Cox proportional hazards model, patients with high CD44 expression had worse overall survival compared with patients with low CD44 expression (hazard ratio = 0.312; 95% confidence interval, 0.125-0.778; *P* = 0.012) (**Table 2**), after adjusting for tumor size, grade, location, lymph node metastasis and so on. These data suggest that the CD44s protein level in pancreatic cancer acts as an independent prognostic factor for patient survival.

Knockdown of CD44 expression inhibits proliferation and migration of pancreatic cancer cells

Western blot analysis of CD44 indicted that CD44 shRNA (CD44i) efficiently knockdowned CD44 expression (**Figure 4**). The clonogenic potential of the cells was assessed by the colony formation assay. The results showed that the stable expression of CD44 shRNA in PANC-1 cells had a significantly decreased capacity for the formation of colonies, compared with the Ctrli cells (**Figure 3A**). Compared to control, the soft agar colonies in CD44 knockdown PANC-1 cells were less in frequency and also smaller in size (**Figure 3B**). The transwell chamber cell migration assay was conducted to assess the cell migration capacity of PANC-1 cells. The results showed that the number of migrated cells decreased significantly in CD44 knockdown PANC-1 cells, compared to that in control cells (**Figure 3C**). These data suggest that CD44 regulates cell migration and overexpression of CD44 may contribute to cancer metastasis.

p-AKT and p-ERK is a target of CD44 and may be one of the mechanisms

We studied the relationship between CD44, p-AKT and p-ERK to determine the possible

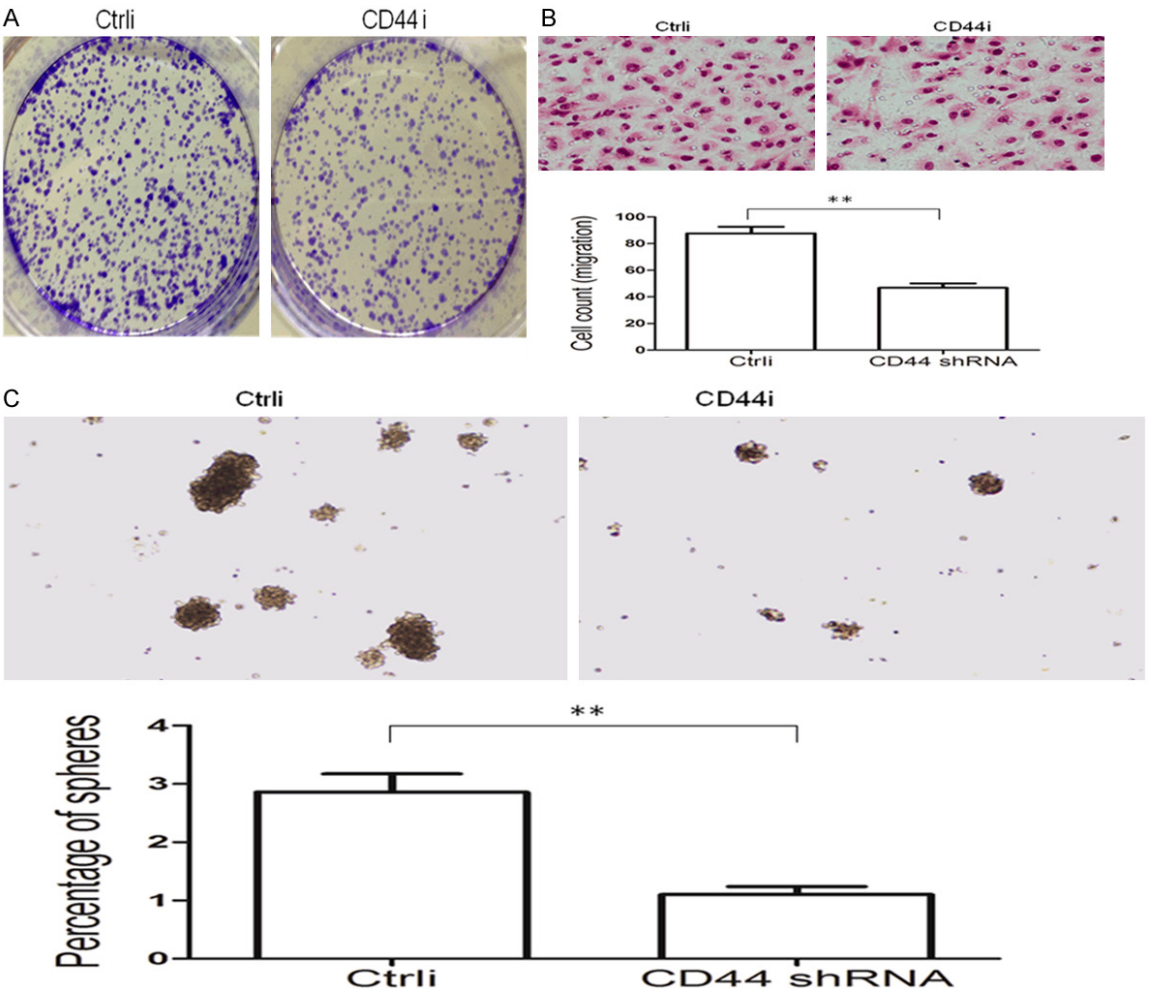

Figure 3. The interference plasmid transfection significantly reduce the proliferation and the invasion capacity of pancreatic cancer cells $^{**}P < 0.01$.

mechanisms of CD44 in pancreatic carcinogenesis. Firstly, we analyzed the expression of p-AKT and p-ERK in pancreatic cancer tissues. By IHC analysis, 23 (31.1%), 25 (33.8%) pancreatic tumor tissues showed a positive staining for p-AKT and p-ERK respectively (**Figure 1B**, **1C**). We found the correlation between the expression of CD44, p-AKT and p-ERK in pancreatic cancer tissue samples by IHC analyses was significant (**Table 3**). Then, to explore the potential role of CD44 in the regulation of pancreatic cancer cell phenotypes and functions, we conducted the functional loss of expression study of CD44 by transfection of its shRNA into the PANC-1 cell. We found that knockdown of CD44 resulted in decreased p-AKT and p-ERK expression (**Figure 4**). The evidence from in vitro and in vivo experimental studies suggests that CD44 may play an important role in tumorigenesis and tumor development by the regulation of p-AKT and p-ERK.

Discussion

CD44 has been studied for three decades, but no consensus opinion on cancer progression has been reached until now. Emerging evidence suggests that CD44 may have an important function during tumorigenesis and tumor progression mediated by deregulation of several signaling pathways such as epithelial-to-mesenchymal transition (EMT). Recent clinical studies have shown high levels of CD44 expression in gastric cancer, colorectal cancer, and non-small cell lung cancer [13]. Moreover, several in vitro and in vitro experimental studies have shown that CD44 was overexpressed in pancreatic cancer cell lines and pancreatic tumors

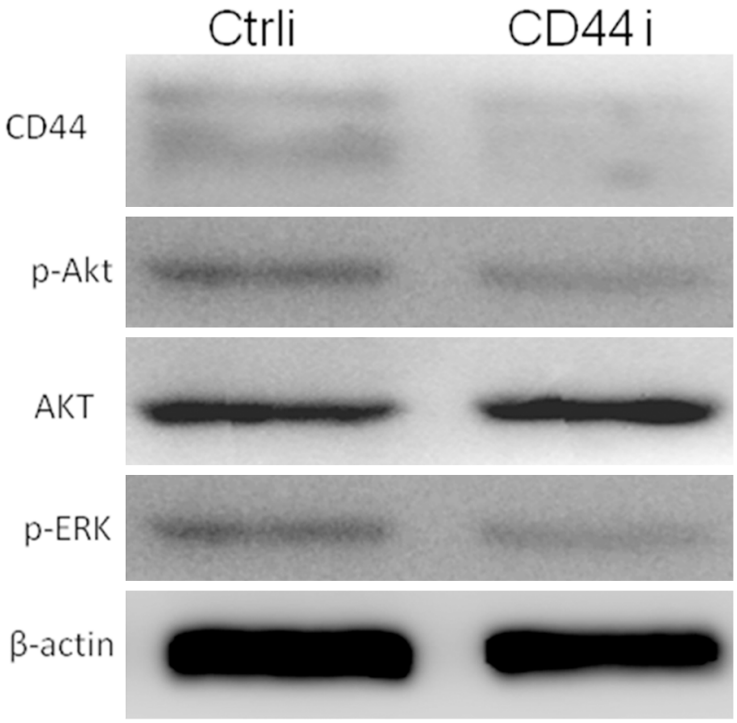

Figure 4. The expression of proteins after interference plasmid transfection in PANC-1 cell detected by Western blot.

Table 3. Analysis of the relationship between the expression of pancreatic cancer prognosis factor

		CD44	p-AKT	p-ERK
CD44	R	-	0.438	0.468
	P	-	0.002*	0.001*
p-AKT	R	0.438	-	0.937
	P	0.002*	-	0.01*
p-ERK	R	0.468	0.937	-
	P	0.001*	0.01*	-

Abbreviations: R: Spearman correlation coefficient. *P*: *P* value.*$P < 0.05$ was considered to be significant.

and plays an important role in the carcinogenesis and progression of pancreatic cancer [14]. The function of CD44 in the carcinogenesis and progression of pancreatic cancer needs to be studied. Li [15] reported that increased CD44v expression was found in metastatic pancreatic carcinoma in human tumor tissue. Clinical analysis showed that CD44v6+ and CD44v9+ were correlated with lymph node metastasis, liver metastasis and TNM stage.

In the present study we provide in vitro and in vivo evidences to support the oncogenic role of CD44 in pancreatic cancer development. Here we show that CD44 is overexpressed in pancreatic cancer tissues. Importantly, we found that overexpression of CD44 correlated with advanced clinical stage and positive lymph node metastasis. The blockage of CD44 by its siRNA inhibitor has been found to inhibit cell invasion and metastasis in vitro in prostate cancer cells [16]. Our in vitro study also showed that stable knockdown of CD44 expression in pancreatic cancer cells can inhibit proliferation and migration in pancreatic cancer cells. This provide preliminary direct evidence for the possibility of CD44 regulating the metastasis of pancreatic cancer. These data clearly suggest that CD44 not only plays a key role in tumorigenesis, but may also be involved in the progression and metastasis of pancreatic cancer. In the present study, using the patient samples, we also found that patients with positive CD44 expression survived significantly shorter than those with negative CD44 expression. Multivariate Cox proportional hazards model analysis showed that CD44 is an independent prognosis factor.

Increased numbers of in vitro and in vivo experimental studies have confirmed the evidence that CD44 plays an important role in tumorigenesis and tumor progression mediated by promoting cell proliferation and migration via several signaling pathways/networks, including p-AKT or p-ERK [17]. The relationship between the expression of p-AKT and tumor prognosis remains controversial [18, 19]. The related research in most of pancreatic cancer, high expression of p-AKT is correlated with poor prognosis [20], but there are also some research considered p-AKT positive staining is correlated with better prognosis [21]. The prognostic role of p-ERK in pancreatic cancer is sure [22]. Pancreatic cancer cells' migration and apoptosis resistance, which both can be initiated via CD44 and c-Met ligand binding and proceed via PI3K/Akt and RAS/MAPK activation, respectively [23]. In the present study we found correlation between CD44 and p-AKT,

p-ERK expression in pancreatic tumor tissues. The correlation coefficients were 0.438, 0.468 respectively. There is a significant positive correlation and worth further exploration. p-AKT and p-ERK expression was also positively correlated (correlation coefficient 0.937, P = 0.01), which was consistent with previous studies.

We also found that knockdown of CD44 resulted in decreased p-AKT and p-ERK expression and was accompanied by decreased transformed phenotype and migration ability, which suggested regulation of p-AKT and p-ERK might be one of the important mechanisms of CD44 in pancreatic cancer. The mechanisms of CD44 in pancreatic cancer still need to be further studied.

In summary, overexpression of CD44 was associated with poor overall survival in patients with pancreatic cancer, and it may regulate tumorigenesis, cell migration and cancer metastasis partially via p-AKT or p-ERK regulatory pathway. However, more prospective studies are needed to explore the prognostic value of CD44 in pancreatic cancer. With the thoroughly research in the mechanism and regulation pathway, CD44 will play a greater role in tumor diagnosis, treatment, prognosis.

Disclosure of conflict of interest

None.

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