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Original Article

Activated leukocyte cell adhesion molecule (ALCAM)/CD166 in pancreatic cancer, a pivotal link to clinical outcome and vascular embolism

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Abstract: Activated leukocyte cell adhesion molecule (ALCAM, or CD166) is a cell adhesion molecule and one of potential tumour metastasis 'soil' receptors that via homotypic and heterotypic interactions, mediates cancer cell adhesion. The present study investigated clinical, pathological and prognostic values of ALCAM in patients with pancreatic cancer. Human pancreatic cancer (PANC-1 and Mia PaCa-2) and human vascular endothelial cell lines were used to construct cell models differentially expressing levels of ALCAM. Tumour-endothelial interaction and tumour migration were assessed by a DiI-based method and electric cell-substrate impedance sensing (ECIS) assay. Pancreatic cancer tissues (n=223), collected immediately after surgery, were analysed for levels of the ALCAM transcripts, which were also analysed against clinical, pathological and clinical outcomes of the patients. ALCAM protein was assessed by immunohistochemistry on a tissue array. Our study demonstrate that pancreatic cancer tissues had significantly higher levels of ALCAM transcripts than normal tissues (P<0.00001). There were no significant differences with staging, differentiation and tumour locations. Tumours from patients who died of pancreatic cancer had significantly high levels of ALCAM compared with those who lived (P=0.018), and this finding was further supported by ROC analysis (P=0.016). Multivariant analysis showed that ALCAM is an independent prognosis factor for overall survival (HR=5.485), with both nodal status and TNM staging contributing to the model (HR=2.578 and 3.02, respectively). A surprising finding was the relationship between ALCAM expression and microvessel embolism of tumour cells (P=0.021, with vs without tumour embolism). Levels of ALCAM were found to be a determinant factor to adherence of the pancreatic cancer cells to vascular endothelial cells, as demonstrated by pancreatic cancer cell models genetically engineered to express differential levels of ALCAM. The tumour-endothelial interaction mediated by ALCAM was readily blocked by addition of soluble ALCAM. Our data supports the conclusion that ALCAM expression is aberrant in pancreatic cancer and its raised expression is an independent prognostic factor for the survival of the patients and the microvascular embolism by cancer cells. Our results suggest that ALCAM plays a key role in mediating tumour-endothelial cell interactions and enhancing tumour embolism in pancreatic cancer, and targeting ALCAM represents a potential therapeutic strategy for treating human pancreatic cancer.

Keywords: Pancreatic cancer, ALCAM, activated leukocyte cell adhesion molecule, CD166, survival, progression, metastasis, embolism, tumour-endothelial interaction

Introduction

Activated leukocyte cell adhesion molecule (ALCAM), also known as CD166 is a transmembrane protein that is widely expressed in the variety of cell (epithelial, endothelial, and majority of cell types) and tissue types, offering a pivotal cell adhesion mechanism [1]. ALCAM confers cell-cell adhesion by interacting with another ALCAM molecule on the opposing cells (homotypic interactions) [2]. It also interacts with other proteins on the opposing cells including CD6 (heterotypical interactions) [3, 4]. Such interactions offer cell-cell adhesion strength in the tissues. Since expressed in multiple cell types, ALCAM is able to mediate cell-cell adhe-

sion both in the same cell, namely epithelial-epithelial or cancer-cancer cell interactions and between different cell types, namely leukocyte-endothelial interactions.

In solid tumours, the expression of ALCAM and the impact of the ALCAM expression pattern in cancer cells have been widely explored [5]. High levels of ALCAM in cancer cells and tissues often lead to poor clinical outcome of the patients and increased tendency of distant metastases. This connection seems to exist in selective tumour types, including cancers derived from squamous cell lineages (namely squamous cell carcinoma) in skin and oesophagus [6, 7] as well as in malignant melanoma [8], gastrointestinal cancers [9-11] and neurological malignancies [12]. It seems that in most endocrine-related cancers, the relationships between ALCAM and the clinical course of the patients are in contrast to other cancers. For example, high levels of ALCAM in breast [13, 14], prostate [15], thyroid [16] and pituitary tumours [5] are linked to favourable outcome of the patients, and less bony metastasis. The reason behind the difference between endocrine-related cancers and other solid cancers remains unknown.

Given that both cancer cells and endothelial cells are amongst cell types that express high levels of ALCAM, the potential role of ALCAM in cancer-endothelial cells is recognised and hypothesized to be important for the vascular spread of cancer cells. Traditionally, it was thought that upon entering the vascular stream, cancer cells would utilise surface molecules and adopt the 'docking, locking and invading' steps to develop a vascular invasion through to the tissues. In this extravasation process, cancer cells use the abundant carbohydrate branches to interact with the same molecules on vascular endothelial cells and rapidly form the weak cancer-endothelial interaction, a process to slow the travelling of cancer cells in the blood stream, and hence cancer cells would dock over the endothelial surface in a weak matter. The docking gives rise to opportunities for cancer cells to later firmly lock onto the endothelial cells by protein-mediated cell adhesion mechanism, which happens slower, but stronger. The protein-protein interactions between cancer cells and endothelium pave the way for cancer to penetrate the endothelium and initiate the extravasation process. The protein-mediated cancer-endothelial interactions are regulated by cell adhesion molecules including homotypic and heterotypic cell adhesion interactions. One such adhesion molecules is ALCAM. Whilst it has been recognised that ALCAM-mediated cancer-endothelial adhesion is important, it has rarely been demonstrated in a clinical setting.

Pancreatic cancer is one of the most aggressive solid cancers with long term survival amongst the poorest in all the cancer types. Whilst the anatomical location of pancreas is hidden and difficult to reach, the lack of specific symptoms often leads to delays in patients seeking medical attention and hence diagnosis at a late stage. Even with the improved imaging technologies, early diagnosis still remains a challenge. Lack of effective therapies is another contributing factor to the poor prognosis of pancreatic cancer. The pancreas resides in a location that is rich in both lymphatic and vascular circulation and the organ itself is highly rich in blood supply owing to its highly active exocrine and endocrine activities. One consequence of such histological and anatomical structures is its early and ready status of metastasis, locally, regionally and to distant locations.

Studies on the role of ALCAM in human pancreatic cancer are limited and tend to be small in cohort size. An early study by Kahlert et al. [17] using a single method, namely immunohistochemistry on 97 pancreatic tumour patients reported the ALCAM staining pattern difference between normal and tumour cells, in which normal pancreatic tissues had membranous staining pattern while tumour tissues had mainly cytoplasmic. The study showed that strong ALCAM staining, as well as the cellular location of the staining, had significant links to recurrence free survival and overall survival. However, it appears that only the level of staining in cancer cells is an independent prognostic factor for overall survival in multivariate analysis [17]. In another cohort of 20 patients with metastatic or locally advanced pancreatic adenocarcinoma, it was reported that circulating cancer cells had higher levels of ALCAM message, along with a few other markers, than tumour cells at the primary site and that when circulating cancer cells had high levels of

ALCAM, patients tended to have significantly shorter survival [18]. In a sharp contrast to that in pancreatic adenocarcinoma, ALCAM in endocrine tumours from the pancreas, namely pancreatic neuroendocrine tumours (PNET), were found to have mainly cytoplasmic staining and a favourable prognostic factor for both recurrence free survival and disease specific survival (n=38) [20]. In a larger cohort of 264 tissues and 115 sera of patients with pancreatic adenocarcinoma, Tachezy et al. [21] did not find any survival correlation between tissue ALCAM staining and the survival of the patients, although the levels of circulating ALCAM had bearing with the clinical outcomes, arguing for further investigations. The impact of ALCAM on the adhesion and migration of pancreatic cancer cells have also been reported [19, 22].

In the present study, we have explored the expression pattern of ALCAM in pancreatic adenocarcinoma and the link with the clinical outcome. One of our striking findings was that the levels of ALCAM in pancreatic cancer are linked with the presence of vessel embolism, and that vessel embolism and ALCAM are clearly linked with the survival of the patients. We went on to demonstrate, using genetically constructed cell models, that expression of ALCAM in both pancreatic cancer cells and endothelial cells are a key determination factor for cancer-endothelial cell adhesion. Our study further showed that the ALCAM-ALCAM interaction-mediated cancer-endothelial cell adhesion can be controlled by soluble ALCAM, arguing a potential value of targeting ALCAM in this cancer type as a strategy for therapeutic intervention.

Materials and methods

Cell lines

Human pancreatic cancer cell lines PANC-1 and Mia PaCa-2 were purchased from ECACC and Human vascular endothelial cells HECV was purchased from Interlab, Naples, Italy.

Key materials

Recombinant human ALCAM-Fc chimera, containing ALCAM Trp28-Ala526 and the human IgG Fc region was purchased from R&D systems (Abingdon, UK). A monoclonal antibody to human ALCAM was purchased from Novacastra, Milton Keynes, UK.

Anti-ALCAM shRNA and ALCAM expression constructs

To modify the expression of ALCAM in target cell lines multiple plasmid systems were designed and purchased from VectorBuilder (Chicago, USA). A number of shRNAs targeting ALCAM and control scramble sequences were designed in order to affect ALCAM expression. Additionally, the ALCAM expression sequence or stuffer control sequence was used for ALCAM over-expression models. Plasmids were transfected into cell lines using Fugene HD (Promega, Southampton, UK).

Establishing pancreatic cancer and endothelial cell models with differential expression of ALCAM

The anti-ALCAM shRNA plasmids were used to transfect PANC-1 and HECV cells and, through a selection process, we established ALCAM knockdown PANC-1 (designated as $PANC1^{ALCAMkd}$) and HECV (designated $HECV^{ALCAMkd}$) submodels together with the respective scramble controls. Mia PaCa-2 which expressed low levels of ALCAM was transfected with ALCAM expression construct, resulting in the establishment of the ALCAM over-expression submodel, $MiaPaCa2^{ALCAMexp}$. These models were then used for the *in vitro* investigations.

Tumour-endothelial interaction assay

Tumour-endothelial interaction was assessed using a method we previously described [23]. Briefly, pancreatic cancer cells, control and transfected were cultured to subconfluence. On collection of the cell suspension, they were stained with 5 μM DiI (1,1'-Dioctadecyl-3,3,3',3'Tetramethylindocarbocyanine Perchlorate) for 30 minutes. After extensive washing to remove the free dyes, fixed number of cells were added to an endothelial cell monolayer (control or ALCAM knockdown), precoated on the floor of the 96-well plates. After 20 minutes, the culture wells were carefully washed with PBS to remove the non-adherent cancer cells. The remaining cells that adhered to the endothelial cell monolayer were fixed with 4% formalin. Representative bright field and fluorescence images were captured on a Leica fluorescent inverted microscope (Leica Microsystems Ltd., Milton Keynes, UK) at ×20 objec-

tive magnification, and the images of merged and attached cancer cells were quantified.

Cell adhesion and migration assay by ECIS

ECIS assay was applied to investigate cellular behaviour based on the impedance parameter detected from gold electrodes coated on the bottom of a 96-well array (Applied Biophysics Inc., NJ, USA). The assay was modified from the previously descried method [24, 25]. In brief, prior to cell seeding, ECIS arrays containing growth medium were stabilised using the stabilisation function within the system and washed. Cells were seeded at an appropriate density before the 96-well array was equipped in the incubated array station and changes in resistance/impedance measured over the course of the experiment. The first 4 hours of data was analysed for initial attachment and spreading. Once the resistance curve reached plateau electrical wounding was applied for 20 seconds at 2000 μA and 60,000 Hz, to generate a wound in the monolayer before recording the change in resistance following recovery of the monolayer to analyse cellular migration over a four-hour period. The second ECIS assay used was the automated tracking of cancer-endothelial interactions. Here, HECV cells, control or $HECV^{ALCAMkd}$ were plated in the ECIS array and allowed to reach confluence. Pancreatic cancer cells were added to the endothelium and the interaction was monitored immediately after adding at a frequency of 4,000 Hz.

Pancreatic cancer tissue arrays (TMA) and staining of ALCAM by immunohistochemical assay

A pancreatic tissue array (No. PA2081c) was obtained from Biomax Inc. Rockville, MD, USA. Following antigen retrieval, the TMA was blocked for 2 hours with 10% horse serum before being incubated with ALCAM primary antibody (2 μg/ml). We used a universal secondary antibody and biotin tertiary reagents to bind to the ALCAM antibody and to conjugate peroxidase (Vectastain Elite Universal ABC kit, Vector Laboratories ltd., Peterborough, UK). DAB (diaminobenzidine, 5 mg/ml; Sigma-Aldrich, Dorset, UK) was used to develop colour and the TMA was counterstained with Gill's hematoxylin (Vector Laboratories ltd., Peterborough, UK). The staining was assessed by three independent assessors and scored as no (0), weak (1), moderate (2), or strong (3) staining, based on method previously reported [26, 27].

Fresh pancreatic cancer and normal tissue collation and clinical information

Pancreatic cancer tissues, together with normal unaffected normal tissues were collected immediately after surgery at Peking University Cancer Hospital and Institute. Tissues obtained from surgery theatre were immediately stored in liquid nitrogen until use. Ethical approval was granted by the Ethics Research Committee of Peking University Cancer Hospital and is fully in accordance with the Helsinki declarations. Consent was obtained from the patients. In total, 223 patients were recruited to the study. Patients were followed in the clinics and the current study has a median follow-up period of 12 months. Clinical, pathological information and follow-up information were collected retrospectively.

Extraction of RNA from tissues and quantitative analysis of ALCAM gene transcript

Tumour and normal tissues were retrieved from liquid nitrogen storage vessels and subsequently homogenised in an RNA isolation buffer. Total RNA was extracted using an ether-based method. After extensive washing, total RNA was purified and quantified by a UV spectrophotometer and standardised to the same concentration. Reverse transcription was carried out using a reverse transcription kit (Promega, Southampton, UK). Quantitative analysis of ALCAM gene transcript was performed on a StepOne Plus thermocycler (Fisher Scientific, UK). The primers used in the study are listed in **Table 1**. The chemistry employed here was the FAM tagged Uniprimer™ and worked with the reverse primer via a unique sequence (z-sequence, underlined in **Table 1**). GAPDH was used as a house keeping control.

Statistical analysis

Pairwise comparisons were made by Mann-Whitney U test and Kruskal-Wallis H test. RUC model by way of the Receiver Operating Characteristic method was used to categorise patients into groups with differential expression in accordance with clinical outcome and presence of tumour emboli. Survival analysis was carried out with the Kaplan Meier's method and Cox regression model. Multiple variate

Table 1. Primers

Gene name	Forward primer	Reverse primer
ALCAM (PCR)	TTATCATACCTTGCCGACTT	GGGTGGAAGTCATGGTATAG
ALCAM (qPCR)	CAGGAGGTTGAAGGACTAAA	ACTGAACCTGACCGTACAGGGATCAGTTTTCTTTGTCA
GAPDH (PCR)	GGCTGCTTTTAACTCTGGTA	GACTGTGGTCATGAGTCCTT
GAPDH (qPCR)	AAGGTCATCCATGACAACTT	ACTGAACCTGACCGTACAGCCATCCACAGTCTTCTG

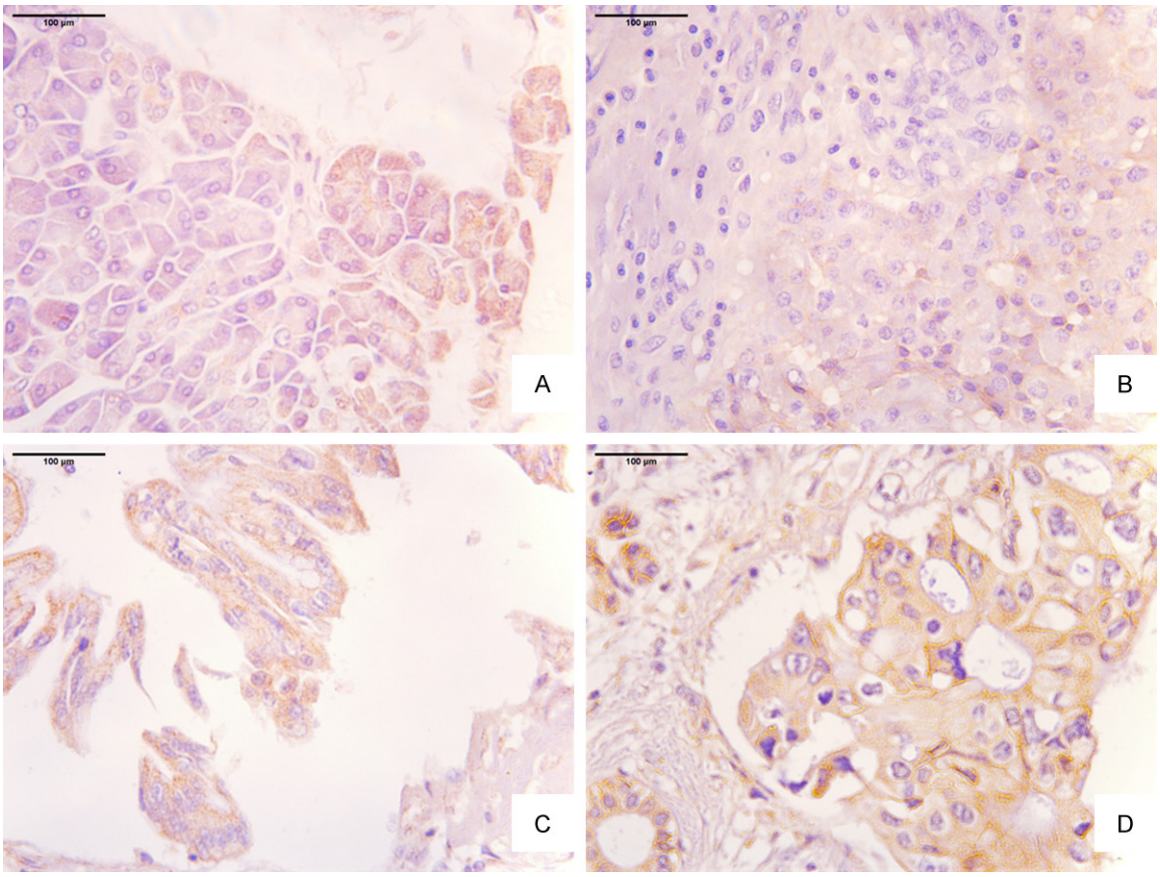

Figure 1. ALCAM staining in normal and tumour pancreatic tissues. Shown are representative staining from normal pancreatic tissues (A), Islet cell tumour (B) and pancreatic ductal adenocarcinoma (C and D). The scale bar represent 100 μm.

analysis and logistic regression were used. All the analyses were carried out using SPSS version 26.

Results

Levels of ALCAM expression in normal pancreatic tissues and pancreatic cancer tissues determined by immunohistochemistry (IHC)

Pancreatic tissues, normal and tumour, all stained positive for ALCAM (**Figure 1** and **Table 2**). The staining was of membranous and cytoplasmic nature. Most of the tissues had both cytoplasmic and membrane ALCAM staining except for several cases in which only cytoplasmic staining could be observed. The intensity of ALCAM staining was different among different pathology types of tissues ($P<0.001$). Therein, adjacent normal pancreas tissue ($P=0.028$) and Islet cell tumour ($P=0.017$) had significantly lower levels of ALCAM staining compared to duct adenocarcinoma, whilst metastatic tumours ($P=0.732$) and chronic pancreatitis ($P=0.126$) showed no statistical difference compared to duct adenocarcinoma. There was no difference in ALCAM staining among different differentiation grade ($P=0.408$), TNM staging ($P=0.241$), tumour staging

Table 2. ALCAM staining score in pancreatic tissues

Group	N	Distribution			Intensity			Statistical significance	
		Both	Cytoplasm	Membrane	1	2	3	Chi value	P
Pathology									
Duct adenocarcinoma	54	53	1	0	6	37	11	42.492	<0.001[a]
Adjacent normal pancreas tissue	48	37	11	0	13	32	3	7.185	0.028[b]
Metastatic	10	10	0	0	2	6	2	0.625	0.732[b]
Islet cell tumour	20	20	0	0	8	10	2	8.120	0.017[b]
Chronic pancreatitis	12	10	2	0	4	7	1	4.135	0.126[b]
Differentiation Grade									
1	11	11	0	0	1	7	3		
2	19	19	0	0	0	13	6	1.794	0.408[c]
3	2	2	0	0	2	0	0	NA	NA
TNM stage									
I	22	22	0	0	1	14	7		
II	30	29	1	0	3	23	4	2.844	0.241[d]
III	2	2	0	0	2	0	0	NA	NA
Tumour stage									
T2	28	28	0	0	1	18	9		
T3	24	23	1	0	3	19	2	5.205	0.074[e]
T4	2	2	0	0	2	0	0	NA	NA
Node metastasis									
N0	42	42	0	0	4	30	8	0.825	0.662
N1	12	11	1	0	2	7	3		

[a]Overall chi-square test among all pathology types; [b]Compared with Duct adenocarcinoma; [c]Compared with differentiation Grade 1; [d]Compared with TNM stage I; [e]Compared with Tumour stage T2.

(P=0.074) and node metastasis (P=0.662). In general, despite the small sample size, tumour tissues stained stronger than normal tissues and benign tumour tissues.

Expression of ALCAM gene transcript in pancreatic cancer

Pancreatic cancer tissues had markedly high levels of ALCAM transcript compared with normal tissues (P<0.000001) (**Figure 2A** and **Table 3**). Whilst the difference between different groups of histological types, differentiation, tumour staging, TNM staging, and nodal metastasis did not reach significant difference (**Table 3**), tumour from patients who died of pancreatic cancer during the follow-up period had significantly higher levels of ALCAM than tumours from those who remained alive (P=0.018) (**Figure 2B** and **Table 3**).

Levels of ALCAM transcript and clinical outcomes

Using the RUC model, it was demonstrated that levels of ALCAM have significant power in predicting the mortality of the patients (RUC value 0.614, P=0.016) and that high levels of ALCAM indicate high probability of pancreatic cancer related death. Based on the cut-off value from the RUC model, we divided patients into two groups, with high level expression and low levels of expression, respectively. Using the Kaplan-Meier model, it was found that patients with high levels of ALCAM had a significantly shorter survival, compared with those of low ALCAM levels with median survival time being 19.7 vs 24.7, respectively (P=0.041) (**Figure 3**). Although in univariate analysis, ALCAM expression level and nodal status are significant factors for the clinical outcome, only ALCAM was found to be an independent prognostic factor for the mortality of the patients by using univariate analysis (P=0.005, HR=3.000) and multivariate analysis (P=0.023, HR=5.485) (**Table 4**).

We have also explored the TCGA dataset (RNAseq) which has a smaller number of patients than the present study [28, 29]. As shown in Figures S1 and S2, the links between

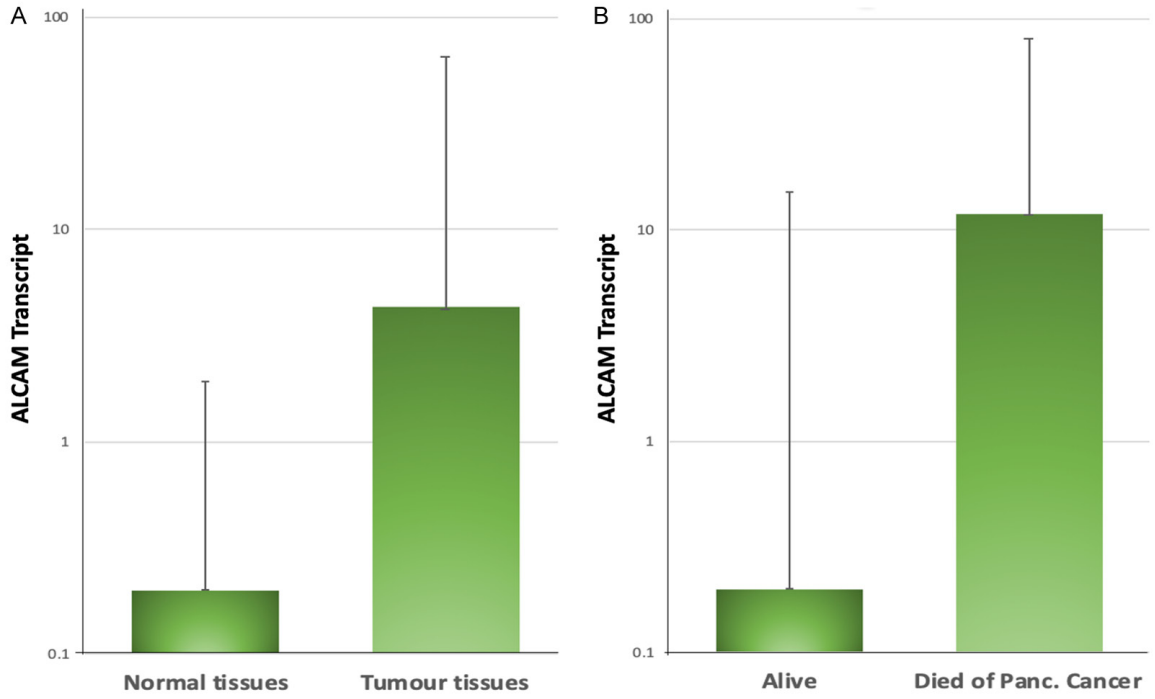

Figure 2. A: Levels of expression of ALCAM transcript in normal and pancreatic cancer tissues. B: Expression of ALCAM in those who died and remain alive. Statistical method was Mann-Whitney U test. Shown are median and IQR.

ALCAM gene transcript and patients' survival, OS (overall survival) and RFS (relapse free survival), are not significant. However, it is noteworthy that high levels of ALCAM were seen to be associated with a shorter RFS survival but longer OS survival, although neither was statistically significant.

ALCAM expression and link to vascular embolism

Our data has further revealed that tumours with cancer emboli in microvessel had significantly higher levels of ALCAM compared those without (P=0.021) (**Figure 4A**). It was further revealed that the presence of microvessel emboli had a significant impact on the overall survival of the patients (**Figure 4B**). RUC model analysis showed that the levels of ALCAM had a significant value in predicting the presence of embolism (RUC value 0.613, P=0.013).

Creation of cell models from pancreatic cancer cells and vascular endothelial cells that differentially express ALCAM and the impact on pancreatic cancer cells

With the findings that both ALCAM and tumour embolism were significantly linked to clinical outcome and that levels of ALCAM had significant connection with the presence of embolism, we sought to explore if levels of ALCAM in pancreatic cancer cells and in vascular endothelial cells may contribute to the tumour-endothelial interactions, a significant factor leading to formation of tumour embolism. PANC-1 cancer cells had positive expression of ALCAM, in contrast to Mia PaCa-2 which was weakly positive for ALCAM. Using anti-ALCAM shRNA, we created PANC-1 knockdown submodel, designated here as $PANC1^{ALCAMkd}$, together with a transfection control (designated as $PANC1^{control}$) (**Figure 5**). Similarly, we created an ALCAM over-expression subline from Mia PaCa-2 cell line with a respectively control that are designated as $MiaPaCa\text{-}2^{ALCAMexp}$ and $MiaPaCa\text{-}2^{control}$.

In vitro, it was shown that levels of ALCAM expression in these submodels did not influence the rate of cell growth. However, using an automated cell analyser, it was clearly demonstrated that loss of ALCAM in $PANC1^{ALCAMkd}$, resulted in significant reduction in both cell adhesion (left) and migration (right) (**Figure 6**, top). In a less striking contrast to PANC-1 cells, over expression of ALCAM in Mia PaCa-2 cells resulted in cells being more adhesive (left) and less mobile (right), although the difference is not highly significant (**Figure 6**, bottom).

Table 3. Expression level of ALCAM transcript and the clinical and pathological groupings

Group	Median	Q1	Q3	P[a]
Tissue types				<0.001
Tumour	68.2	0.1	60.2	
Normal	15.5	0	1.7	
Sex				0.452
Male	2.43	0.09	76.3	
Female	7.8	0	56	
Differentiation				0.312
High	0	0	162.9	
High-Medium	16.5	0.1	165	
Medium	0.76	0.07	53.64	
Medium-Low	6	0.09	52.373	
Low	11	2	34	
Anatomical site				0.427
Head	3.3	0.1	50.97	
Body	24.4	0	76.4	
Body/Tail	13.2	0	87.57	
Tail	19.3	11.4	113.3	
Other locations	108.3	21.2	176.3	
Tumour staging				0.315
T1	18.1	8.3	89.2	
T2	7.6	0	108.1	
T3	0.3	0	28.6	
T4	14.3	0.1	84.1	
Lymph nodes				0.73
Negative	15.85	0.08	85.5	
Positive	18.1	8.3	89.2	
Metastatic at diagnosis				0.996
No	3.5	0.1	61.51	
Yes	6	0.12	16.62	
TNM stage				0.924
1	13	0	135	
2	0.4	0.1	50.33	
3	11.6	0.1	84.1	
4	10.9	0.1	33.5	
Combined				0.473
TNM1-2	0.4	0.1	52.7	
TNM3-4	11.6	0.1	68.8	
Clinical Outcome				0.018
Alive	0.2	0	14.9	
Died	11.9	0.1	67.9	

[a]by Mann-Whitney U test.

ALCAM expression in endothelial cells and in pancreatic cancer cells, the impact on tumour-endothelial interactions

We conducted a direct tumour-endothelial interaction assay to determine how expression of ALCAM in vascular endothelial cells influences the interaction and adhesiveness between the two cell populations. As shown in **Figure 7**, knockdown of ALCAM from PANC-1 cells significantly reduced the adhesion of cancer cells to endothelial cells. In contrast, over-expression of ALCAM in Mia PaCa-2 cells significantly increased the adhesiveness between the two cell types. It was also demonstrated that soluble ALCAM (sALCAM) at a higher concentration blocked this interaction to a significant degree.

The tumour-endothelial interactions affected by ALCAM expression was similarly reproduced in the ECIS based cell-cell interaction assay in that loss of ALCAM in endothelial cells resulted in a rapid rise of capacitance when the PANC-1 ALCAM knock-down cancer cells were added. It is noteworthy that when both PANC-1 and HECV cells were knocked down for ALCAM, the capacitance reached the maximum (**Figure 8**). To confirm the reproducibility of the knockdown, we tested two separate shRNAs, which showed a reproducible outcome.

Discussion

The present study reports that in a large pancreatic cancer cohort, the expression of ALCAM, particularly at the transcript levels, was highly aberrant in tumour tissues and is a strong prognostic factor. This is linked to the tumour embolism in pancreatic tumours, attributable to the expression of ALCAM in pancreatic cancers, as demonstrated by *in vitro* cell models.

ALCAM and clinical outcome of pancreatic cancer

ALCAM transcript levels were increased in pancreatic tumours compared with normal pancreas tissues, and this increase in tumour tissues was particularly high in tumours from patients who died from pancreatic cancer. Collectively, raised levels of ALCAM transcript presented an independent prognostic factor for overall survival of the patients. This finding is important as it is the first large cohort investigation using the quantitative approach to assess the ALCAM transcript and on its link to the clinical and pathological factors of the tumour type. This finding is in line with some of the earlier studies

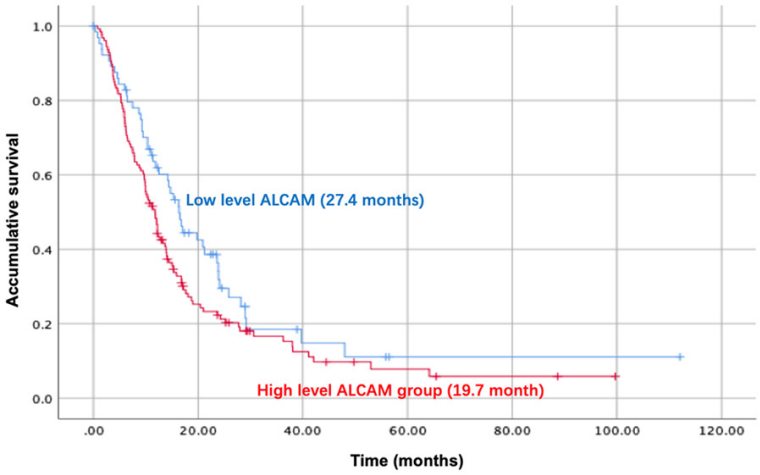

Figure 3. ALCAM levels and overall survival of the patients by Kaplan-Meier survival analysis. Patients with high levels of ALCAM transcript had significantly shorter survival (P=0.041).

Table 4. ALCAM expression, clinical and pathological factors in relationship with the survival of the patients

Factor	Univariate analysis		Multivariate analysis[a]	
	P value	HR	*P* value	HR[b]
ALCAM expression	0.005	3.000	0.023	5.485
Gender	0.268	1.456	0.915	1.075
Age	0.264	1.016	0.397	1.025
Tumour differentiation	0.138	1.293	0.930	1.029
Location of tumours	0.906	1.024	0.875	0.940
Local invasion	0.855	0.946	0.713	0.666
Nodal involvement	0.005	2.750	0.187	2.578
TNM staging	0.147	1.509	0.441	3.020

[a]Cox Regression model against pancreatic related death; [b]Hazard Ratio.

showing high levels of ALCAM protein staining in pancreatic adenocarcinoma are associated with a poor survival [17].

The present study of immunohistochemical staining analysis of the pancreatic tissue array did not show a marked difference amongst the clinical and pathological factors. This is in line with an early study on pancreatic cancer tissues using a similar immunohistochemical method [21]. The reason for the discrepancies between different studies with the same methods, namely immunohistochemistry, and between different methods, namely transcript analysis vs protein staining, are not entirely clear but could be due to the following possibilities. Firstly, the presence of an antagonistic mechanism to ALCAM could make assessment of ALCAM protein alone insufficient. This is likely to relate to the presence of circulating soluble ALCAM, a truncated form of ALCAM shed from the cell surface. Indeed, the study by Tachezy et al. [21] that measured both cellular and circulating ALCAM did not find a prognostic value of either in pancreatic cancer, suggesting that a more viable approach is required to assess this protein. Secondly, the discordance between levels of mRNA and protein is another reason for potential discrepancy. Although mRNA and translation into protein are highly connected, the fate of protein post-translation is however highly variable. For the same reason as stated earlier, a mature and functional ALCAM may be cleaved, shed from cell surface and may become an antagonist. Thus, to evaluate a total level of a molecule, mRNA in this case may be a more suitable option.

Pancreatic adenocarcinoma versus other tumour types

The relationship between ALCAM and clinical outcome in pancreatic adenocarcinoma reported here are in line with reports of smaller cohorts in the past [17, 18], but clearly in contrast to those reported on PNET in that high levels of ALCAM indicated a favourable clinical outcome [20]. The observation seen with PNET is not alone and has been reported in breast, prostate and thyroid cancers in which patients with high tumour levels of ALCAM had good clinical outcome. It is noted that a possible reason is the endocrine nature of the tumour types. Tachezy et al. [20] reported that PNET tumour with hormone production tended to have stronger ALCAM staining (100%) compared with non-hormone production tumours (63% strong and 37% weak) (P=0.037).

In a context of pancreatic cancer, it has been reported that ALCAM is involved in the pancre-

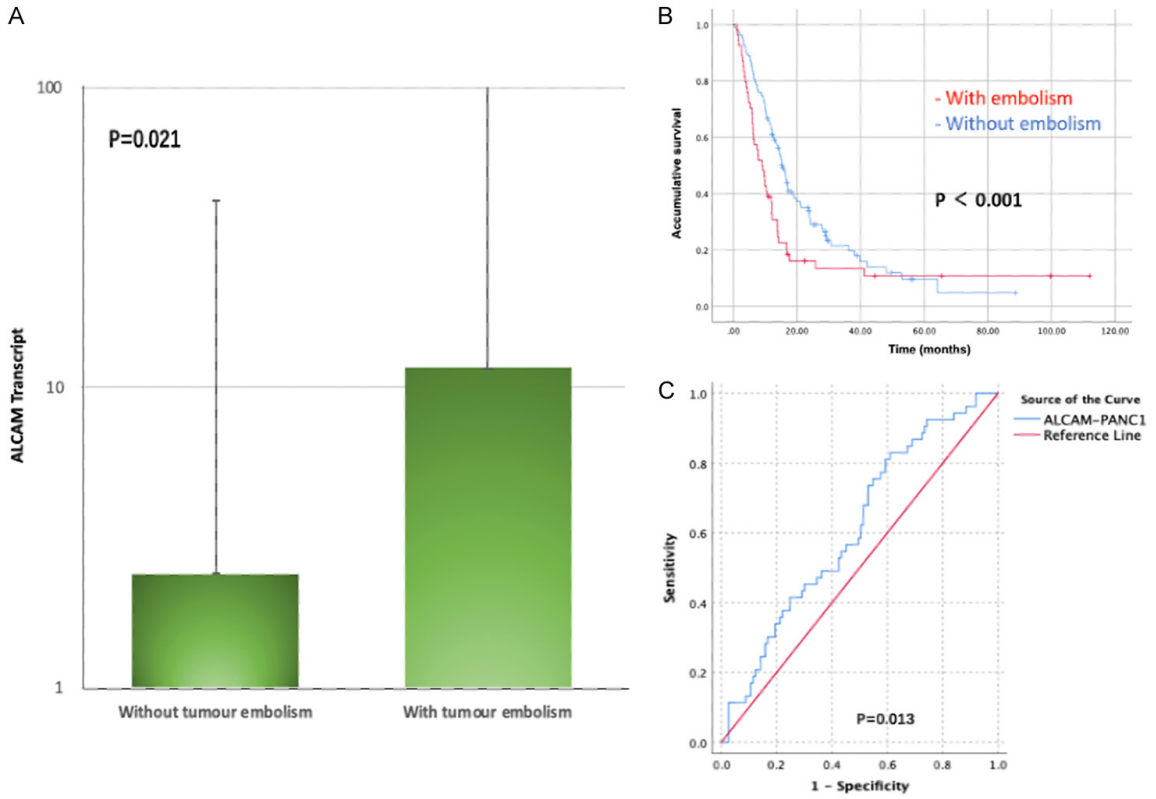

Figure 4. A: Levels of ALCAM in tumour without and with microvessel emboli. P=0.021 by Mann-Whitney U test. B: Relationship between the presence of microvessel embolism and survival of the patients. C: Levels of ALCAM had a significant value in predicting tumour microvessel embolism (RUC value 0.613, P=0.013).

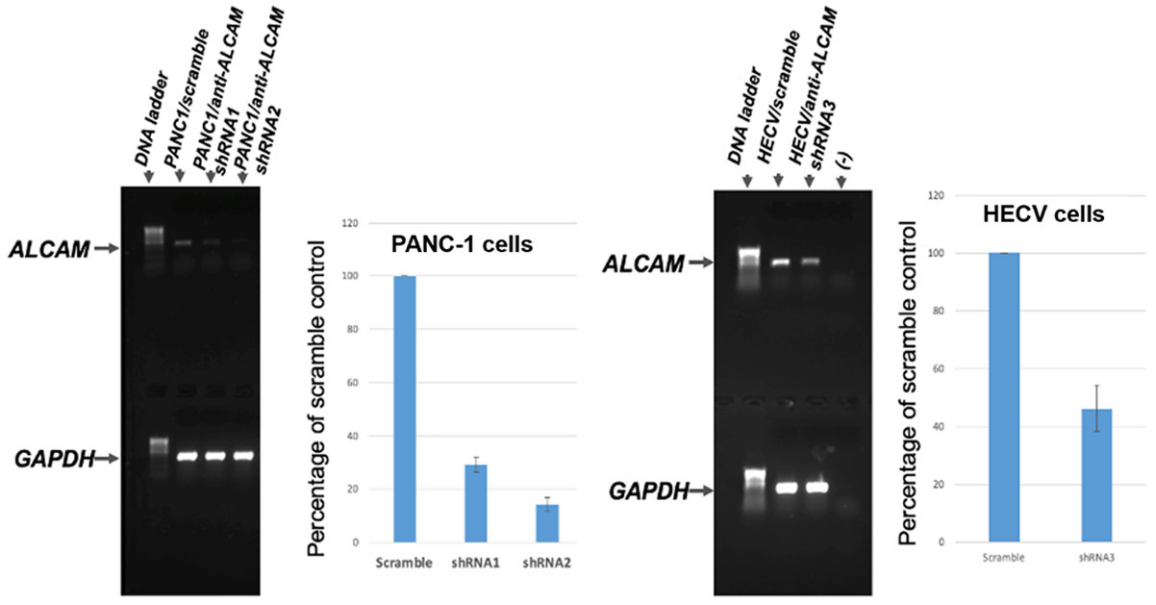

Figure 5. Creation of pancreatic and endothelial cell models. Expression of ALCAM in the cells were verified by PCR.

atic interaction with the stellate cells of the pancreas and knocking down ALCAM in pancreatic cancer cells reduces this contact [30]. Pancreatic stellate cells are important stromal

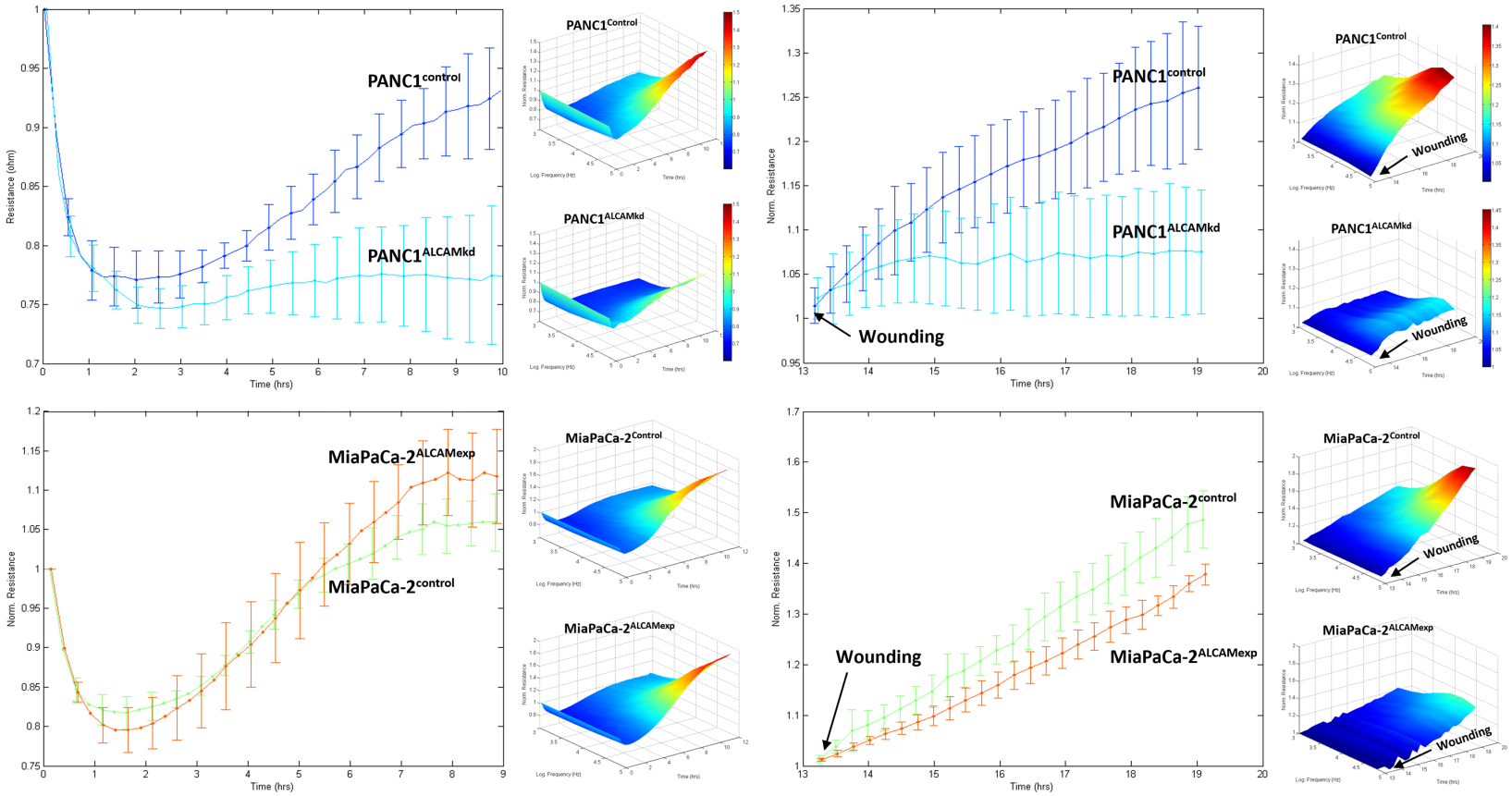

Figure 6. ALCAM differential expression, cell adhesiveness and migration as detected by ECIS. Top panel: PANC1 cells; Bottom Panel: Mia PaCa-2 cells. Left: cell adhesiveness; Right: Wounding assay for cellular migration.

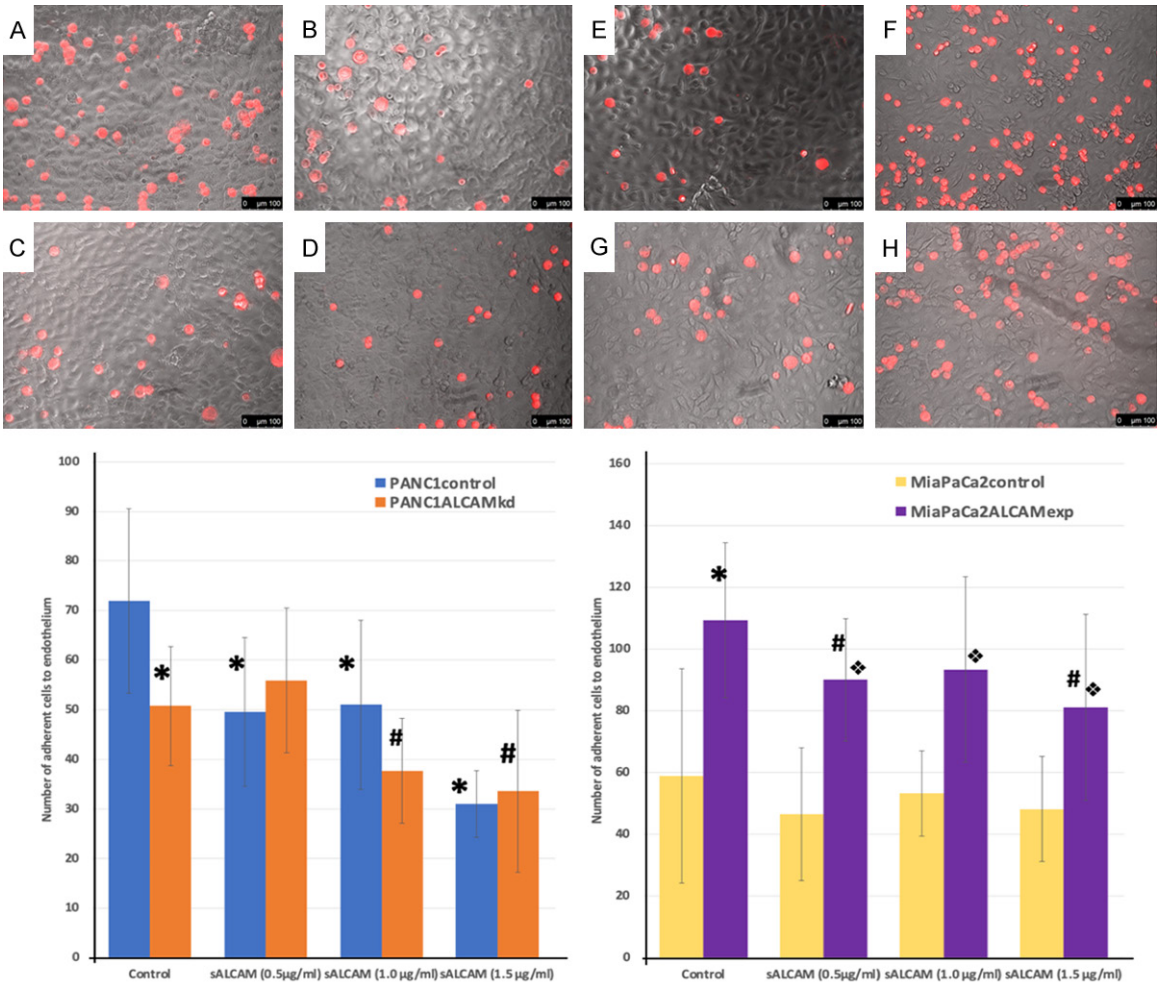

Figure 7. Top panel: images showing adherence of PANC1 (A-D) and Mia PaCa-2 (E-H) cells to endothelial cells. (A/C and E/G) are the respective control cells. (B/D) are ALCAM knockdown cells and (F/H) are ALCAM over-expression cells. Top rows (A/B/E/F) are control treatment; Bottom rows (C/D/G/H) are cells treated with 1.0 µg/ml sALCAM. Red coloured cells are the respective cancer cells labelled with DiI. The background cells are endothelial cells. Bottom: Graphical representation of pancreatic cancer cells adherence to endothelial cells. Left: * vs $PANC1^{control}$ without treatment; # vs $PANC1^{ALCAMkd}$ without treatment. Right: * vs $MiaPaCa2^{control}$ without treatment; # vs $MiaPaCa2^{ALCAMexp}$ without treatment; ❖ vs $MiaPaCa2^{control}$ with similar sALCAM concentration.

and periductal cells that can facilitate fibrosis and some aspect of tumour progression. Although brief, the finding does indicate that ALCAM is important for the communication between pancreatic cancer cells and other cells such as stellate and mutually influence each other to facilitate the progression of pancreatic cancer.

ALCAM and tumour-endothelial interactions and the clinical value of tumour microvessel embolism

ALCAM is known to be expressed in both lymphatic and vascular endothelial cells [31] and is a key to the tubule formation and the integrity of endothelium by, in this case, ALCAM-mediated endothelial-endothelial interactions. The present study clearly demonstrated that under a static culture condition, ALCAM is an important factor for the cancer-endothelial interaction, an important part in the locking stage of tumour circulation and extravasation. It has been established that ALCAM on breast cancer cells is also able to interact with Galectin-8 (Gal-8) of endothelial cells [32]. Thus, ALCAM on pancreatic cancers is likely to adopt multiple forms of interaction with its partners on endothelial cells, including homotypic (ALCAM-ALCAM) and heterotypic (ALCAM-galectin,

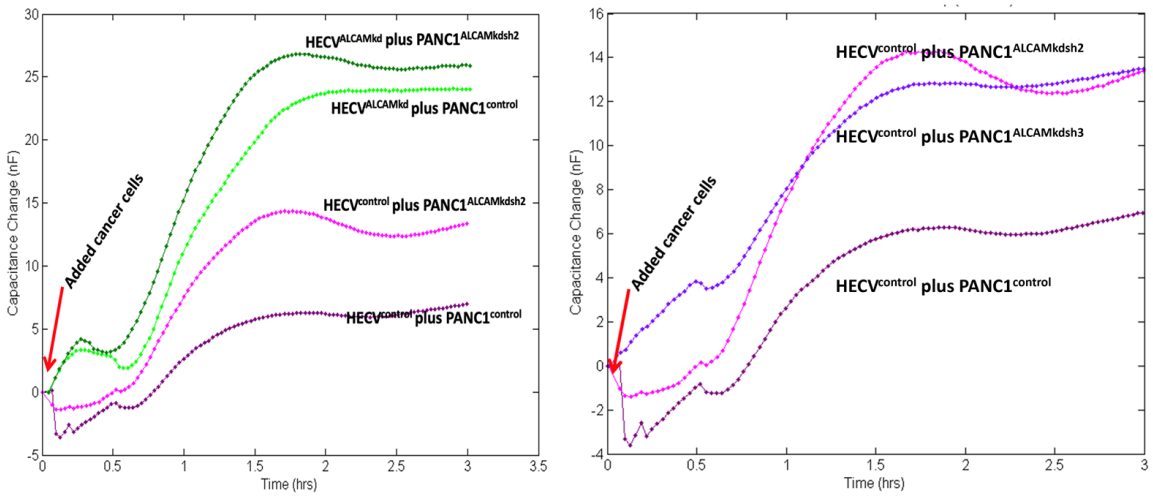

Figure 8. ECIS based analyses of tumour-endothelial cell interaction. Showing are two ALCAM knockdown cell models over endothelial cells which also had ALCAM expression modified.

ALCAM-CD6) interactions to facilitate the aggregation and adhesion of cancer cells on endothelium. It is interesting to note a similar report in non-small cell lung cancers (NSCLC) showing that ALCAM on lung cancer cells facilitates the adhesion of cancers with cerebral endothelial cells and subsequent formation of brain metastases [33]. This role of ALCAM during tumour-endothelial interaction and during cancer cell extravasation bears resemblance of its role in monocyte extravasation [34]. These known functions between ALCAM, cancer cells and endothelial cells have important bearing to the findings of the present study. We found that loss of ALCAM in pancreatic cancer cells and in endothelial cells substantially weakened the adhesiveness between pancreatic cancer cells and vascular endothelial cells. This would render pancreatic cancer cells less likely to settle on the endothelium to complete an essential step of metastasis, to cluster and extravasate, hence hindering the probability of vascular spread. This finding also supports well the clinical findings that the presence of cancer embolism is highly correlated with the level of ALCAM in pancreatic cancer and that the presence of tumour embolism is linked well with the poor clinical outcome. Collectively, this study has shown that levels of ALCAM in pancreatic cancer cells are closely linked with tumour-endothelial adhesion, and arguably subsequent clustering of cancer cells over the endothelium. This would support a well-established hypothesis that clustering of cancer cells over the vascular endothelium bed results in the slowdown of flow, locking of cancer cells over endothelium and subsequent extravasation of cancer cells. One must also bear in mind that ALCAM is only one of the many proteins involved in this process. Other proteins include Galectins, CD6, and CD44, etc, should also be considered. The present study provides sufficient evidence that ALCAM is amongst the most important ones in this context in pancreatic adenocarcinoma.

Soluble ALCAM and potential therapeutic value

Soluble ALCAM or sALCAM, a truncated form of ALCAM as the result of enzymatic cleavage of mature ALCAM, is antagonistic to mature ALCAM by competing for the homotypic binding. sALCAM as a potential therapeutic target for cancer intervention has been suggested [35]. The present study has demonstrated that sALCAM can indeed block ALCAM-mediated tumour-endothelial interactions and pancreatic cancer adhesiveness to matrix, two important factors contributing to the metastatic process. Limited by the availability of serum samples from the patients in the present study, we were unable to assess the levels of the circulating soluble ALCAM in the same patients. However, in a previous study, patients with high levels of soluble ALCAM, as opposed to tissue levels of ALCAM, had a significant link to the poor clinical outcome of the patients [18]. Similar relationships have been reported in other cancer types including oesophageal cancer [7], prostate cancer [26], breast cancer [36], to name a few (for a comprehensive summary, please refer to a recent review [5]). Together, with previous

reports on soluble ALCAM in antagonizing the functions of ALCAM, it would be plausible to suggest that sALCAM has an important therapeutic role in this particular cancer type and is worthy of further exploration.

As an important member of cell adhesion molecules, ALCAM has been shown to participate in the progression of various tumour types, including pancreatic cancer, as reported here. The impact of ALCAM on tumour-endothelial cells and on the presence of tumour embolism is of particular interest. Together with the findings of potential role of sALCAM in this context argues a therapeutic implication. It is clear that there is a need to further explore how the adhesion mediated tumour-endothelial and indeed tumour-tumour interaction are governed outside and inside the cells, the latter most probably requires the understanding of the signalling events triggered by the interaction. To our knowledge, a series of vital signalling molecules and pathways are involved in the regulation of ALCAM, including the primary promoter element of ALCAM gene transcription nuclear factor-kappa B (NF-κB) and Sp1, actin cytoskeleton and binding partner CD9 which both have fixation effect on membrane ALCAM, and proteolysis ADAM17 and MMP14 which can regulate the shed of ALCAM extracellular domain, etc [1]. They are clearly important and a fertile area to explore in the future and are indeed ongoing research in our institute.

In conclusion, the present study has shown that levels of ALCAM transcript in human pancreatic adenocarcinoma have a significant prognostic value and are also an important predictor for the development of tumour microvessel embolism, which is likely to be mediated by highly levels of ALCAM protein in pancreatic cancer cells and endothelial cells.

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Disclosure of conflict of interest

None.

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References

[1] von Lersner A, Droesen L and Zijlstra A. Modulation of cell adhesion and migration through regulation of the immunoglobulin superfamily member ALCAM/CD166. Clin Exp Metastasis 2019; 36: 87-95.

[2] van Kempen LC, Nelissen JM, Degen WG, Torensma R, Weidle UH, Bloemers HP, Figdor CG and Swart GW. Molecular basis for the homophilic activated leukocyte cell adhesion molecule (ALCAM)-ALCAM interaction. J Biol Chem 2001; 276: 25783-25790.

[3] Bowen MA, Bajorath J, D'Egidio M, Whitney GS, Palmer D, Kobarg J, Starling GC, Siadak AW and Aruffo A. Characterization of mouse ALCAM (CD166): the CD6-binding domain is conserved in different homologs and mediates cross-species binding. Eur J Immunol 1997; 27: 1469-1478.

[4] Starling GC, Whitney GS, Siadak AW, Llewellyn MB, Bowen MA, Farr AG and Aruffo AA. Characterization of mouse CD6 with novel monoclonal antibodies which enhance the allogeneic mixed leukocyte reaction. Eur J Immunol 1996; 26: 738-746.

[5] Yang Y, Sanders AJ, Dou QP, Jiang DG, Li AX and Jiang WG. The clinical and theranostic values of activated leukocyte cell adhesion molecule (ALCAM)/CD166 in human solid cancers. Cancers (Basel) 2021; 13: 5187.

[6] Verma A, Shukla NK, Deo SV, Gupta SD and Ralhan R. MEMD/ALCAM: a potential marker for tumor invasion and nodal metastasis in esophageal squamous cell carcinoma. Oncology 2005; 68: 462-470.

[7] Tachezy M, Effenberger K, Zander H, Minner S, Gebauer F, Vashist YK, Sauter G, Pantel K, Izbicki JR and Bockhorn M. ALCAM (CD166) expression and serum levels are markers for poor survival of esophageal cancer patients. Int J Cancer 2012; 131: 396-405.

[8] Donizy P, Zietek M, Halon A, Leskiewicz M, Kozyra C and Matkowski R. Prognostic significance of ALCAM (CD166/MEMD) expression in cutaneous melanoma patients. Diagn Pathol 2015; 10: 86.

[9] Tachezy M, Zander H, Gebauer F, Marx A, Kaifi JT, Izbicki JR and Bockhorn M. Activated leukocyte cell adhesion molecule (CD166)-its prognostic power for colorectal cancer patients. J Surg Res 2012; 177: e15-20.

[10] Hansen AG, Freeman TJ, Arnold SA, Starchenko A, Jones-Paris CR, Gilger MA, Washington

MK, Fan KH, Shyr Y, Beauchamp RD and Zijlstra A. Elevated ALCAM shedding in colorectal cancer correlates with poor patient outcome. Cancer Res 2013; 73: 2955-2964.
[11] Ye M, Du YL, Nie YQ, Zhou ZW, Cao J and Li YF. Overexpression of activated leukocute cell adhesion molecule in gastric cancer is associated with advanced stages and poor prognosis and miR-9 deregulation. Mol Med Rep 2015; 11: 2004-2012.
[12] Kijima N, Hosen N, Kagawa N, Hashimoto N, Nakano A, Fujimoto Y, Kinoshita M, Sugiyama H and Yoshimine T. CD166/activated leukocyte cell adhesion molecule is expressed on glioblastoma progenitor cells and involved in the regulation of tumor cell invasion. Neuro Oncol 2012; 14: 1254-1264.
[13] Ihnen M, Wirtz RM, Kalogeras KT, Milde-Langosch K, Schmidt M, Witzel I, Eleftheraki AG, Papadimitriou C, Janicke F, Briassoulis E, Pectasides D, Rody A, Fountzilas G and Muller V. Combination of osteopontin and activated leukocyte cell adhesion molecule as potent prognostic discriminators in HER2- and ER-negative breast cancer. Br J Cancer 2010; 103: 1048-1056.
[14] Burandt E, Bari Noubar T, Lebeau A, Minner S, Burdelski C, Janicke F, Muller V, Terracciano L, Simon R, Sauter G, Wilczak W and Lebok P. Loss of ALCAM expression is linked to adverse phenotype and poor prognosis in breast cancer: a TMA-based immunohistochemical study on 2,197 breast cancer patients. Oncol Rep 2014; 32: 2628-2634.
[15] Minner S, Kraetzig F, Tachezy M, Kilic E, Graefen M, Wilczak W, Bokemeyer C, Huland H, Sauter G and Schlomm T. Low activated leukocyte cell adhesion molecule expression is associated with advanced tumor stage and early prostate-specific antigen relapse in prostate cancer. Hum Pathol 2011; 42: 1946-1952.
[16] Chaker S, Kak I, MacMillan C, Ralhan R and Walfish PG. Activated leukocyte cell adhesion molecule is a marker for thyroid carcinoma aggressiveness and disease-free survival. Thyroid 2013; 23: 201-208.
[17] Kahlert C, Weber H, Mogler C, Bergmann F, Schirmacher P, Kenngott HG, Matterne U, Mollberg N, Rahbari NN, Hinz U, Koch M, Aigner M and Weitz J. Increased expression of ALCAM/CD166 in pancreatic cancer is an independent prognostic marker for poor survival and early tumour relapse. Br J Cancer 2009; 101: 457-464.
[18] Amantini C, Morelli MB, Nabissi M, Piva F, Marinelli O, Maggi F, Bianchi F, Bittoni A, Berardi R, Giampieri R and Santoni G. Expression profiling of circulating tumor cells in pancreatic ductal adenocarcinoma patients: biomarkers predicting overall survival. Front Oncol 2019; 9: 874.
[19] Hong X, Michalski CW, Kong B, Zhang W, Raggi MC, Sauliunaite D, De Oliveira T, Friess H and Kleeff J. ALCAM is associated with chemoresistance and tumor cell adhesion in pancreatic cancer. J Surg Oncol 2010; 101: 564-569.
[20] Tachezy M, Zander H, Marx AH, Gebauer F, Rawnaq T, Kaifi JT, Sauter G, Izbicki JR and Bockhorn M. ALCAM (CD166) expression as novel prognostic biomarker for pancreatic neuroendocrine tumor patients. J Surg Res 2011; 170: 226-232.
[21] Tachezy M, Zander H, Marx AH, Stahl PR, Gebauer F, Izbicki JR and Bockhorn M. ALCAM (CD166) expression and serum levels in pancreatic cancer. PLoS One 2012; 7: e39018.
[22] Fujiwara K, Ohuchida K, Sada M, Horioka K, Ulrich CD 3rd, Shindo K, Ohtsuka T, Takahata S, Mizumoto K, Oda Y and Tanaka M. CD166/ALCAM expression is characteristic of tumorigenicity and invasive and migratory activities of pancreatic cancer cells. PLoS One 2014; 9: e107247.
[23] Hiscox S and Jiang WG. Quantification of tumour cell-endothelial cell attachment by 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI). Cancer Lett 1997; 112: 209-217.
[24] Jiang WG, Ablin RJ, Kynaston HG and Mason MD. The prostate transglutaminase (TGase-4, TGaseP) regulates the interaction of prostate cancer and vascular endothelial cells, a potential role for the ROCK pathway. Microvasc Res 2009; 77: 150-157.
[25] Keese CR, Bhawe K, Wegener J and Giaever I. Real-time impedance assay to follow the invasive activities of metastatic cells in culture. Biotechniques 2002; 33: 842-844, 846, 848-850.
[26] Sanders AJ, Owen S, Morgan LD, Ruge F, Collins RJ, Ye L, Mason MD and Jiang WG. Importance of activated leukocyte cell adhesion molecule (ALCAM) in prostate cancer progression and metastatic dissemination. Oncotarget 2019; 10: 6362-6377.
[27] Xin L, Liu C, Liu Y, Mansel RE, Ruge F, Davies E, Jiang WG and Martin TA. SIKs suppress tumor function and regulate drug resistance in breast cancer. Am J Cancer Res 2021; 11: 3537-3557.
[28] Nagy A, Munkacsy G and Gyorffy B. Pancancer survival analysis of cancer hallmark genes. Sci Rep 2021; 11: 6047.
[29] Uhlen M, Zhang C, Lee S, Sjostedt E, Fagerberg L, Bidkhori G, Benfeitas R, Arif M, Liu Z, Edfors F, Sanli K, von Feilitzen K, Oksvold P, Lundberg E, Hober S, Nilsson P, Mattsson J, Schwenk JM, Brunnstrom H, Glimelius B, Sjoblom T, Edqvist

PH, Djureinovic D, Micke P, Lindskog C, Mardinoglu A and Ponten F. A pathology atlas of the human cancer transcriptome. Science 2017; 357: eaan2507.

[30] Zhang WW, Zhan SH, Geng CX, Sun X, Erkan M, Kleeff J and Xie XJ. Activated leukocyte cell adhesion molecule regulates the interaction between pancreatic cancer cells and stellate cells. Mol Med Rep 2016; 14: 3627-3633.

[31] Iolyeva M, Karaman S, Willrodt AH, Weingartner S, Vigl B and Halin C. Novel role for ALCAM in lymphatic network formation and function. FASEB J 2013; 27: 978-990.

[32] Fernandez MM, Ferragut F, Cardenas Delgado VM, Bracalente C, Bravo AI, Cagnoni AJ, Nunez M, Morosi LG, Quinta HR, Espelt MV, Troncoso MF, Wolfenstein-Todel C, Marino KV, Malchiodi EL, Rabinovich GA and Elola MT. Glycosylation-dependent binding of galectin-8 to activated leukocyte cell adhesion molecule (ALCAM/CD166) promotes its surface segregation on breast cancer cells. Biochim Biophys Acta 2016; 1860: 2255-2268.

[33] Munsterberg J, Loreth D, Brylka L, Werner S, Karbanova J, Gandrass M, Schneegans S, Besler K, Hamester F, Robador JR, Bauer AT, Schneider SW, Wrage M, Lamszus K, Matschke J, Vashist Y, Uzunoglu G, Steurer S, Horst AK, Oliveira-Ferrer L, Glatzel M, Schinke T, Corbeil D, Pantel K, Maire C and Wikman H. ALCAM contributes to brain metastasis formation in non-small-cell lung cancer through interaction with the vascular endothelium. Neuro Oncol 2020; 22: 955-966.

[34] Lyck R, Lecuyer MA, Abadier M, Wyss CB, Matti C, Rosito M, Enzmann G, Zeis T, Michel L, Garcia Martin AB, Sallusto F, Gosselet F, Deutsch U, Weiner JA, Schaeren-Wiemers N, Prat A and Engelhardt B. ALCAM (CD166) is involved in extravasation of monocytes rather than T cells across the blood-brain barrier. J Cereb Blood Flow Metab 2017; 37: 2894-2909.

[35] Kinoshita R, Sato H, Yamauchi A, Takahashi Y, Inoue Y, Sumardika IW, Chen Y, Tomonobu N, Araki K, Shien K, Tomida S, Torigoe H, Namba K, Kurihara E, Ogoshi Y, Murata H, Yamamoto KI, Futami J, Putranto EW, Ruma IMW, Yamamoto H, Soh J, Hibino T, Nishibori M, Kondo E, Toyooka S and Sakaguchi M. exSSSRs (extracellular S100 soil sensor receptors)-Fc fusion proteins work as prominent decoys to S100A8/A9-induced lung tropic cancer metastasis. Int J Cancer 2019; 144: 3138-3145.

[36] Witzel I, Schroder C, Muller V, Zander H, Tachezy M, Ihnen M, Janicke F and Milde-Langosch K. Detection of activated leukocyte cell adhesion molecule in the serum of breast cancer patients and implications for prognosis. Oncology 2012; 82: 305-312.

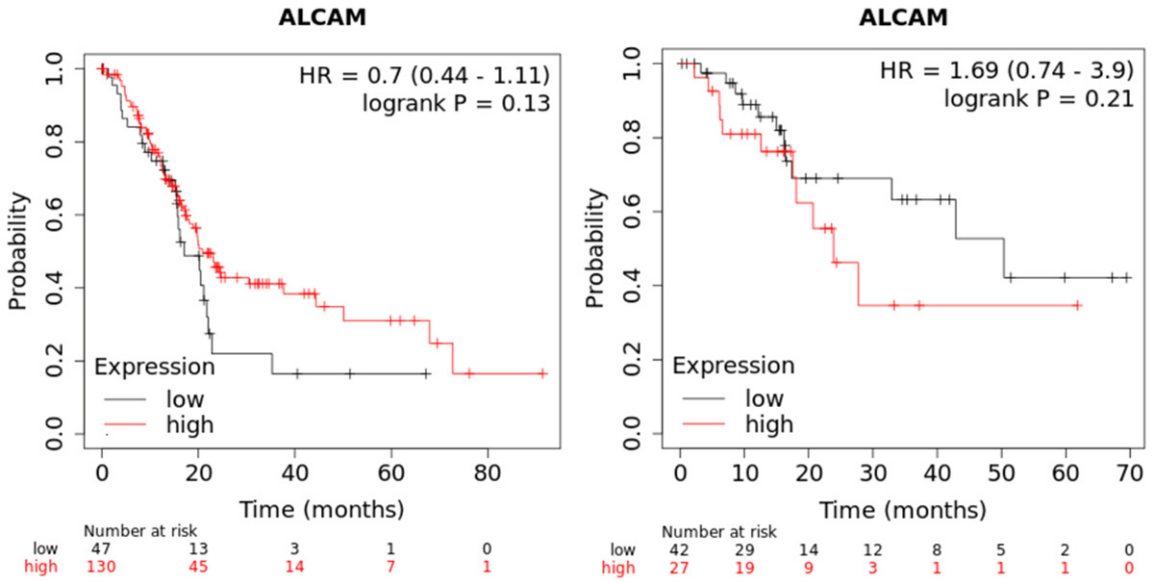

Figure S1. TCGA dataset of ALCAM transcription expression in human pancreatic cancer detected by RNAseq (www.kmplot.org) for overall survival (OS) (n=207) (Left) and relapse free survival (RFS) (n=69) (right). Patients had high levels of ALCAM had a median overall survival of 20.9 months compared with 17.03 months of those with low levels (P=0.21). In contrast and statistically non-significant fashion, patients had high levels of ALCAM had a median relapse free survival of 23.9 months compared with 50.4 months of those with low levels (P=0.21) [28].

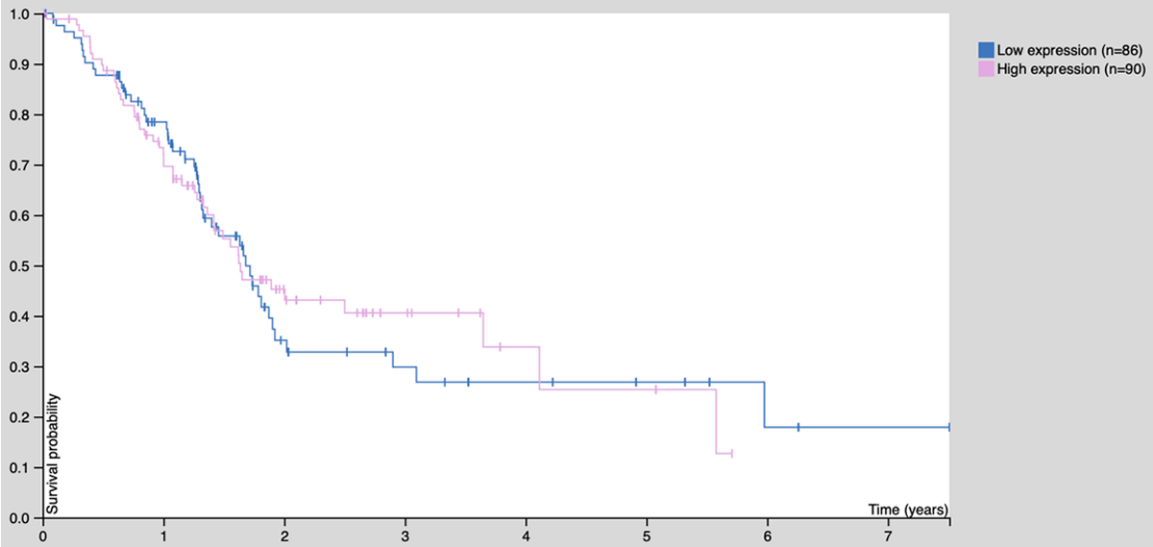

Figure S2. ALCAM protein expression in pancreatic adenocarcinoma from the proteinatlas dataset (n=176) [29] (www.proteinatlas.org). Cut-off point was median level. P=0.88.