

Original Article

Membranous co-expression of EpCAM and CD44s predicts poor prognosis in pancreatic cancer patients

Bao-Qing Xu^{1*}, Yao Meng^{1*}, Xin-Yu Fan¹, Lan-Xiang Ma², Jun Qin³, Zhi-Nan Chen¹, Ling Li¹

¹Cell Engineering Research Center and Department of Cell Biology, State Key Laboratory of Cancer Biology, Fourth Military Medical University, Xi'an 710032, Shaanxi, P. R. China; ²Department of Cardiovascular Medicine, Shaanxi General Hospital of CAPF, Xi'an 710054, Shaanxi, P. R. China; ³Department of Urology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi, P. R. China. *Equal contributors.

Received July 3, 2016; Accepted September 1, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: The identification of reliable prognostic markers and valid pharmacological targets can improve the clinical outcomes of pancreatic cancer patients. Metastasis associated molecules EpCAM and CD44s both were reported as potential therapeutic targets and tumor-initiating cells markers for pancreatic cancer. However, the co-expression of EpCAM and CD44 and the corresponding function have not been evaluated in human pancreatic cancer yet. Hence, we investigated whether EpCAM and CD44 were co-expressed in human pancreatic cancer and what the relationship between their co-expression and the clinic-pathological characteristics was. We evaluated and determined the co-expression of EpCAM and CD44 in 146 pairs of resected pancreatic cancer specimens, and the relationship between the co-expression and the patient's overall survival or other biological variables by a well-established immunohistochemical staining using Kaplan-Meier method and χ^2 test. Compared with normal pancreas tissues, pancreatic cancer tissues had higher co-expression of membranous EpCAM with CD44s rather than with CD44v6; and their expressions were significantly correlated. Furthermore, the membranous EpCAM and CD44s co-expression acted as an independent prognosis factor on patients' survival time. These data suggested a potential role of co-expression of EpCAM and CD44s in predicting patient's poor prognosis.

Keywords: EpCAM, CD44s, co-expression, pancreatic cancer, prognosis

Introduction

Pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic cancer, is a deadly disease. 80% patients present with advanced-stage disease at the time of diagnosis, due to the fact that no effective screening tests for PDAC are available [1]. Patient survival after resection of PDAC tumors is poor, and 5-year survival rate remains only 15-20% [2]. Recent approval of FOLFIRINOX is an only accepted standard of care for approximately 30-40% PDAC patients, with modest improvements in patient survival [3]. It is imperative to find new reliable prognostic markers and valid pharmacological targets for more tailored therapies.

EpCAM is a type-I single-span transmembrane glycoprotein with "oncogenic" features. Highly expressed in most carcinomas, EpCAM is an

attractive target for diagnostic and therapeutic intervention [4]. Besides, EpCAM is also expressed in human normal stem/progenitor cells, and acted as a marker for cancer stem cells (CSCs) or tumor-initiating cells (TICs) in several solid tumors including PDAC [5]. Normally, EpCAM displays a dynamic expression during tumor progression [6]. In primary tumors and overt metastases, EpCAM is over-expressed; whereas in intermediates of the metastatic cascade, i.e., circulating and disseminating tumor cells (CTCs/DTCs), EpCAM expression is down-regulated accompanied by the gain of migratory and invasive properties.

The "oncogenic" function of EpCAM includes cell-cell adhesion, cell proliferation, maintaining a pluripotent state as well as regulation of differentiation, migration and invasion. These functions can be mediated by three kinds of molecule pattern of EpCAM: membranous

bound full-length EpCAM, cytoplasm full-length EpCAM derived by endocytosis from membrane, and truncated EpCAM variant (EpICD) generated from regulated intramembrane proteolysis (RIP). For cell proliferation, EpCAM mainly acted through RIP signaling and/or its associated binding partners in specialized membrane microdomains, such as lipid rafts, or tetraspanin-enriched microdomains (TEMs) [7]. Actually, EpCAM-mediated cell proliferation via RIP also requires cell-to-cell contacts mediated by plasma membrane cell-adhesion molecules, which represents the initial trigger events for EpCAM cleavage [8]. Therefore, EpCAM's appearance at the cell surface, especially its localization in specific subdomains of plasma membrane, is essential for cell proliferation signaling.

It has been reported that in metastasizing rat pancreatic carcinoma cell lines, EpCAM forms a complex with tetraspanins CD9, CO-029 and CD44 variant isoforms (CD44v4-7) in TEMs [9]. Overexpression of these molecules, especially the complex formed by metastasis associated molecules CD44v4-v7 and EpCAM, is of crucial importance in the process of tumor progression such as cell-cell or cell-matrix adhesion, apoptosis resistance. When located in TEMs, the role of the CD44v-EpCAM complex differs from that of the individual molecules. In colorectal cancer, for example, the cells with EpCAM/claudin-7/CO-029/CD44v6 complex display a higher degree of apoptosis resistance than cells devoid of any one of the four molecules [10]. Individual expression of EpCAM, claudin-7, CO-029, and CD44v6 alone cannot be considered as prognostic markers for colorectal cancer patients; however, the co-expression of four molecules correlates with disease-free survival. In anaplastic thyroid carcinoma, EpCAM together with CD44v6 but not with CD44s was associated in the clinical specimens, although direct interaction between EpCAM and CD44v6 was not examined [11]. All these studies suggest that the complex, rather than the individual molecules, facilitates aggressive phenotype and promotes tumor metastasis. Therefore, the role of EpCAM in cancer progression should be further explored together with its associated or interacted molecules.

CD44 is also a family of single-span transmembrane glycoproteins involving in many cellular

processes including survival, proliferation, migration and metastasis. CD44 family members differ in their extracellular domain, where 10 variant exons are either completely excluded as in CD44s or included in various combinations within the CD44 ectodomain, giving rise to the CD44 variant isoforms (CD44v1-v10) [12, 13]. There are ample evidences that overexpression of various CD44 isoforms promotes tumor progression and metastasis, suggesting it is one of the main players in tumor development [13]. We previously reported that both CD44s and CD44v6 were up-regulated in pancreatic adenocarcinoma [14]. However, CD44s but not CD44v6 is associated with poor overall survival and acts as an independent prognostic factor for patient survival, although others reported the relevance of CD44v6 signaling to tumor growth and metastasis [15]. In addition, CD44s is present on several types of cancer stem cells [13]. CD44s promotes tumor-initiation and postradiation recurrence through directly affecting TICs [14, 16].

To the best of our knowledge, the association between EpCAM and CD44 isoforms has not been evaluated in human pancreatic cancer tissues. The purpose of this study is to evaluate the co-expression of these two molecules and explore their relationship with clinic-pathological features in pancreatic cancer patients.

Materials and methods

Patient samples and tissue microarray

For tissues mRNA level analysis, seven pairs of fresh human pancreatic adenocarcinoma specimens and adjacent non-tumor pancreatic tissues were collected from patients who underwent surgery at the University of Michigan Comprehensive Cancer Center (Ann Arbor, MI). For tissues protein expression analysis, a total number of 146 consecutive patients with pancreatic adenocarcinoma diagnosed between Sep 2004 and Nov 2011 were included in this retrospective study. Among them, 73 patients' survival information of 87 months postoperative follow-up was received. All the patients (100%) underwent primary surgical intervention. The median age of patients was 59.1 years (range: 34-85 years) with 56 women and 90 men. Patients' characteristics, such as gender, age, tumor size, type, grade and location, TNM stage, AJCC stage, patient survival and

history of smoking, drinking, and diabetes were obtained from the medical records [31]. This study was reviewed and approved by the Institutional Review Board of the Fourth Military Medical University.

A tissue microarray (TMA) constructed from formalin-fixed, paraffin-embedded tissue blocks from 146 patients with pancreatic adenocarcinoma was purchased from the National Engineering Center for Biochip (Shanghai, China). All procedures of construction TMA were performed as previously described [32]. Briefly, representative tumor areas and paired surrounding non-tumor tissues (normal pancreas or chronic pancreatitis) within the edge of 5 cm were carefully selected by a trained pathologist. Each tissue sample was represented by three cylindrical core tissue biopsies (diameter 0.6 mm). For each tissue block, 4 μ m thick sections were cut and transferred to an adhesive-coated slide, and then verified by a hematoxylin-eosin-stained section.

Antibodies and cell lines

The following antibodies were used in this study: mouse anti-CD44s (MAB3838, clone MEM-263, Abnova, Taipei City, Taiwan), mouse anti-CD44v6 (MAB4073, clone VFF-18, Millipore, Billerica, MA) and rabbit anti-EpCAM (ab-124825, clone EPR677, Epitomics, Burlingame, CA, USA).

Immunohistochemistry staining and scoring

TMA slides were dried at 63°C for 1 hour before staining. All procedures were performed at room temperature as previously described [17, 32]. Briefly, sections were dewaxed in xylene and rehydrated in a graded alcohol series, and washed in water before antigen retrieval using a Leica ST5010 Autostainer (Leica Microsystems Inc., Buffalo Grove, IL, USA) with 10 mM sodium citrate buffer (pH 5.96) at 100°C in an autoclave for 5 minutes. The sections were then treated with 3% hydrogen peroxide for 15 minutes to block endogenous peroxidase. After 30 minutes of incubation with primary antibody in a humid chamber, the slides were incubated for 30 minutes with an EnVision™/HRP anti-rabbit or anti-mouse solution (Dako, Glostrup, Denmark). Reaction products were visualized with diaminobenzidine plus substrate-chromogen solution treat-

ing for 5 minutes. The slides were counter-stained with Meyer's hematoxylin and mounted. To confirm the specificity of the primary antibodies, tissue sections were incubated in the absence of the primary antibodies.

The Immunohistochemistry staining results were recorded by counting at least 400 cells in five areas of each tissue section. The number of positively stained cells and the intensity of positive staining on epithelial cells were independently graded by two experienced pancreatic pathologists in a blinded manner. Briefly, the intensity of positively stained cells was arranged into four groups: group 0 displayed no visible difference compared to the negative control; the positively stained cells of group 1, 2 and 3 were stained in light brown, mid-brown and dark brown, with the same intensity covering more than 75% of the staining area. The total number of cells and the stained ones were counted; the positive rate was calculated and categorized according to percentage: 0, no stained cells; 1, 1-9%; 2, 10-50%; 3, 51-80%; 4, 81-100%. The total immunostaining score was calculated by multiplying intensity score by positive rate score, ranging from 0 to 12, and divided into four subgroups: zero, total score 0; weak, total score 1-4; moderate, total score 5-8; strong, total score 9-12. A total score >4 was defined as EpCAM over-expression [17]. For statistical analysis, the EpCAM positive tumor tissues were divided into two groups: the low expression group (categories as zero and weak, corresponding to a total score 0-4) and the high expression group (categories as moderate and strong, corresponding to a total score >4). For membranous CD44s, the samples with 1+ staining in >50% of cells or 2+ staining in >20% of cells or all 3+ staining of cells were defined as high expression [14].

Statistical analysis

Spearman rank correlation was conducted to analyze the correlations between EpCAM and CD44 co-expression. The association between the concurrent EpCAM and CD44 expression and clinicopathological factors was assessed by student *t* test and χ^2 test. Overall survival, defined as the time from surgery until death (living patients were censored at the time of their last follow-up), was estimated by the Kaplan-Meier method and evaluated by the log-rank

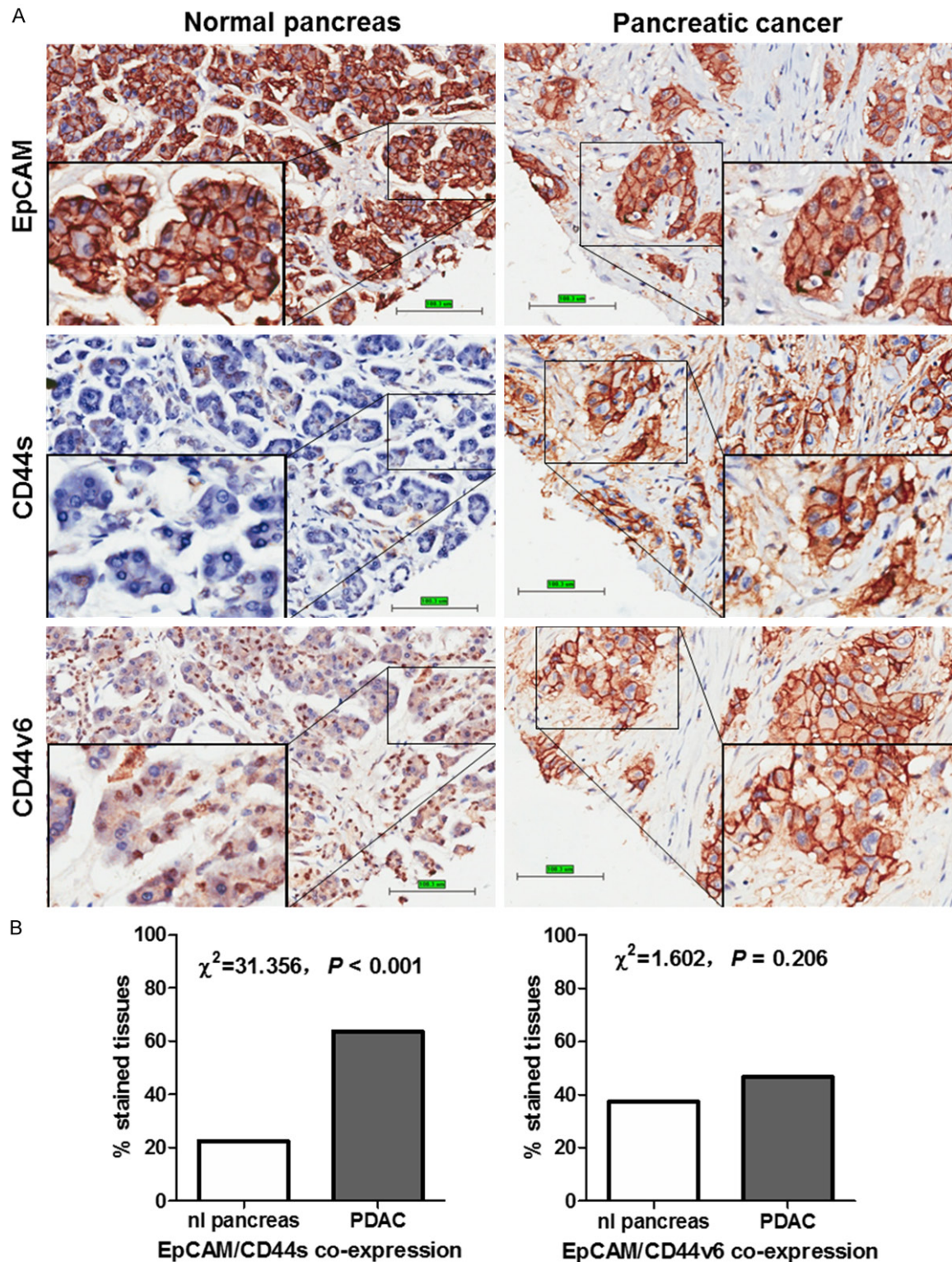


Figure 1. Concurrent EpCAM and CD44 expression in pancreatic tissues. A: Representative morphology of EpCAM and CD44 immunohistochemistry staining in pancreatic normal and tumor tissues. Immunostaining showed a fine granular pattern. EpCAM positive expression was seen both on plasma membrane and in cytoplasm, while CD44s and CD44v6 was mainly seen on plasma membrane (Bar =100.3 mm, original magnification $\times 200$). The inserts provide details of expression patterns. B: The percentage for co-expression of EpCAM and CD44s or CD44v6 in pancreatic normal and tumor tissue samples. Estimated by χ^2 test, compared between normal pancreas (nl pancreas) and pancreatic cancer (PDAC).

Co-expression of EpCAM and CD44s predicts poor prognosis

Table 1. The co-expression of EpCAM and CD44s in normal pancreas and pancreatic cancer tissues

Tissues	EpCAM ^{low} CD44s ^{low}	EpCAM ^{low} CD44s ^{high}	EpCAM ^{high} CD44s ^{low}	EpCAM ^{high} CD44s ^{high}	P value*	r (95% CI)
Normal pancreas	16.42% (11/67)	4.48% (3/67)	73.13% (49/67)	5.97% (4/67)	0.311	0.1257 (-0.1253-0.3616)
Pancreatic cancer	28.77% (42/146)	18.49% (27/146)	17.81% (26/146)	34.93% (51/146)	0.003	0.2444 (0.0804-0.3954)

*Spearman rank correlation was conducted to analyze the correlation between membranous expression of EpCAM and CD44s.

Table 2. The co-expression of EpCAM and CD44v6 in normal pancreas and pancreatic cancer tissues

Tissues	EpCAM ^{low} CD44v6 ^{low}	EpCAM ^{low} CD44v6 ^{high}	EpCAM ^{high} CD44v6 ^{low}	EpCAM ^{high} CD44v6 ^{high}	P value*	r (95% CI)
Normal pancreas	11.94% (8/67)	8.96% (6/67)	53.73% (36/67)	25.37% (17/67)	0.353	-0.1153 (-0.3523-0.1357)
Pancreatic cancer	6.16% (9/146)	41.10% (60/146)	12.33% (18/146)	40.41% (59/146)	0.365	-0.0852 (-0.2694-0.1049)

*Spearman rank correlation was conducted to analyze the correlation between membranous expression of EpCAM and CD44v6.

test. A Cox's proportional hazards model was performed to analyze the correlation of EpCAM and/or CD44s expression with patient survival and determine the independent prognostic factors. All statistical analysis was performed using the Statistical Package of Social Sciences (SPSS, V.16.0, Chicago, Illinois, USA) and GraphPad Prism 5.0 (GraphPad Software, <http://www.graphpad.com>).

Results

Membranous EpCAM and CD44s were co-expressed in pancreatic cancer

The expression of EpCAM and CD44 was reported to associate with pancreatic cancer progression separately. However, the co-expression of membranous EpCAM and CD44 has not been described in human pancreatic cancer tissues yet. To determine the co-expression of EpCAM and CD44 in pancreatic cancer, we divided patients into three distinct subgroups identified by the expression of EpCAM and/or CD44 antigens, including CD44s and CD44v6. We found that the degree of membranous EpCAM and CD44s co-expression was significantly higher in pancreatic cancer than in normal pancreas (**Figure 1A, 1B**, $P < 0.001$). The concurrent EpCAM and CD44s expression was found in 93 of 146 (63.7%) postoperative tumor specimens, which included concurrent high expression (51/146, 34.93%) and concurrent low expression (42/146, 28.77%, **Table 1**). In normal pancreas, the concurrent EpCAM and CD44s expression was only found in 15 of 67 (22.4%).

Meanwhile, we investigated the concurrent EpCAM and CD44v6 expression in the sa-

me patients' tissue samples. Different from CD44s, the concurrent EpCAM and CD44v6 expression was only found in 68 of 146 (46.6%) tumor specimens (**Table 2**); and their co-expression was not statistically higher than that in normal pancreas (37.31%, $P = 0.206$, **Figure 1B**). The above results indicated that membranous EpCAM was highly co-expressed with CD44s, but not with CD44v6, in pancreatic cancer tissues.

Membranous EpCAM was significantly correlated with CD44s in pancreatic cancer

Next, we investigated whether membranous expression of EpCAM and CD44 were correlated with each other in pancreatic cancer tissues. Our results showed that high expression of membrane bound EpCAM was significantly correlated with high expression of CD44s in pancreatic cancer ($r = 0.2444$, $P = 0.003$, **Figure 2A**), but not in normal pancreas ($r = 0.1257$, $P = 0.311$, **Figure 2B**). On the mRNA level, EpCAM was also correlated with CD44s, though did not reach a statistical significance ($r = 0.5819$, $P = 0.167$, **Figure 2E**). In contrast, high expression of membrane bound EpCAM was conversely associated with high expression of CD44v6 both in pancreatic cancer and in normal pancreas (**Figure 2C, 2D**). And, these inverse associations had not statistical significance. Additionally, either CD44s or CD44v6 was not correlated with cytoplasm EpCAM in pancreatic cancer (**Supplementary Figure 1**). Thus, these results suggested that CD44s, but not CD44v6, was significantly correlated with membranous EpCAM expression in patients with pancreatic cancer.

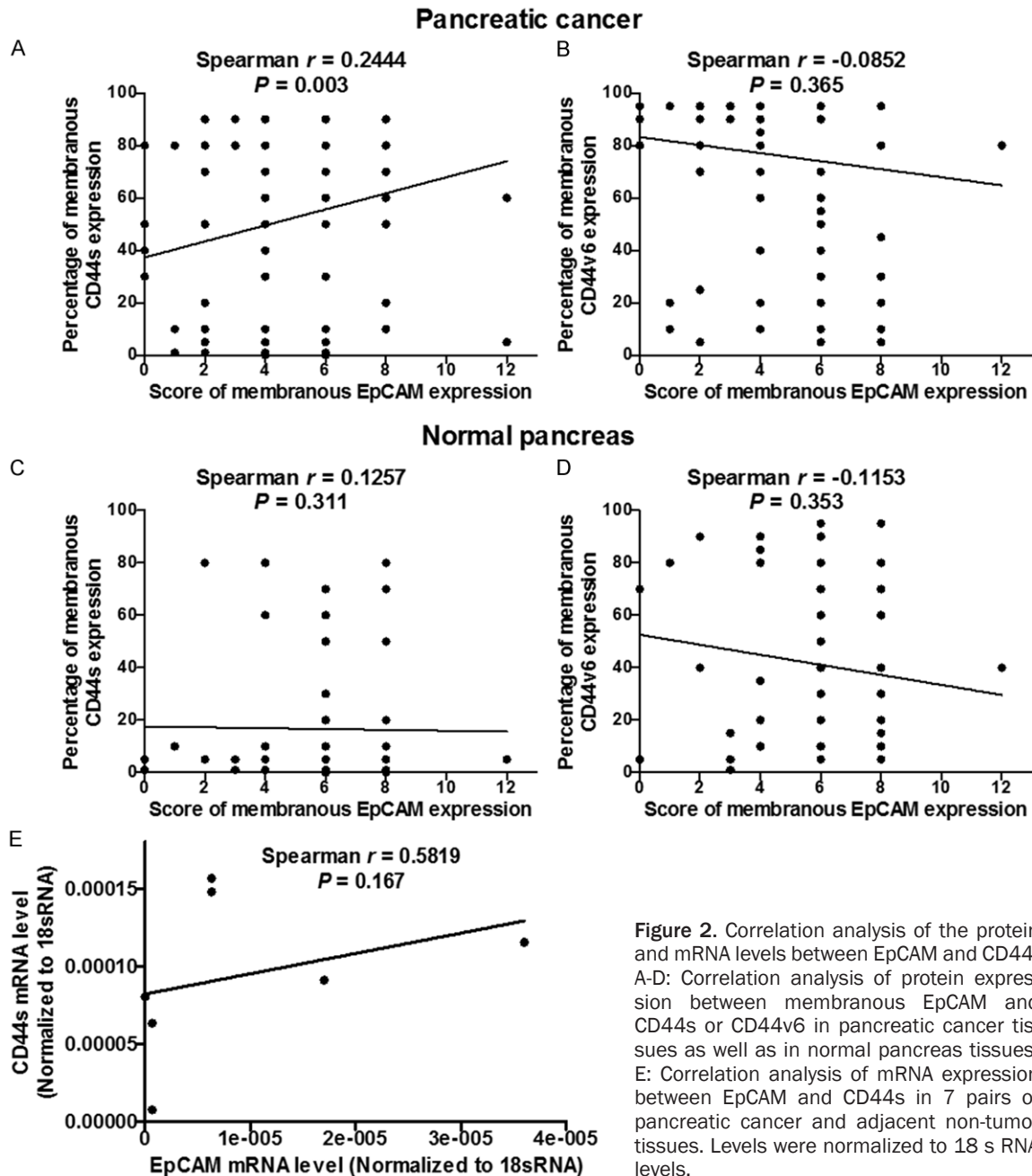


Figure 2. Correlation analysis of the protein and mRNA levels between EpCAM and CD44. A-D: Correlation analysis of protein expression between membranous EpCAM and CD44s or CD44v6 in pancreatic cancer tissues as well as in normal pancreas tissues. E: Correlation analysis of mRNA expression between EpCAM and CD44s in 7 pairs of pancreatic cancer and adjacent non-tumor tissues. Levels were normalized to 18 s RNA levels.

Membranous EpCAM and CD44s co-expression was significantly correlated with poor prognosis in pancreatic cancer patients

To explore the possible role of the concurrent EpCAM and CD44s expression, we investigated the correlation between their co-expression and patients' clinic-pathological characteristics. We found that the concurrent EpCAM and CD44s expression was significantly associated with higher tumor grade ($P < 0.001$) and tumor location in the body and tail of the pancreas or

($P = 0.060$, Table 3). There were no significant differences among patients with concurrent high expression, individual high expression or concurrent low expression with respect to gender, age, tumor size and type, pathological depth of tumor (pT1/T2/T3), pathological lymph node metastasis (pN0/N1/M1), pathological stage (pStage IA/IB/IIA/IIB/IV) as well as smoking, drinking and diabetes.

The association of concurrent EpCAM and CD44s expression with patient's survival was

Co-expression of EpCAM and CD44s predicts poor prognosis

Table 3. Correlation between EpCAM/CD44s co-expression and clinic-pathological features

Clinic-pathological features	EpCAM ^{low} CD44s ^{low,†} (n=42)	EpCAM ^{high} or CD44s ^{high,†} (n=53)	EpCAM ^{high} CD44s ^{high,†} (n=51)	P value*
Gender, n (male/female)	27/15	32/21	31/20	0.916
Age, mean ± s.d. (years)	58.4±11.0	59.8±10.6	59.1±11.2	0.887
Tumor size, mean ± s.d. (cm)	3.95±1.3	4.09±1.96	4.36±1.84	0.686
Tumor type, n (ductal/glandular)	37/5	44/9	44/6	0.700
Tumor grade, n (I+II, III)	39/2	43/9	24/22	<0.001
Tumor location, n (head, tail + others)	34/7	34/18	31/20	<0.006
pT stage, n (T1+T2, T3)	33/8	42/11	45/6	0.432
pN stage, n (N0, N1+M1)	20/18	29/21	23/24	0.667
pStage, n (IA+IB, IIA+IIB+IV)	15/27	23/30	20/31	0.746
Patient survival [#] , n (live/dead)	7/14	11/15	5/21	0.197
Smoking, n (no, few + heavy)	24/17	32/15	28/19	0.587
Drinking, n (no, few + heavy)	29/12	31/16	30/15	0.878
Diabetes, n (no/yes)	31/6	34/11	39/8	0.565

[†]Low (negative to weak expression), high (moderate to strong expression); *Estimated by student t test and χ^2 test; [#]Only 73 cases have available survival data, and only 61 cases have available survival time.

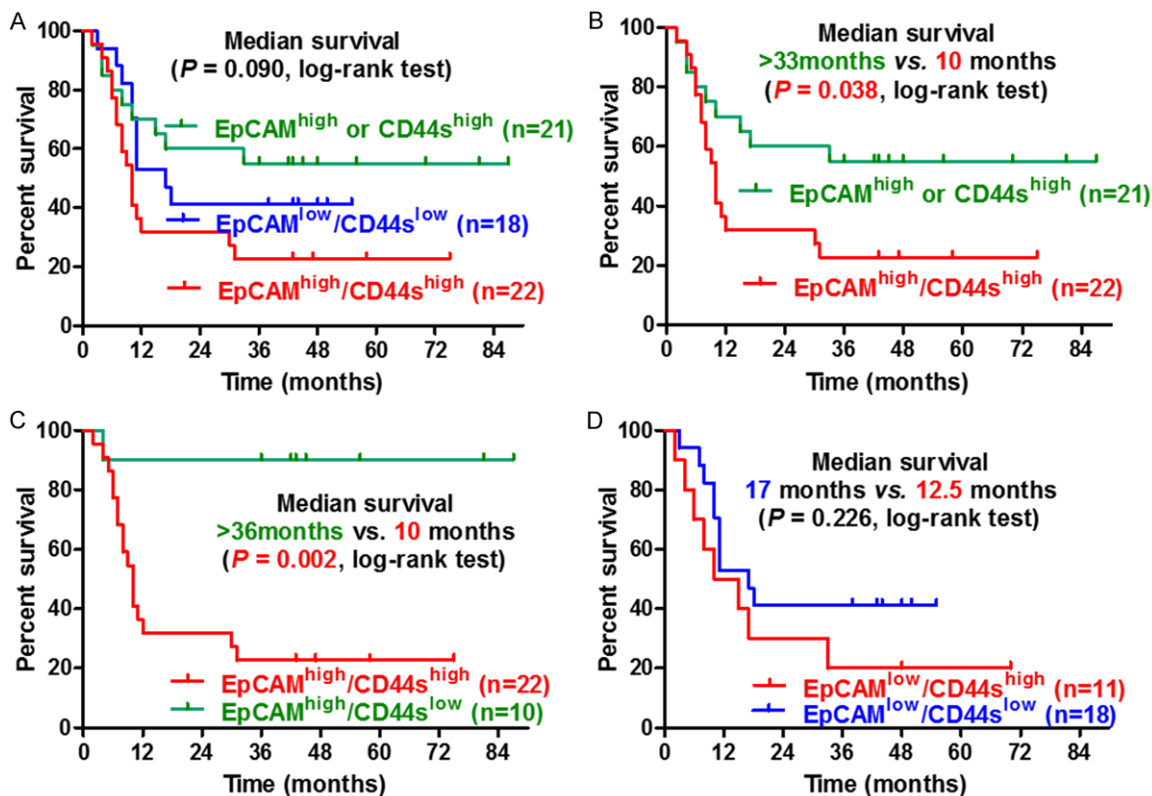


Figure 3. Kaplan-Meier analysis of overall survival in pancreatic cancer patients according to the expression level of membranous EpCAM and/or CD44s. A: Kaplan-Meier analysis of overall survival in 61 patients comparing high and low EpCAM and/or CD44s expression groups. B: Kaplan-Meier analysis of overall survival in 43 patients comparing the EpCAM and CD44s co-expression and their individual high expression groups. C: Kaplan-Meier analysis of overall survival in 32 patients comparing CD44s high and low expression subgroups in EpCAM high expression group. D: Kaplan-Meier analysis of overall survival in 29 patients comparing CD44s high and low expression subgroups in EpCAM low expression group.

Co-expression of EpCAM and CD44s predicts poor prognosis

Table 4. Prognostic factors for overall survival by Cox proportional hazard model

Clinic-pathological features	HR (95% CI) [†]	P value
Gender (male/female)	0.961 (0.362-2.554)	0.937
Age (years)	1.043 (0.983-1.107)	0.167
Tumor size (cm)	1.1.03 (0.802-1.515)	0.547
Tumor type (ductal, glandular + others)	0.489 (0.102-2.343)	0.371
Tumor grade (I+II, III)	4.060 (1.254-13.149)	0.019*
Tumor location (head, tail + others)	0.703 (0.228-2.169)	0.540
pT stage (T1+T2, T3)	0	0.913
pN stage (N0, N1+M1)	0	0.926
pStage (IA+IB, IIA+IIB+IV)	0	0.905
Smoking (no, few + heavy)	1.930 (1.055-3.531)	0.033*
Drinking (no, few + heavy)	1.382 (0.706-2.706)	0.345
Diabetes (no/yes)	2.798 (0.876-8.933)	0.082
EpCAM/CD44s co-expression (EpCAM ^{high} CD44s ^{high} /EpCAM ^{high} or CD44s ^{high} /EpCAM ^{low} CD44s ^{low})	2.128 (1.067-4.244)	0.032*

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

estimated by Kaplan-Meier analysis and the log-rank test from available censored survival time of 61 pancreatic cancer patients. In our previous study, EpCAM alone not significantly affected overall survival and prognosis in total population of pancreatic cancer patients, although it was significantly correlated clinic-pathological parameters [17]. In this paper, however, patients with tumors highly expressing both EpCAM and CD44s carried the poorest clinical outcomes among three groups (**Figure 3A**). The median survival time in patients with highly expressing both EpCAM and CD44s was only 10 months, in contrast to the median survival time over 33 months in patients highly expressing either EpCAM or CD44s ($P=0.038$, **Figure 3B**).

Furthermore, in the subgroups of patients with high expression of EpCAM in their tumors, patients with high expression of both EpCAM and CD44s had a significantly worse overall survival than patients with high expression of EpCAM but low expression of CD44s (10 months vs. >36 months, $P=0.002$, log-rank test, **Figure 3C**). Within 7 years' follow-up with median follow-up time of 45 months, 90% patients with their tumor bearing high levels of EpCAM but low levels of CD44s were still alive, and their median survival time was not reached yet. In the subgroups of patients with low expression of EpCAM in their tumors, the median survival times had no difference between patients with additional high or low expression of CD44s (**Figure 3D**). Taken together, these data indicated that concurrent EpCAM

and CD44s expression may predict poor clinical outcomes of patients.

In the multivariate analysis of the total cohort of pancreatic cancer patients using Cox's proportional hazards model, tumor grade ($P=0.019$), smoking ($P=0.033$) and EpCAM/CD44s co-expression ($P=0.032$, HR 2.128; CI 1.067-4.244) were proved to be independent prognostic variables for overall survival (**Table 4**). In conclusion, the above data supported that the concurrent membranous EpCAM and CD44s expression was a independent prognostic factor for the poor prognosis of patients with pancreatic cancer.

Discussion

In the present study, we demonstrated that membranous EpCAM, together with CD44s but not with CD44v6, was co-expressed and acted as an independent factor indicating a poor prognosis for post-surgical patients with pancreatic cancer. Patients with co-expression of EpCAM and CD44s had more advanced tumor grade and shorter survival time than patients with expression of individual molecule. Thus, our results suggested that the association of EpCAM and CD44s contributed to the aggressive phenotype of pancreatic cancer in clinical specimens. The co-expression of EpCAM and CD44s in PDAC may help to predict patient's prognosis.

EpCAM and CD44 variants (CD44v4-v7 or CD44v6) have been reported to be co-expressed

and associated in carcinoma-specific complexes, and then promote the carcinogenesis and metastasis in rat pancreatic adenocarcinoma cell lines [9], colorectal cancer [10] and anaplastic thyroid carcinoma [11]. However, the co-expression of EpCAM and CD44 has not been studied in human pancreatic cancer yet. This is the first study that investigated the association between EpCAM and CD44 and their corresponding function in human pancreatic cancer. Although CD44v6 is reported to promote tumor growth and metastasis [15, 20], the identification of CD44 as a CSCs (TICs) marker is mainly based on standard isoforms of CD44 (CD44s) [13, 21]. Our previous study showed that CD44s, but not CD44v6, had an important function in TICs and TICs-related tumor recurrence and metastasis [14]. In this study, we showed that EpCAM and CD44s, but not CD44v6, were co-expressed and significantly correlated in pancreatic cancer tissues. The concurrent expression of EpCAM and CD44s was associated with patient's poor clinical outcomes. Our results in this paper implied that EpCAM may have different functions due to different interacting protein partners in different types of cancer, cancer cells or its subpopulation. As far as pancreatic cancer is concerned, EpCAM may associate with CD44s and then mediate proliferation signaling.

Recently, loss of membrane expression of EpCAM which accompanied by activated proliferative signaling and stemness genes has been proposed as a biomarker for poor prognosis and more aggressive phenotype [6, 22]. Furthermore, our previous results and others' indicated that endocytosis and RIP were two ways of withdrawing EpCAM from the cell surface, and two means of regulating the EpCAM availability and functionality on cell surface [17, 22]. However, EpCAM-dependent proliferation, tumorigenic and pluripotency features all rely on its existence at the cell surface or in specific subdomains of the plasma membrane [7, 8]. It was reported that retention of EpCAM on the cell surface of colon epithelium cells allowed for a rapid, EpCAM-dependent response when necessary [23]. When located in TEM, EpCAM has the potential to organize signaling complexes at the membrane surface of cancer cells or CSCs probably [9, 24], and to participate in important signaling pathways, such as Wnt, Notch, and Hippo [5]. Thus, the

unraveling of EpCAM interaction molecules, such as CD44s in pancreatic CSCs with the capacity to fine-tune EpCAM function, is therefore of great importance.

CSCs are responsible for tumor formation, progression, metastasis, drug resistance and recurrence. At present, the increasing numbers of CSCs markers were identified in pancreatic cancer, for instance, CD44, CD24, EpCAM, CD133, c-MET, CXCR4, ALDH1, and more recently DclK1 and Lgr5 [25, 26]. However, all these different CSCs markers are not completely overlapped with each other; and thus their co-expression might have higher probability of connecting to the CSCs properties [27]. For instance, we have reported that pancreatic cancer cells co-expressing CD44⁺CD24⁺EpCAM⁺ or CD44⁺CD133⁺ show CSCs properties enabling them to resist to radiotherapy [14]. Based on these findings, drugs targeting CSCs and their corresponding markers or signaling pathway might have chance of eliminating CSCs. Thus, the effective therapeutic strategy should combine conventional chemotherapeutic drugs with the agents that specifically deplete pancreatic CSCs.

Currently, cancer targeting therapeutic strategies primarily focus on one key "driver gene or signaling pathway" of oncogenesis. However, the development of PDAC is a multistep process resulting from the accumulation of multiple genetic lesions in normal cells. Recent genomics sequencing results revealed that there is heterogeneity in the intratumor genetics and phenotypics as well as plasticity of diverse spatial and temporal [28, 29]. This complicated heterogeneity and plasticity may explain why the targeted therapy is failing in PDAC. For example, Kras mutations play a crucial role in early stages of pancreatic carcinogenesis, but accumulation of other cooperative genetic alterations is also required for full oncogenic transformation [29]. Kapoor reported that PDAC tumor cells acquired a by-pass growth mechanism of oncogene Kras addiction by activating Yap1 oncogene [30]. Likewise, for PDAC metastasis and recurrence, tumor cells require cooperative activities between the tumor and the surrounding tissue and a combination of several classes of molecules are involved, like cell-cell and cell-matrix adhesion molecules or matrix-degrading enzyme [3].

Thus for PDAC, a promising therapeutic strategy might be the combined targeting of several key cancer functions such as metabolism, mitochondrial activity, autophagy, and CSCs subpopulations. We and other groups have provided evidence that both CD44s and EpCAM are central players in PDAC carcinogenesis and progression, and both are metastasis associated adhesion molecules that can be therapeutically targeted [4, 12, 14, 17]. In this study, we reported that CD44s and EpCAM were significantly correlated, and their association greatly contributed to tumor progression post-surgery rather than their individual molecules. Our results further suggested that it is needed to target multiple molecules for cancer therapy, such as EpCAM and CD44s in pancreatic cancer. By far, the exact mechanism of the CD44s-EpCAM complex promoting tumor progression is unknown. We are now undertaking further detailed experiments to uncover it.

In conclusion, our present data demonstrated that membranous co-expression of EpCAM and CD44s is an independent poor prognostic factor in pancreatic patients. And, combined targeting EpCAM and CD44s might be an efficient strategy for overcoming PDAC therapeutic resistance post-surgery.

Acknowledgements

This study was supported by China National Science and Technology Major Project (2015-ZX09501-009) and National Natural Science Foundation of China (31571469).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Ling Li and Zhi-Nan Chen, Cell Engineering Research Center and Department of Cell Biology, State Key Laboratory of Cancer Biology, Fourth Military Medical University, Xi'an 710032, Shaanxi, China. Tel: 86-29-84774547; Fax: 86-29-83293906; E-mail: liling25@fmmu.edu.cn (LL); Tel: 86-29-84773243; Fax: 86-29-83293906; E-mail: znchen@fmmu.edu.cn (ZNC)

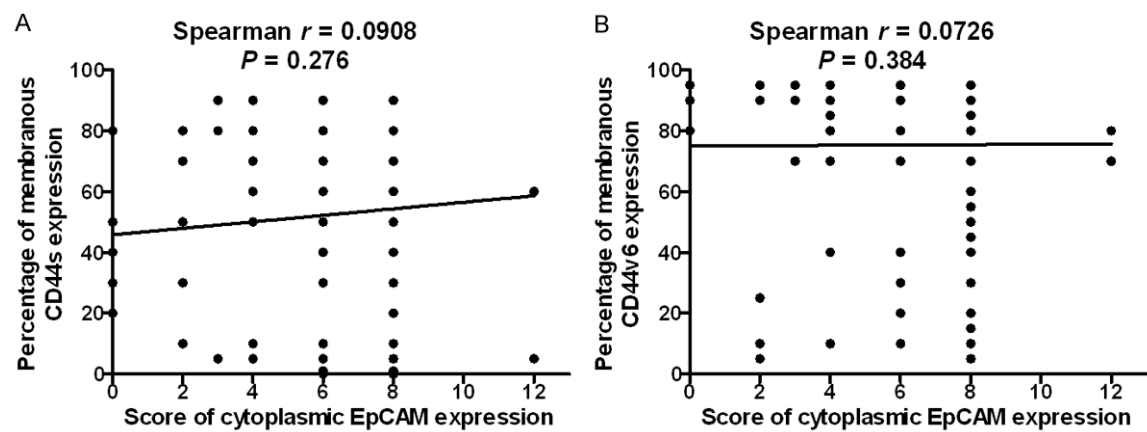
References

- [1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016; 66: 7-30.
- [2] Garrido-Laguna I, Hidalgo M. Pancreatic cancer: From state-of-the-art treatments to prom-

- ising novel therapies. *Nat Rev Clin Oncol* 2015; 12: 319-334.
- [3] Falasca M, Kim M, Casari I. Pancreatic cancer: Current research and future directions. *Biochim Biophys Acta* 2016; 1865: 123-132.
- [4] Martowicz, A, Seeber A, Untergasser G. The role of epcam in physiology and pathology of the epithelium. *Histol Histopathol* 2016; 31: 349-355.
- [5] Dolle L, Theise ND, Schmelzer E, Boulter L, Gires O, van Grunsven LA. Epcam and the biology of hepatic stem/progenitor cells. *Am J Physiol Gastrointest Liver Physiol* 2015; 308: G233-250.
- [6] Gires O, Stoecklein NH. Dynamic epcam expression on circulating and disseminating tumor cells: Causes and consequences. *Cell Mol Life Sci* 2014; 71: 4393-4402.
- [7] Schnell U, Cirulli V, Giepmans BN. Epcam: Structure and function in health and disease. *Biochim Biophys Acta* 2013; 1828: 1989-2001.
- [8] Denzel S, Maetzel D, Mack B, Eggert C, Barr G, Gires O. Initial activation of epcam cleavage via cell-to-cell contact. *BMC Cancer* 2009; 9: 402.
- [9] Schmidt DS, Klingbeil P, Schnolzer M, Zoller M. Cd44 variant isoforms associate with tetraspanins and epcam. *Exp Cell Res* 2004; 297: 329-347.
- [10] Kuhn S, Koch M, Nübel T, Ladwein M, Antolovic D, Klingbeil P, Hildebrand D, Moldenhauer G, Langbein L, Franke WW, Weitz J, Zöller M. A complex of epcam, claudin-7, cd44 variant isoforms, and tetraspanins promotes colorectal cancer progression. *Mol Cancer Res* 2007; 5: 553-567.
- [11] Okada T, Nakamura T, Watanabe T, Onoda N, Ashida A, Okuyama R, Ito K. Coexpression of epcam, cd44 variant isoforms and claudin-7 in anaplastic thyroid carcinoma. *PLoS One* 2014; 9: e94487.
- [12] Orian-Rousseau V. Cd44 acts as a signaling platform controlling tumor progression and metastasis. *Front Immunol* 2015; 6: 154.
- [13] Zoller M. Cd44: Can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer* 2011; 11: 254-267.
- [14] Li L, Hao X, Qin J, Tang W, He F, Smith A, Zhang M, Simeone DM, Qiao XT, Chen ZN, Lawrence TS, Xu L. Antibody against cd44s inhibits pancreatic tumor initiation and postirradiation recurrence in mice. *Gastroenterology* 2014; 146: 1108-1118.
- [15] Matzke-Ogi A, Jannasch K, Shatirishvili M, Fuchs B, Chiblak S, Morton J, Tawak B, Lindner T, Sansom O, Alves F, Warth A, Schwager C, Mier W, Kleeff J, Ponta H, Abdollahi A, Orian-Rousseau V. Inhibition of tumor growth and

- metastasis in pancreatic cancer models by interference with cd44v6 signaling. *Gastroenterology* 2016; 150: 513-525, e510.
- [16] Molejon MI, Tellechea JI, Loncle C, Gayet O, Gilabert M, Duconseil P, Lopez-Millan MB, Moutardier V, Gasmi M, Garcia S, Turrini O, Ouaisi M, Poizat F, Dusetti N, Iovanna J. Deciphering the cellular source of tumor relapse identifies cd44 as a major therapeutic target in pancreatic adenocarcinoma. *Oncotarget* 2015; 6: 7408-7423.
- [17] Meng Y, Xu BQ, Fu ZG, Wu B, Xu B, Chen ZN, Li L. Cytoplasmic epcam over-expression is associated with favorable clinical outcomes in pancreatic cancer patients with hepatitis b virus negative infection. *Int J Clin Exp Med* 2015; 8: 22204-22216.
- [18] Zhao J. Cancer stem cells and chemoresistance: The smartest survives the raid. *Pharmacol Ther* 2016; 160: 145-58.
- [19] Oskarsson T, Batlle E, Massague J. Metastatic stem cells: Sources, niches, and vital pathways. *Cell Stem Cell* 2014; 14: 306-321.
- [20] Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, Apuzzo T, Sperduti I, Volpe S, Cocorullo G, Gulotta G, Dieli F, De Maria R, Stassi G. Cd44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. *Cell Stem Cell* 2014; 14: 342-356.
- [21] Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, Apuzzo T, Sperduti I, Volpe S, Cocorullo G, Gulotta G, Dieli F, De Maria R, Stassi G. Cd44 splice isoform switching in human and mouse epithelium is essential for epithelial-mesenchymal transition and breast cancer progression. *J Clin Invest* 2011; 121: 1064-1074.
- [22] Fong D, Moser P, Kasal A, Seeber A, Gastl G, Martowicz A, Wurm M, Mian C, Obrist P, Mazzoleni G, Spizzo G. Loss of membranous expression of the intracellular domain of epcam is a frequent event and predicts poor survival in patients with pancreatic cancer. *Histopathology* 2014; 64: 683-692.
- [23] Maetzel D, Denzel S, Mack B, Canis M, Went P, Benk M, Kieu C, Papior P, Baeuerle PA, Munz M, Gires O. Nuclear signalling by tumour-associated antigen epcam. *Nat Cell Biol* 2009; 11: 162-171.
- [24] Ladwein M, Pape UF, Schmidt DS, Schnölzer M, Fiedler S, Langbein L, Franke WW, Moldenhauer G, Zöller M. The cell-cell adhesion molecule epcam interacts directly with the tight junction protein claudin-7. *Exp Cell Res* 2005; 309: 345-357.
- [25] Rao CV, Mohammed A. New insights into pancreatic cancer stem cells. *World J Stem Cells* 2015; 7: 547-555.
- [26] Raj D, Aicher A, Heeschen C. Concise review: Stem cells in pancreatic cancer: From concept to translation. *Stem Cells* 2015; 33: 2893-2902.
- [27] Kure S, Matsuda Y, Hagio M, Ueda J, Naito Z, Ishiwata T. Expression of cancer stem cell markers in pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinomas. *Int J Oncol* 2012; 41: 1314-1324.
- [28] Iacobuzio-Donahue CA. Genetic evolution of pancreatic cancer: Lessons learnt from the pancreatic cancer genome sequencing project. *Gut* 2012; 61: 1085-1094.
- [29] Neuzillet C, Tijeras-Raballand A, Bourget P, Cros J, Couvelard A, Sauvanet A, Vullierme MP, Tournigand C, Hammel P. State of the art and future directions of pancreatic ductal adenocarcinoma therapy. *Pharmacol Ther* 2015; 155: 80-104.
- [30] Kapoor A, Yao W, Ying H, Hua S, Liewen A, Wang Q, Zhong Y, Wu CJ, Sadanandam A, Hu B, Chang Q, Chu GC, Al-Khalil R, Jiang S, Xia H, Fletcher-Sananikone E, Lim C, Horwitz GI, Viale A, Pettazoni P, Sanchez N, Wang H, Protopopov A, Zhang J, Heffernan T, Johnson RL, Chin L, Wang YA, Draetta G, DePinho RA. Yap1 activation enables bypass of oncogenic kras addiction in pancreatic cancer. *Cell* 2014; 158: 185-197.
- [31] Li L, Tang W, Wu X, Karnak D, Meng X, Thompson R, Hao X, Li Y, Qiao XT, Lin J, Fuchs J, Simeone DM, Chen ZN, Lawrence TS, Xu L. Hab18g/cd147 promotes pstat3-mediated pancreatic cancer development via cd44s. *Clin Cancer Res* 2013; 19: 6703-6715.
- [32] Qin J, Yang B, Xu BQ, Smithc A, Xu L, Yuan JL, Li L. Concurrent cd44s and stat3 expression in human clear cell renal cellular carcinoma and its impact on survival. *Int J Clin Exp Pathol* 2014; 7: 3235-3244.
- [33] Wu X, Tang W, Marquez RT, Li K, Highfill CA, He F, Lian J, Lin J, Fuchs JR, Ji M, Li L, Xu L. Overcoming chemo/radio-resistance of pancreatic cancer by inhibiting stat3 signaling. *Oncotarget* 2016; 7: 11708-11723.

Co-expression of EpCAM and CD44s predicts poor prognosis



Supplementary Figure 1. Correlation analysis of cytoplasmic EpCAM and CD44 in pancreatic cancer tissues. A: CD44s; B: CD44v6. Spearman rank correlation was conducted to analyze the correlations between EpCAM and CD44 co-expression.