Original Article
Cytoplasmic EpCAM over-expression is associated with favorable clinical outcomes in pancreatic cancer patients with Hepatitis B virus negative infection

Yao Meng[1*], Bao-Qing Xu[1*], Zhi-Guang Fu[1], Bo Wu[1], Bo Xu[2], Zhi-Nan Chen[1], Ling Li[1]

[1]Cell Engineering Research Center and Department of Cell Biology, State Key Laboratory of Cancer Biology, Fourth Military Medical University, Xi'an, Shaanxi 710032, P.R. China; [2]Department of Cardiovascular Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, P.R. China. *Equal contributors.

Abstract: The identification of reliable prognostic markers that distinguish patients' status and predict therapeutic response can improve the clinical outcomes of pancreatic cancer patients. The epithelial cell adhesion molecule (EpCAM) is known to be highly expressed in cancers and serves as a prognosis factor. Generally, membranous EpCAM expression in cancer cells and its clinical significance are evaluated. However, there is also an evidence of cytoplasmic EpCAM distribution in cancer cells. Hence, we investigated which kind of the immunostaining pattern in pancreatic cancer patients was, and whether membranous or cytoplasmic immunostaining had clinical significance. We determined the cytoplasmic or membranous EpCAM expression by a well-established immunohistochemical staining protocol in 157 pairs of carcinoma and paired adjacent non-tumor pancreatic tissue samples using the EpCAM-specific antibody. Furthermore, we evaluated the relationship between tumoral EpCAM expression of resected specimens and patient's overall survival as well as other biological variables like clinical prognosis by Kaplan-Meier method and χ^2 test. We found that pancreatic cancer patients had expressed higher level of cytoplasmic EpCAM but lower level of membranous EpCAM, and their expressions were significantly correlated. Cytoplasmic EpCAM acted as a favorable prognosis factor on survival time in patients with HBV negative infection. Pancreatic cancer patients with cytoplasmic EpCAM over-expression and negative Hepatitis B virus infection might benefit further from post-surgery chemotherapy. These data suggested a potential role of cytoplasmic EpCAM in predicting patient's prognosis and determining therapeutic strategy.

Keywords: cytoplasmic EpCAM, pancreatic cancer, HBV, prognosis, survival

Introduction

Pancreatic ductal adenocarcinoma (PDAC), the fourth commonest cause of cancer-related death in both male and female, is usually diagnosed at an advanced stage in 80% of cases [1]. Complete surgical resection is the only treatment with survival benefit. However, even patients with resectable disease will promptly develop into metastatic and/or local relapses with 15-20% 5-year survival rate and their median survival after curative resection is 20-24 months [2]. Recent studies show that FOLFIRINOX or nab-paclitaxel plus gemcitabine may improve the outcome in patients with good performance status [3, 4]. Therefore, the identification of reliable prognostic markers that

evaluate post-operative status and predict therapeutic response can provide valuable tools for effectively selecting patients who are most likely to benefit from precise therapeutic approaches after surgery.

Epithelial cell adhesion molecule (EpCAM), encoded by the TACSTD1 gene, is a transmembrane carcinoma-associated antigen with oncogenic features. Several biological functions of EpCAM have been described including cell-cell adhesion, proliferation, maintenance of undifferentiated states, as well as regulation of differentiation [5, 6]. EpCAM is mostly expressed by less differentiated and proliferating cells. In cancer, however, EpCAM can be switched on and off through several strategies to fine-tune

Table 1. Patient's characteristics

Features	No. patients (n=157)
Gender: male/female	99/58
Age: mean/median/range (y)	59.31/59/34-85
Tumor size: ≤3/>3 (cm)	53/104
Histological type: ductal/glandular/other/not defined	133/21/2/1
Histopathological grade: I/II/III/not defined	9/105/36/7
pT: T1/T2/T3/not defined	6/122/28/1
pN: N0/N1/not defined	79/63/15
pM: M0/M1	155/2
pStage: IA/IB/I-II/IIA/IIB/IV	4/61/15/13/62/2
*Survival (n=79):	
Mean/median/range (m)	25.2/11.5/0-87
Live/dead	23/56
Smoking: no/few/heavy/not defined	88/34/20/15
Drinking: no/few/heavy/not defined	95/25/20/17
Diabetes: no/yes/not defined	110/26/21
Hepatitis: no/yes/not defined	61/32/64
Heritage: no/yes/not defined	121/11/25

*Only 79 patients had survival data.

EpCAM dependent-function [5]. EpCAM is usually expressed on the basolateral membrane at lower levels in normal epithelial tissues; whereas in many malignant tumors including pancreatic cancer EpCAM is over-expressed and its expression pattern shifts to an intense membranous over-expression, combined with cytoplasmic staining in some cases [7, 8]. Besides, EpCAM is also expressed in human normal stem/progenitor cells and cancer-initiating cells and has been identified as a marker for cancer-initiating cells [5, 9, 10]. In pancreatic cancer, for example, EpCAM positive cancer stem cells (or cancer-initiating cells) show a 100-fold enhanced tumorigenic potential compared with EpCAM-negative pancreatic cancer cells [11].

Due to its high expression in epithelial cancers, EpCAM has attracted attention as a tumor marker for prognosis monitoring. However, the clinical prognostic relevance of EpCAM is a matter of debate, as its expression depends on the environment [6-8]. Besides, the role of EpCAM varies in different cancer types. In renal clear cell carcinoma and thyroid carcinoma, EpCAM is the 'good guy', whose high expression is a good prognostic factor. For many other tumor types including carcinomas of the bladder, gallbladder, ovarian as well as breast, high EpCAM expression predicts poor prognosis.

Even within the same cancer type, such as pancreatic cancer, contradictory results have been found. Fong reported that EpCAM overexpression suggested worse survival in patients with advanced stage of carcinomas [12]. In contrast, Akita found that high EpCAM expression was a good prognostic factor in patients receiving the curative resection [13]. Following this discrepancy, the clinical significance of EpCAM in pancreatic cancer needs to be established.

The survival of patients with carcinomas is mainly associated with the over-expression of membranous EpCAM, detected by antibodies targeting the extracellular domain of EpCAM [12, 13]. In addition to the membrane staining, a specific intracellular immunostaining can also be detected in invasive colorectal cancer cells [14] and advanced breast cancer [15]. The cytoplasmic expression of EpCAM is frequently accompanied by the loss of EpCAM on the plasma membrane. Research indicates that the cytoplasmic EpCAM accumulated in intracellular membranous compartments including endoplasmic reticulum, Golgi apparatus and other intra-cytoplasmic vesicles is associated with a favorable outcome, especially in patients with node-positive breast cancer [15]. Thus, EpCAM expression has different clinical implications according to its subcellular localization. The impact of cytoplasmic EpCAM expression on the prognosis of pancreatic cancer patients after resection is not clear yet. In terms of the tremendous heterogeneity in solid tumors, it is important to establish the clinical significance of cytoplasm EpCAM in pancreatic cancer, in order to select patients who may benefit from post-surgery therapy.

In this study, we examined the expression pattern of EpCAM and its relationship with clinicopathological features in pancreatic cancer. Furthermore, we investigated whether cytoplasmic EpCAM immunostaining had an inde-

pendent clinical significance with respect to membranous staining in pancreatic cancer.

Materials and methods

Patient samples

A total number of 157 consecutive patients with pancreatic ductal adenocarcinoma diagnosed between Sep 2004 and Nov 2011 were included in this retrospective study. Among them, 79 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 years (range: 34-85 years) with 58 women and 99 men. Patients' characteristics, such as gender, age, histological type, grade of tumor, tumor size, TNM stage, AJCC stage, patient survival and history of smoking, drinking, diabetes, hepatitis and heritage were obtained from the medical records (**Table 1**). This study was reviewed and approved by the Institutional Review Board of the Fourth Military Medical University.

Tissue microarray

A tissue microarray (TMA) constructed from formalin-fixed, paraffin-embedded tissue blocks from 157 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 [16]. 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 blocks, 4 μm thick sections were cut and transferred to an adhesive-coated slide, and then verified by a hematoxylin-eosin-stained section.

Immunohistochemistry

TMA slides were dried at 63°C for 1 hour before staining. All procedures were performed at room temperature as previously described [17, 18]. Briefly, sections were dewaxed in xylene and rehydrated in a graded alcohol series. Sections were then washed in water before antigen retrieval using a Leica ST5010 Auto-

stainer (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. Primary antibody rabbit anti-EpCAM monoclonal antibody (clone EPR677, Epitomics, Burlingame, CA, USA), which binds an epitope in the extracellular region of the protein, was diluted at 1:100 with background-reducing diluents (Dako, Carpinteria, CA, USA). 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 solution (Dako, Glostrup, Denmark). Reaction products were visualized with diaminobenzidine plus substrate-chromogen solution treating for 5 minutes. The slides were counterstained with Meyer's hematoxylin and mounted. Careful rinses with several changes of phosphate-buffered saline (PBS) were performed between each stage of the procedure. To confirm the specificity of the primary antibodies, tissue sections were incubated in the absence of the primary antibodies and with control rabbit IgG.

TMA scoring

The Immunohistochemistry staining results were recorded by counting at least 400 cells in five areas of each tissue sections. 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 positive stained cells was arranged into four groups: group 0 displayed no visible difference compared to the negative control sample; the positively stained cells of group 1, 2 and 3 were light brown, mid-brown and dark brown, with the same intensity covering more than 75% of the staining area [18]. The total number of cells and the stained ones were counted; the positive rate was calculated and categorized according to a 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 [12]. For statistical analysis, the stained
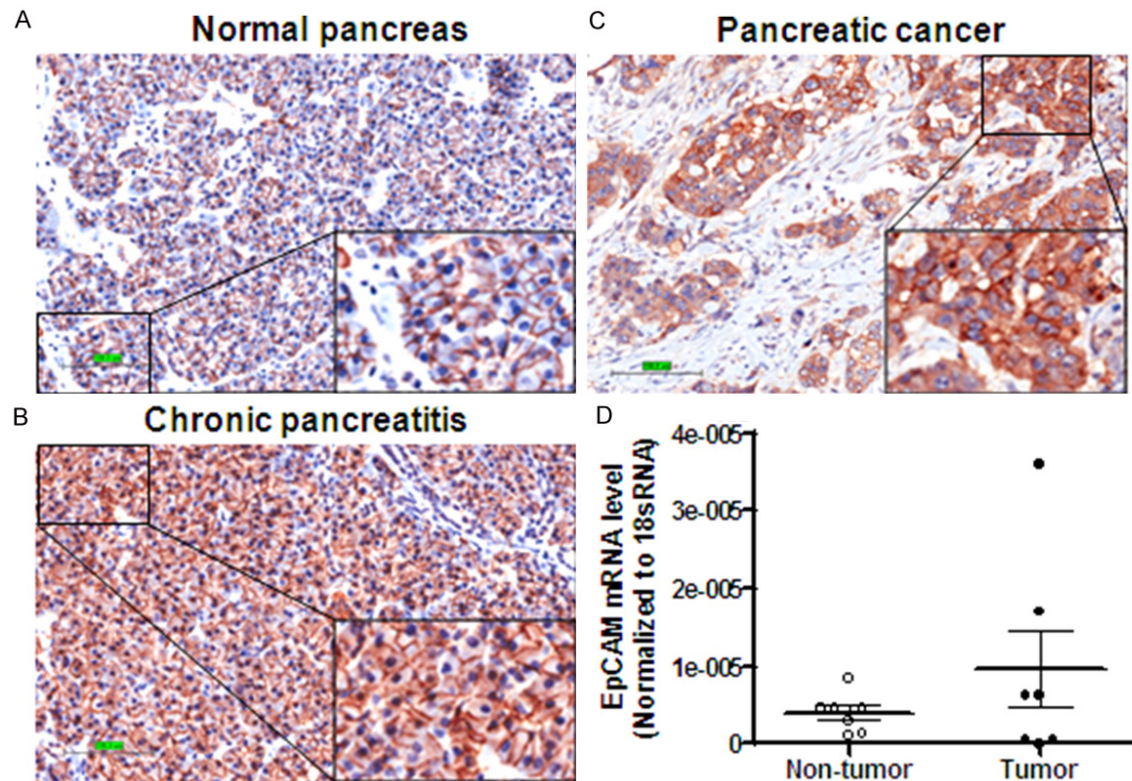
Figure 1. EpCAM expression in pancreatic tissues. (A-C) Immunohistochemistry staining of EpCAM expression in normal pancreas (A), chronic pancreatitis (B) and pancreatic cancer (C). EpCAM positive expression was seen on plasma membrane and in cytoplasm, and immunostaining showed a fine granular pattern (Bar=100.8 µm, original magnification 200 X). The inserts provide details of EpCAM expression patterns. (D) Relative EpCAM mRNA levels in 7 pairs of pancreatic cancer tissues and adjacent non-tumor tissues. Levels were normalized to 18s RNA levels.

Table 2. EpCAM expression in normal pancreas, chronic pancreatitis and pancreatic cancer patients[b]

Over-expression (score >4)	Membranous EpCAM % (n)			Cytoplasmic EpCAM % (n)			P value[#]
	No	Yes	P value[*]	No	Yes	P value[*]	
Normal pancreas	20.2% (18/89)	79.8% (71/89)	/	50.6% (45/89)	49.4% (44/89)	/	<0.001
Chronic pancreatitis	19.1% (13/68)	80.9% (55/68)	0.812	29.4% (20/68)	70.6% (48/68)	0.008	0.162
Pancreatic cancer	47.8% (75/157)	52.2% (82/157)	<0.001, <0.001[a]	17.2% (27/157)	82.8% (130/157)	<0.001, 0.044[a]	<0.001

[*]Chronic pancreatitis or pancreatic cancer was compared with normal pancreas; [a]Pancreatic cancer was compared with chronic pancreatitis; [#]Cytoplasmic EpCAM expression was compared with membranous EpCAM expression; [b]Estimated by χ^2 test.

tumor tissues were divided into two groups: the nil-low expression group (categories as zero and weak, corresponding to a total score 0-4) and the over-expression group (categories as moderate and strong, corresponding to a total score >4).

Quantitative real-time PCR (qPCR)

Total RNA extraction and cDNA synthesis were performed as previously described [18]. qPCR amplification was performed using an ABI 7700 real-time PCR system (Thermo Fisher Scientific, Waltham, MA) with gene-specific primers for EpCAM. Forward: 5'-GGACCTGACAGTAAATGG-GGAAC-3'; reverse: 5'-CTCTTCTTTCTGGAAATAA CCAGCAC-3'. Genes of interest were normalized to the housekeeping gene 18S RNA: forward: 5'-CGCCGCTAGAGGTGAAATTC-3'; reverse: 5'-TTGGCAAATGCTTTCGCT C-3'. Relative mRNA levels are presented as unit values of $2^{-\Delta Ct}=2^{-(Ct\,(HKG)\,-\,Ct\,(GOI))}$.

Immunofluorescence staining

Immunofluorescence staining were performed as previously described [17]. Cells grown on chambered cover slips were fixed, blocked and
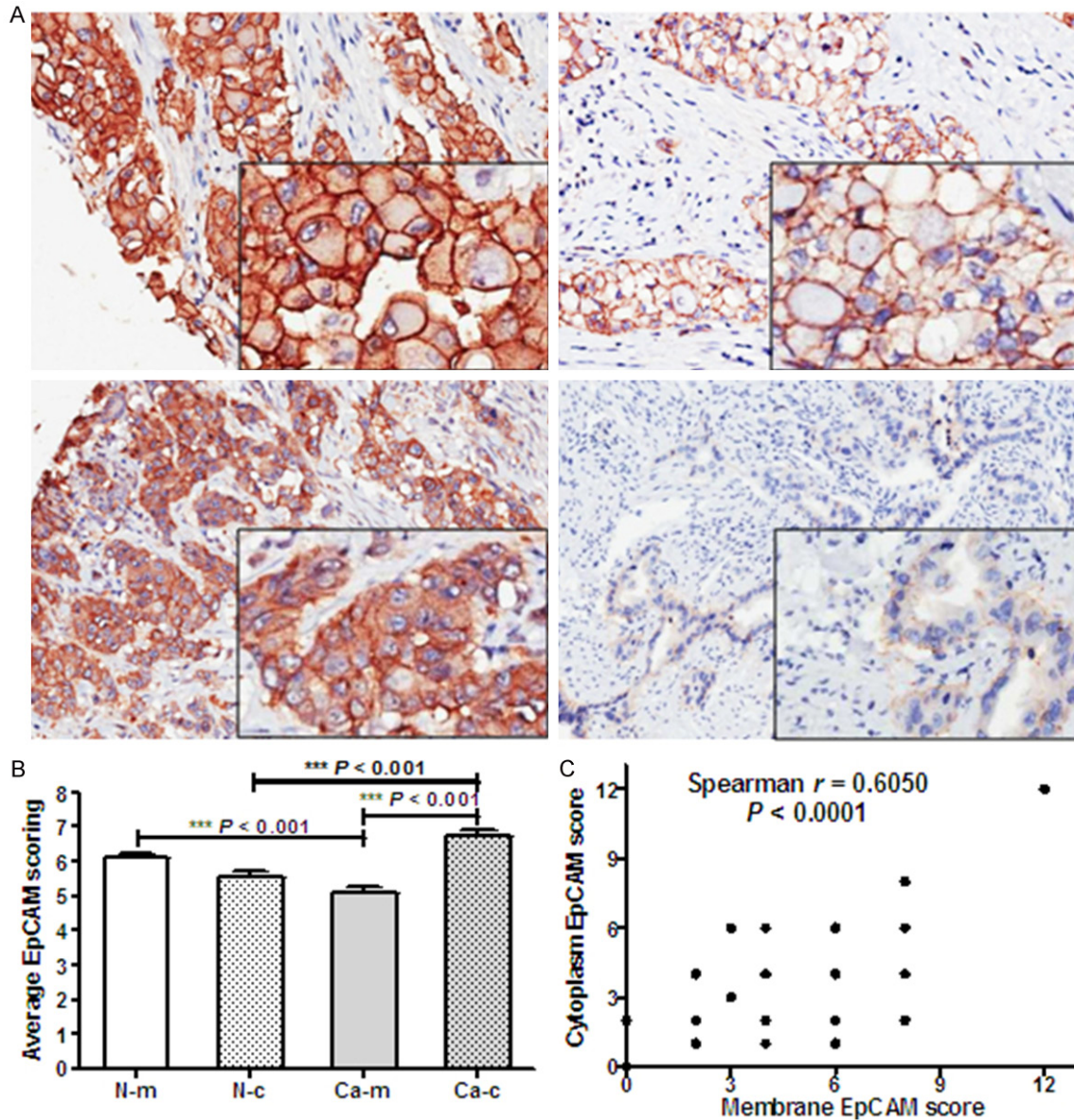
Figure 2. Immunohistochemical analysis of membranous and cytoplasmic EpCAM expression in pancreatic cancer tissues. A. Varied membranous and cytoplasmic EpCAM expression pattern: strong membranous and cytoplasmic expression (*up-left*), strong membranous and weak cytoplasmic expression (*up-right*), weak membranous and strong cytoplasmic expression (*down-left*), weak membranous and cytoplasmic expression (*down-right*). The inserts provide details of EpCAM expression patterns. B. The scatter chart of scoring of EpCAM expression in membrane and cytoplasm from pancreatic cancer tissues and non-tumor tissues, respectively. C. Correlation analysis between membranous and cytoplasmic EpCAM expression in pancreatic cancer patients.

probed with the anti-EpCAM. The signals were detected with fluorochrome-conjugated FITC. Cover slips were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) to visualize the nuclei. Cell images were observed and acquired using a fluorescence microscope (Nikon ECLIPSE Ti, Pudong New District, Shanghai, China).

Western blot analysis

Western blot analysis were performed using standard methods [17, 18]. Whole cell extracts from cultured cells were prepared by adding RIPA lysis buffer (1 M Tris-HCl [pH 7.5], 5 M NaCl, 0.01% NP-40, 0.5 M EGTA, and 10% SDS) (Thermo Fisher Scientific, Rockford, IL)

Table 3. Membranous and cytoplasmic EpCAM expression in pancreatic tissues

Subcellular location	Expression level[†]			
	Zero	Weak	Moderate	Strong
Membranous EpCAM, % (n)	3.2% (5/157)	44.6% (70/157)	51% (80/157)	1.2% (2/157)
Cytoplasmic EpCAM, % (n)	3.8% (6/157)	13.4% (21/157)	81.5% (128/157)	1.3% (2/157)

[†]Total immunostaining score was calculated by multiplying intensity score by positive rate score and categorized as four expression subgroups: zero, negative total score; weak, total score 1-4; moderate, total score 5-8; strong, total score 9-12.

and complete protease inhibitor cocktail (Roche, Indianapolis, IN) to the cell monolayer. Proteins were separated on 10% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in 5% skim milk for 1 hour. Subsequently, the membranes were incubated with each individual antibody overnight at 4°C and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG for 2 hours. Finally, signals were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). To demonstrate equal loading, membranes were stripped and reprobed with a monoclonal antibody against β-actin.

Statistical analysis

Spearman rank correlation was conducted to analyze the expression correlations between cytoplasmic EpCAM and membranous EpCAM. The agreement between membranous EpCAM and cytoplasmic EpCAM levels was assessed by kappa statistic (k). The k value was interpreted as follows: k<0 when the observed agreement was less than that expected by chance (disagreement); 0≤k≤0.2 slight agreement; 0.21≤k≤0.4 fair agreement; 0.41≤k≤0.6 moderate agreement; 0.61≤k≤0.8 substantial agreement; 0.81≤k≤1.0 almost perfect agreement [15].

The association between EpCAM expression and clinicopathological factors was assessed by the χ^2 test. Overall survival was estimated by the Kaplan-Meier method and evaluated by the log-rank test. Factors appeared to be of prognostic significance in the univariate model were evaluated in a multivariate Cox regression model. A *P*-value <0.05 was considered statistically significant. All statistical analysis was performed using the Statistical Package of Social Sciences (SPSS, V.16.0, Chicago, Illinois, USA).

Results

Patient characteristics

The patient's clinopathological characteristics were summarized in **Table 1**. Vast majority of patients suffered from pancreatic ductal carcinoma (133/157, 84.71%) and the tumor size was above 3 cm (104/157, 66.24%). The histopathological grading of tumors showed poorly, moderately and well differentiated adenocarcinoma in 9, 105 and 36 patients, respectively. The TNM classification was 6, 122 and 28 patients with pT1, pT2 and pT3; 79, 63 and 2 patients with pN0, pN1 and pM1; and 4, 61, 13, 62 and 2 patients with pStage IA, IB, IIA, IIB and IV, respectively. At the time of last clinical follow-up (November 2011), 56 patients (71%) were died and 23 patients (29%) were still alive. The median survival time was 11.5 months (range: 0-87 months).

Cytoplasmic EpCAM is over-expressed in pancreatic cancer

To determine the clinical significance of EpCAM in pancreatic cancer, we examined the EpCAM expression in 157 pairs of pancreatic cancer tissues and adjacent non-tumor tissue (normal pancreas or chronic pancreatitis) from surgery. As shown in **Figure 1A-C**, EpCAM was expressed both on plasma membrane and in cytoplasm.

Normally, EpCAM was mainly over-expressed on the plasma membrane (mEpCAM, 71/89, 79.8%) in normal pancreas; while in the cases of chronic pancreatitis, it was over-expressed both on the plasma membrane (55/68, 80.9%) and in the cytoplasm (48/68, 70.6%, **Table 2**). However, in pancreatic cancer, EpCAM was mostly over-expressed in the cytoplasm (cEpCAM, 130/157, 82.8%). Compared with the other two, pancreatic cancer has not only significantly higher ratio of cEpCAM staining (*P*<0.001 and *P*=0.044), but also significantly
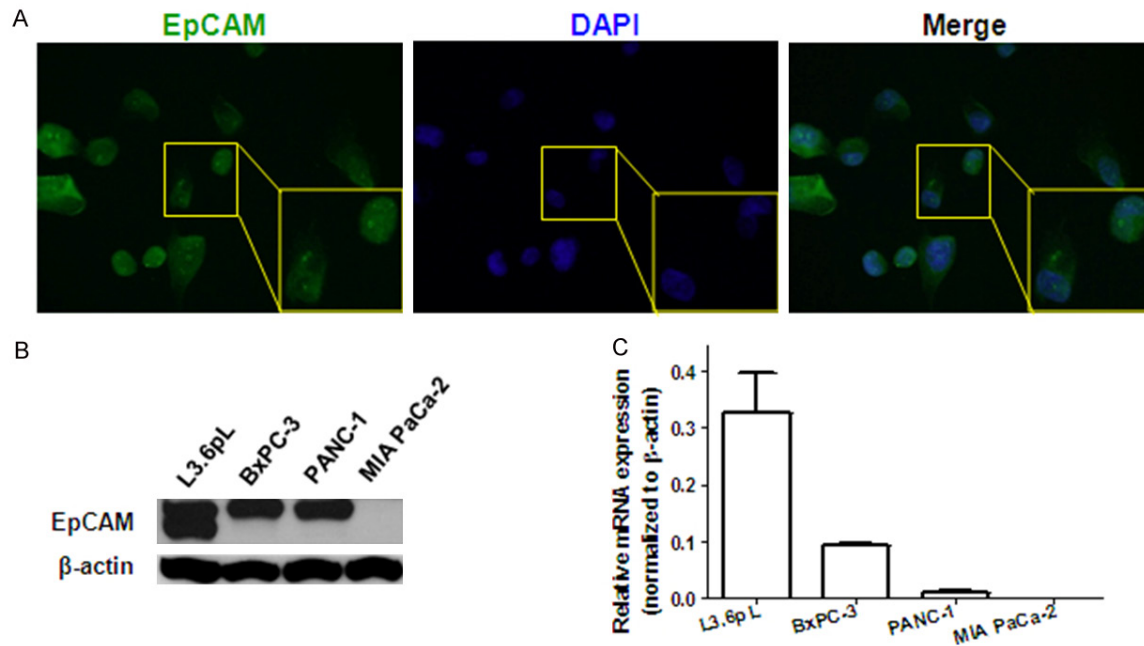
Figure 3. EpCAM expression in pancreatic cancer cell lines. A. EpCAM protein subcellular distribution in PANC-1 was assessed by immunofluorescence staining. Original magnification: 400X. B. EpCAM protein expression in human pancreatic cancer cells. β-actin was included as an internal control for loading. C. EpCAM mRNA expression in human pancreatic cancer cells. Levels were normalized to β-actin levels.

Table 4. The association of membranous and cytoplasmic EpCAM expression in pancreatic tissues

Groups	Percentage
mEpCAM⁻cEpCAM⁻	(15.92%) 25/157
mEpCAM⁺cEpCAM⁻	(1.27%) 2/157
mEpCAM⁻cEpCAM⁺	(31.85%) 50/157
mEpCAM⁺cEpCAM⁺	(50.96%) 80/157

Spearman r = 0.6050 (95% confidence interval: 0.4918-0.6981), $P<0.0001$.

lower ratio of mEpCAM staining ($P<0.001$ and $P<0.001$, **Table 2**). Furthermore, EpCAM mRNA levels in the pancreatic cancer were 2.42-fold higher than those in the paired adjacent non-tumor tissues, though this difference did not reach statistical significance ($P=0.286$, n=7, **Figure 1D**).

Figure 2A showed representative pictures for sole and/or concurrent membranous and cytoplasmic EpCAM immunostaining in pancreatic cancer tissues. As for EpCAM subcellular location, the percentage of moderate and strong expression of cEpCAM (82.8%) was significantly higher than that of mEpCAM (52.2%, $P<0.001$)

(**Tables 2, 3**). According to the previously defined scoring criteria calculated by intensity and percentage, in pancreatic cancer, the score of cEpCAM was significantly higher than that of mEpCAM (6.75±2.22 *vs.* 5.10±2.23, $P<0.001$, **Figure 2B**). In cell line for investigating the EpCAM subcellular distribution *in vitro*, we also found that EpCAM had positive immunofluorescence signaling in the cytoplasm of PANC-1 cells (**Figure 3A**). These data indicated that the EpCAM protein levels in cancer tissues or cancer cells were connected to its subcellular distribution. In pancreatic cancer for example, cEpCAM was over-expressed while mEpCAM was nil-low expressed irrespective of the activation of EpCAM mRNA transcription.

Cytoplasmic EpCAM expression is significantly correlated with membranous EpCAM expression in pancreatic cancer

We next investigated that whether membranous and cytoplasmic EpCAM immunostaining were related to each other. Remarkably, high expression of mEpCAM was significantly associated with high expression of cEpCAM, and strong correlation was existed between them

Table 5. The relationship between cytoplasmic EpCAM expression and patients characteristics

Characteristics	No (n=27)	Yes (n=130)	P value[*]
Gender, n (male/female)	17/10	82/48	0.991
Age, mean ± s.d. (years)	60.8±8.7	59±11.2	0.428
Size, mean ± s.d. (cm)	4±1.3	4.2±1.9	0.594
Type, n (ductal/glandular)	18/8	115/15	0.012
Grade, n (I+II, III)	22/2	93/34	0.052
pT stage, n (T1+T2,T3)	24/3	104/25	0.458
pN, n (N0/N1)	16/11	63/52	0.673
pStage, n (IA+IB, IIA+IIB+IV)	13/14	52/63	0.783
Patient survival, n (live/dead)	2/10	21/46	0.493
Smoking, n (no, few+heavy)	16/9	72/45	0.818
Drinking, n (no, few+heavy)	17/8	78/37	0.987
Diabetes, n (no/yes)	17/7	93/19	0.575
Hepatitis, n (no/yes)	10/9	49/23	0.21
Heritage, n (no/yes)	23/2	98/9	1

[*]Estimated by student t test and χ^2 test.

(r=0.6050, *P*<0.0001, **Figure 2C**). Furthermore, the overall agreement between membrane and cytoplasm with or without EpCAM over-expression in pancreatic cancer accounted for 66.88% of cases (105/157) (**Table 4**). The disagreement included 50 cases (31.85% of the total) of mEpCAM nil-low expression/cEpCAM over-expression and 2 cases (1.27% of the total) of mEpCAM over-expression/ cEpCAM nil-low expression. The Odds Ratio (OR) for association between mEpCAM and cEpCAM was 20 (95% CI 4.539-88.121); and the agreement between mEpCAM and cEpCAM was fair (κ=0.318, *P*<0.001). In the following study, we mainly focused on the relationship between cEpCAM expression and its clinopathological significance.

*The clinopathological significance of cytoplasmic EpCAM over-expression*

Then, we explored the correlation between cytoplasmic EpCAM expression and clinicopathological characteristics. We observed that cEpCAM expression was significantly associated with tumor grade (*P*=0.052) and tumor type (*P*=0.012) (**Table 5**). Similarly, in *in vitro* cell line models (**Figure 3B** & **3C**), both EpCAM protein and mRNA were highly expressed in well or moderate differentiated cell lines like L3. 6 pL, BxPC-3, but lower expressed in poor differentiated cell lines like PANC-1 and MIA PaCa-2 [19].

However, there were no significant differences between patients with over and nil-low tumoral cEpCAM expression with respect to gender, age, tumor size, 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, diabetes, hepatitis and heritage.

The association of EpCAM expression with patient's survival was estimated by Kaplan-Meier analysis and the log-rank test from censored available survival time of 66 pancreatic cancer patients. As shown in **Figure 4A**, the association between EpCAM expression and patient's survival was more remarkably in cEpCAM than in mEpCAM. Patients with cEpCAM over-expression had a median survival of 18 months compared with 11 months in the patients with cEpCAM nil-low expression (*P*=0.297). Furthermore, in the subgroup of patients with an HBV-negative infection, the median survival of cEpCAM over-expression group was greatly prolonged (30 months vs. 10 months, *P*=0.0679, **Figure 4B** & **4C**). The favorable association between cEpCAM expression and patient survival was more evident when the mEpCAM expression was concomitantly considered. As shown in **Figure 4D**, double over-expression of cEpCAM and mEpCAM were associated with the most favorable longer survival (25% survival above 43 months) with respect to single over-expression of cEpCAM (25% survival above 33 months), or double nil-low expression of cEpCAM and mEpCAM (25% survival of 11 months), although not reached a significant difference (*P*=0.283).

In the univariable regression model, cEpCAM over-expression provided a relatively low hazard with respect to nil-low expression (hazard ratio=0.470; 95% confidence interval, 0.167 -1.323; *P*=0.153), although this difference did not reached statistic significance. In a subgroup of HBV negative infection, patients whose tumor has high expression of both cytoplasmic EpCAM and membranous EpCAM had a more favorable outcome when compared to patients whose tumor has single over-expression of cEpCAM or double nil-low expression of
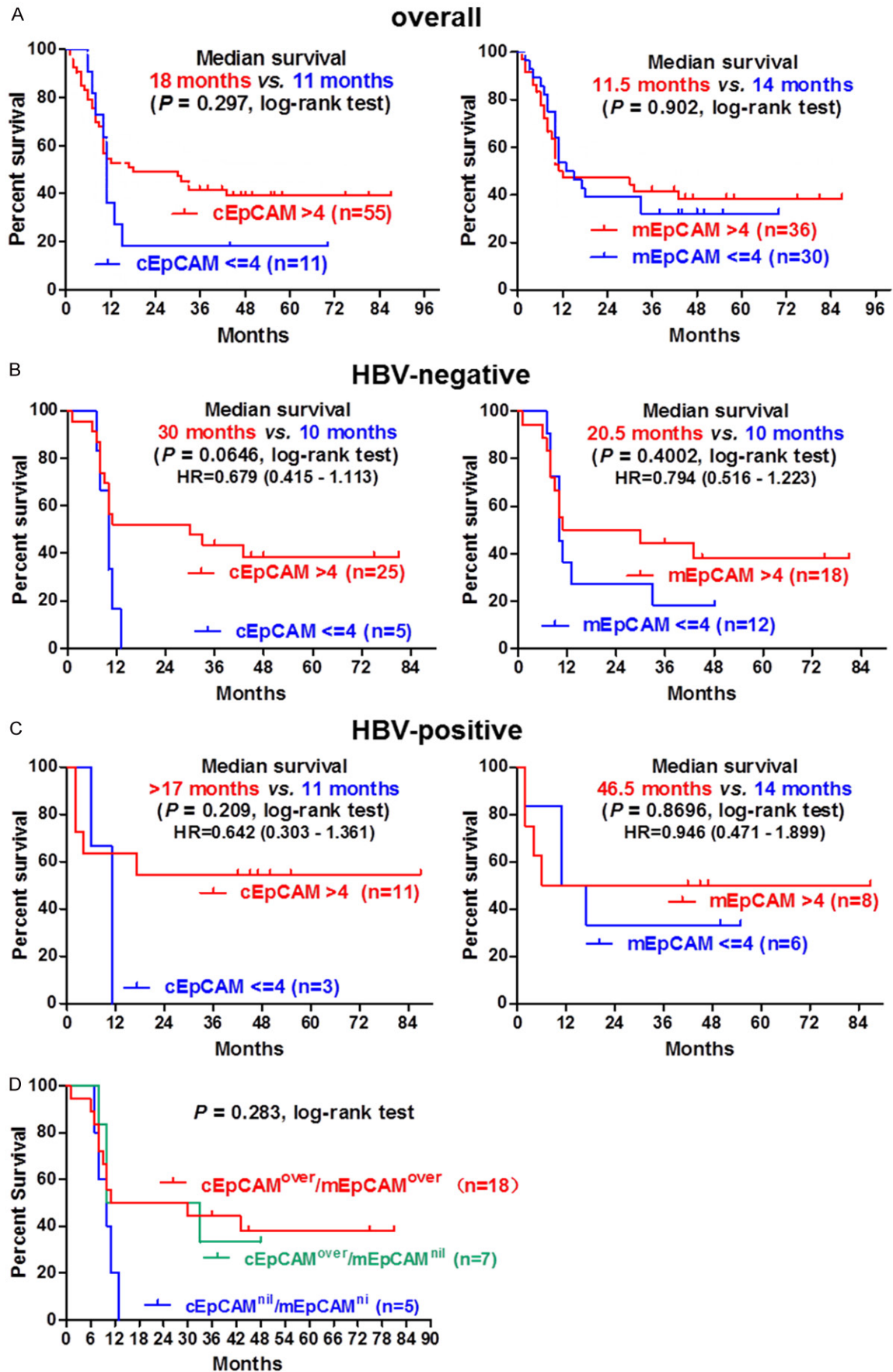
Figure 4. Overall survival by Kaplan-Meier analysis according to EpCAM expression in pancreatic cancer patients. A. Kaplan-Meier analysis of overall survival for 66 patients based on cytoplasmic or membranous EpCAM scores by immunohistochemistry staining. B. Kaplan-Meier overall survival curves stratified according to membranous or cytoplasmic EpCAM expression in the HBV-negative pancreatic cancer subset. C. Kaplan-Meier overall survival curves stratified according to membranous or cytoplasmic EpCAM expression in the HBV-positive pancreatic cancer subset. D. Kaplan-Meier overall survival curves stratified according to membranous and cytoplasmic EpCAM expression in the HBV-negative pancreatic cancer subset.

Table 6. Prognostic factors for overall survival by Cox proportional hazard model

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)*	P value
Gender (male/female)	0.625 (0.33-1.20)	0.158	—	—
Age (years)	1.033 (1.00-1.06)	0.030	—	—
Grade (I+II, III)	2.162 (1.15-4.07)	0.017	15.509 (2.686-89.563)	0.009
pN (N0/N1)	2.526 (1.33-4.79)	0.005	—	—
pStage (IA+IB, IIA+IIB+IV)	1.823 (0.98-3.40)	0.058	—	—
Smoking (no, few+heavy)	1.397 (0.93-2.11)	0.112	3.177 (1.02-9.93)	0.047
Hepatitis (no/yes)	0.829 (0.37-1.88)	0.652	0.017 (0.00-0.816)	0.039
cEpCAM expression (over/nil)	0.470 (0.167-1.323)	0.153	—	—
mEpCAM expression (over/nil)	0.933 (0.69-1.26)	0.652	—	—
cEpCAM/mEpCAM co-expression (mEpCAM^over^ cEpCAM^over^/mEpCAM^nil^ cEpCAM^over^/mEpCAM^nil^ cEpCAM^nil^)	0.784 (0.592-1.040)	0.091#	0.681 (0.395-1.174)	0.166#

*HR, hazard ratio; CI, confidence interval. #The relationship between cEpCAM/mEpCAM co-expression and prognostic factors for overall survival were analyzed in HBV negative patients.

cEpCAM and mEpCAM (P=0.091, HR 0.784; CI 0.592–1.040). However, for the total cohort of pancreatic cancer patients by multivariate analysis, only tumor grade (P=0.009), smoking (P=0.047) and hepatitis (P=0.039) were of prognostic significance (**Table 6**).

Discussion

In this study, we showed that the level of cEpCAM was higher and the level of mEpCAM was lower in pancreatic cancer, and their expressions were significantly correlated. The cEpCAM over-expression was also closely linked to some of the important clinical variables such as tumor grade and tumor type, but not with patient survival time. In patients with HBV-negative infection, cEpCAM over-expression was correlated to prolonged survival, suggesting a possible prognostic role of cEpCAM in pancreatic cancer patients with no HBV infection. These data suggested a potential role of cEpCAM in predicting patients' prognosis and determining therapeutic strategy. At present, there is no valuable biomarker to divide patients into good or bad prognostic group after resection, thus we believe that this study will help to determine the optimal post-surgery therapy for pancreatic cancer patients. Pancreatic cancer patients with cEpCAM over-expression and negative Hepatitis B virus infection may have a favourable clinical outcomes and might be benefit further from chemotherapy post-surgery.

EpCAM expressed on the cellular membrane or in the cytoplasm has different roles and functions. In differentiated normal cells, EpCAM on the cell surface plays essential roles in rapid response to surrounding extracellular signals by forcing the interaction between a ligand on one cell and its receptor on the other cell [5]. However, in cancer cells, the loss of membranous EpCAM expression along with the appearance of cytoplasmic EpCAM expression was frequently observed [14, 15]. Our results also demonstrated that cytoplasmic EpCAM expression was occurred in 80% of cases, which was accompanied by the loss of membranous EpCAM in 50% of cases. From the view of cell biology, the high level of EpCAM in cytoplasm was one way to limit its activity on the membrane, to depress the oncogenic potential of membranous EpCAM, and to maintain epithelial cells in differentiated state [15]. The result that cytoplasmic EpCAM up-regulation associated with a favourable outcome was in line this point, which indicated an inhibiting role of cytoplasmic EpCAM in pancreatic cancer tumorgenesis and progression.

The molecular basis for the rapid down-regulation of membranous EpCAM is as yet unknown. It was reported that EpCAM was endocytosed in a clathrin-dependent way due to NPXY consensus motif within the intracellular domain [20, 21], and then cleaved off by BACE1 located on the membranes of trans-Golgi network and endosomes with acidic pH for optimum enzymatic activity [22]. Our results and others' further pinpoint endocytosis and subsequent degradation of EpCAM in intracellular compartments as a potential mechanism for EpCAM withdrawal from the cell surface [23]. Besides endocytosis, the loss of EpCAM from the cell surface was also mediated by regulated intramembrane proteolysis (RIP), a newly described means to regulate cell surface availability and functionality of EpCAM [24]. RIP activated proliferative signaling via triggering the release of intracellular domain (EpICD) which led to the functional inactivation of transmembrane EpCAM. In human cancer cells, EpICD can translocate into the nucleus and regulate transcription. Recently, proteolysis analysis revealed that intermediate cleavage products as EpICD were found in pancreatic cancer and EpICD had been proposed as a biomarker for poor prognosis and aggressive phenotype [25, 26]. However, the loss of membranous EpICD expression was only observed in one-third of pancreatic cancer patients [25]. Collectively, endocytosis and RIP of EpCAM possibly act as two separate but converging ways as regards EpCAM being removed from the cell surface [27]. Different cleavage pathways are probably determined by the localization of EpCAM and relevant proteases in distinct membrane compartments. Hence, further studies are required to explore the detailed pattern of membranous EpCAM down-regulation in pancreatic cancer, particularly in Chinese patients.

The infection rate of hepatitis B virus (HBV) is especially high in China. Chronic HBV infection is a major risk factor for hepatocellular carcinoma (HCC) with HBV X protein (HBx) acting as a cofactor. Recently, HBV infection has been reported to play an important role in the progression of some extrahepatic malignancies, such as colorectal cancer (CRC) [28] and pancreatic cancer [29-31]. Iloeje identified chronic HBV infection as a risk for PDAC patients under the age of 50 years [32]. Besides, patients with non-HBV infection had significantly longer overall survival compared with inactive HBsAg carri-

ers in Chinese patients with pancreatic cancer [29], which was consistent with our results. For patients who underwent resection for PDAC, gemcitabine treatment has been reported to extend patients survival. As the exposure to HBV could potentially compromise the outcome of gemcitabine treatment, prevention of viral reactivation before post-surgery gemcitabine chemotherapy for pancreatic cancer patients is highly recommended.

EpCAM expression is up-regulated in HBV-infected cancer cells as its DNA is activated by HBx [33, 34]. HBx also activates β-catenin and epigenetic up-regulation of miR-181, both of which target EpCAM, thus endows HCC cells with stem cell properties, or "stemness" [35]. For these reasons, EpCAM is presumed to associate with HBV-mediated carcinogenesis. EpCAM is also involved in HBV related tumor development and progression. Therefore a combined method of EpCAM scoring and HBV status monitoring offers a better view in predicting patient's clinical outcomes. As shown in this paper, cytoplasmic EpCAM over-expression is associated with a favorable clinical outcomes in pancreatic cancer patients with no Hepatitis B virus infection.

In summary, we show that cytoplasmic EpCAM is highly expressed in pancreatic cancer and acts as a favorable prognosis factor on survival time in patient with HBV negative infection. Evaluation of the level of cytoplasmic EpCAM can predict good prognosis, and provides a reference on further treatment.

Acknowledgements

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, Shaanxi 710032, 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 and Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.

[2] Ryan DP, Hong TS and Bardeesy N. Pancreatic adenocarcinoma. N Engl J Med 2014; 371: 1039-1049.

[3] Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardiere C, Bennouna J, Bachet JB, Khemissa-Akouz F, Pere-Verge D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C and Ducreux M. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 2011; 364: 1817-1825.

[4] Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J and Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 2013; 369: 1691-1703.

[5] Dolle L, Theise ND, Schmelzer E, Boulter L, Gires O and van Grunsven LA. EpCAM and the biology of hepatic stem/progenitor cells. Am J Physiol Gastrointest Liver Physiol 2015; 308: G233-250.

[6] Schnell U, Cirulli V and Giepmans BN. EpCAM: structure and function in health and disease. Biochim Biophys Acta 2013; 1828: 1989-2001.

[7] Patriarca C, Macchi RM, Marschner AK and Mellstedt H. Epithelial cell adhesion molecule expression (CD326) in cancer: a short review. Cancer Treat Rev 2012; 38: 68-75.

[8] van der Gun BT, Melchers LJ, Ruiters MH, de Leij LF, McLaughlin PM and Rots MG. EpCAM in carcinogenesis: the good, the bad or the ugly. Carcinogenesis 2010; 31: 1913-1921.

[9] Imrich S, Hachmeister M and Gires O. EpCAM and its potential role in tumor-initiating cells. Cell Adh Migr 2012; 6: 30-38.

[10] Yamashita T, Honda M, Nakamoto Y, Baba M, Nio K, Hara Y, Zeng SS, Hayashi T, Kondo M, Takatori H, Mizukoshi E, Ikeda H, Zen Y, Takamura H, Wang XW and Kaneko S. Discrete nature of EpCAM+ and CD90+ cancer stem cells in human hepatocellular carcinoma. Hepatology 2013; 57: 1484-1497.

[11] Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF and Simeone DM. Identification of pancreatic cancer stem cells. Cancer Res 2007; 67: 1030-1037.

[12] Fong D, Steurer M, Obrist P, Barbieri V, Margreiter R, Amberger A, Laimer K, Gastl G, Tzankov A and Spizzo G. Ep-CAM expression in pancreatic and ampullary carcinomas: frequency and prognostic relevance. J Clin Pathol 2008; 61: 31-35.

[13] Akita H, Nagano H, Takeda Y, Eguchi H, Wada H, Kobayashi S, Marubashi S, Tanemura M, Takahashi H, Ohigashi H, Tomita Y, Ishikawa O, Mori M and Doki Y. Ep-CAM is a significant prognostic factor in pancreatic cancer patients by suppressing cell activity. Oncogene 2011; 30: 3468-3476.

[14] Gosens MJ, van Kempen LC, van de Velde CJ, van Krieken JH and Nagtegaal ID. Loss of membranous Ep-CAM in budding colorectal carcinoma cells. Mod Pathol 2007; 20: 221-232.

[15] Alberti S, Ambrogi F, Boracchi P, Fornili M, Querzoli P, Pedriali M, La Sorda R, Lattanzio R, Tripaldi R, Piantelli M, Biganzoli E and Coradini D. Cytoplasmic Trop-1/Ep-CAM overexpression is associated with a favorable outcome in node-positive breast cancer. Jpn J Clin Oncol 2012; 42: 1128-1137.

[16] Qin J, Yang B, Xu BQ, Smithc A, Xu L, Yuan JL and 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.

[17] 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 and Xu L. HAb18G/CD147 promotes pSTAT3-mediated pancreatic cancer development via CD44s. Clin Cancer Res 2013; 19: 6703-6715.

[18] Li L, Hao X, Qin J, Tang W, He F, Smith A, Zhang M, Simeone DM, Qiao XT, Chen ZN, Lawrence TS and Xu L. Antibody against CD44s inhibits pancreatic tumor initiation and postradiation recurrence in mice. Gastroenterology 2014; 146: 1108-1118.

[19] Kallifatidis G, Rausch V, Baumann B, Apel A, Beckermann BM, Groth A, Mattern J, Li Z, Kolb A, Moldenhauer G, Altevogt P, Wirth T, Werner J, Schemmer P, Buchler MW, Salnikov AV and Herr I. Sulforaphane targets pancreatic tumour-initiating cells by NF-kappaB-induced antiapoptotic signalling. Gut 2009; 58: 949-963.

[20] Tsaktanis T, Kremling H, Pavsic M, von Stackelberg R, Mack B, Fukumori A, Steiner H, Vielmuth F, Spindler V, Huang Z, Jakubowski J, Stoecklein NH, Luxenburger E, Lauber K, Lenarcic B and Gires O. Cleavage and Cell

Adhesion Properties of Human Epithelial Cell Adhesion Molecule (HEPCAM). J Biol Chem 2015; 290: 24574-24591.

[21] Winkler J, Martin-Killias P, Pluckthun A and Zangemeister-Wittke U. EpCAM-targeted delivery of nanocomplexed siRNA to tumor cells with designed ankyrin repeat proteins. Mol Cancer Ther 2009; 8: 2674-2683.

[22] Cole SL and Vassar R. The Alzheimer's disease beta-secretase enzyme, BACE1. Mol Neurodegener 2007; 2: 22.

[23] Driemel C, Kremling H, Schumacher S, Will D, Wolters J, Lindenlauf N, Mack B, Baldus SA, Hoya V, Pietsch JM, Panagiotidou P, Raba K, Vay C, Vallbohmer D, Harreus U, Knoefel WT, Stoecklein NH and Gires O. Context-dependent adaption of EpCAM expression in early systemic esophageal cancer. Oncogene 2014; 33: 4904-4915.

[24] Maetzel D, Denzel S, Mack B, Canis M, Went P, Benk M, Kieu C, Papior P, Baeuerle PA, Munz M and Gires O. Nuclear signalling by tumour-associated antigen EpCAM. Nat Cell Biol 2009; 11: 162-171.

[25] Fong D, Moser P, Kasal A, Seeber A, Gastl G, Martowicz A, Wurm M, Mian C, Obrist P, Mazzoleni G and 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.

[26] Fong D, Seeber A, Terracciano L, Kasal A, Mazzoleni G, Lehne F, Gastl G and Spizzo G. Expression of EpCAM (MF) and EpCAM (MT) variants in human carcinomas. J Clin Pathol 2014; 67: 408-414.

[27] Gires O and Stoecklein NH. Dynamic EpCAM expression on circulating and disseminating tumor cells: causes and consequences. Cell Mol Life Sci 2014; 71: 4393-4402.

[28] Song E, Chen J, Ou Q and Su F. Rare occurrence of metastatic colorectal cancers in livers with replicative hepatitis B infection. Am J Surg 2001; 181: 529-533.

[29] Wei XL, Qiu MZ, Chen WW, Jin Y, Ren C, Wang F, Luo HY, Wang ZQ, Zhang DS, Wang FH, Li YH and Xu RH. The status of HBV infection influences metastatic pattern and survival in Chinese patients with pancreatic cancer. J Transl Med 2013; 11: 249.

[30] Andersen ES, Omland LH, Jepsen P, Krarup H, Christensen PB, Obel N and Weis N. Risk of all-type cancer, hepatocellular carcinoma, non-Hodgkin lymphoma and pancreatic cancer in patients infected with hepatitis B virus. J Viral Hepat 2015; 22: 828-834.

[31] Dumitrascu T, Pineau P, Dima S, Stroescu C, Brasoveanu V, Herlea V, Ionescu M and Popescu I. Impact of Hepatitis B Virus on Clinicopathological Features and Outcomes After Resection for Pancreatic Adenocarcinoma. Anticancer Res 2015; 35: 5123-5128.

[32] Iloeje UH, Yang HI, Jen CL, Su J, Wang LY, You SL, Lu SN and Chen CJ. Risk of pancreatic cancer in chronic hepatitis B virus infection: data from the REVEAL-HBV cohort study. Liver Int 2010; 30: 423-429.

[33] Fan H, Zhang H, Pascuzzi PE and Andrisani O. Hepatitis B virus X protein induces EpCAM expression via active DNA demethylation directed by RelA in complex with EZH2 and TET2. Oncogene 2015; [Epub ahead of print].

[34] Kimura O, Kondo Y, Kogure T, Kakazu E, Ninomiya M, Iwata T, Morosawa T and Shimosegawa T. Expression of EpCAM increases in the hepatitis B related and the treatment-resistant hepatocellular carcinoma. Biomed Res Int 2014; 2014: 172913.

[35] Arzumanyan A, Friedman T, Ng IO, Clayton MM, Lian Z and Feitelson MA. Does the hepatitis B antigen HBx promote the appearance of liver cancer stem cells? Cancer Res 2011; 71: 3701-3708.