RESEARCH ARTICLE

Prognostic Significance of Annexin A1 Expression in Pancreatic Ductal Adenocarcinoma

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Abstract

Annexin A1 is a 37-kDa calcium- and phospholipid-binding protein of the annexin superfamily considered to play an important role in tumorigenesis. However, associations with clinicopathological features in pancreatic ductal adenocarcinoma (PDAC) cases have yet to be fully defined. We therefore investigated the prognostic value of annexin A1 protein as a PDAC biomarker in 83 tumor and matched non-cancerous tissues or normal pancreas tissues. Expression was analyzed using real-time RT-PCR, Western blotting and immunohistochemistry. In non-tumor tissue, myoepithelial cells showed no or weak expression of annexin A1 while expression was strong and sometimes even located in the nuclei of endothelial cells in tumor tissue. High expression was significantly associated with advanced stage (P <0.05) and a worse overall survival (P <0.05). These results provide new insights to better understand the role of annexin A1 in PDAC survival, and might be relevant to prediction of prognosis and development of more effective therapeutic strategies aimed at improving survival.

Keywords: Annexin A1 - pancreatic ductal adenocarcinoma - prognosis

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Introduction

Pancreatic ductal adenocarcinoma (PDAC), which represents 90% of pancreatic cancers, is among the worst malignancies whose average 5-year survival is a dismal 4% (Taghavi et al., 2011). Late diagnosis of PDAC and the limited response to current treatments result in an exceptionally poor prognosis (Ferrone et al., 2012). At present, prognostic biomarkers of PDAC are relatively lacked which also results in a relatively unavailable monitoring of the progressing (Winter et al., 2012). Therefore, an efficient prognostic marker has an extreme urgency.

Nowadays, more and more studies have made effects to investigate efficient biomarkers for the early detection, diagnosis, and monitoring of cancers (Bhat et al., 2012). As a kind of multifunctional calcium-dependent phospholipid-binding protein found in almost all organisms across all kingdoms (Hayes & Moss., 2004), annexins have received more and more attention as novel biomarkers of cancer. Annexin A1(ANXA1, lipocortinI) is the first characterized member of the annexin superfamily, which participated in important biological processes including inhibition of cell proliferation, anti-inflammatory effects, the regulation of cell migration, differentiation, death (Lim & Pervaiz., 2007). Recent interest in the biological activity of this intriguing molecule has unraveled important functional attributes of annexin A1 in the process of carcinogenesis (Fatimathas & Moss., 2010). Studies have proved that the expression of annexin A1 was in a tissue-specific and tumor-specific manner, which means the expression of annexin A1 is significantly different among various types of tissues, tumor tissues and that in normal counterparts, and also is closely related to the malignant growth of tumor cell.

Previous studies have shown overexpression of annexin A1 in pancreatic cancer, which may be one of the factors that link with the malignant transformation, lower differentiation and poor prognosis of pancreatic cancer (Bai et al., 2004). However, little attention has been paid to the subcellular localization of annexin A1 expression in PDAC, which may also play an important role during tumor development and progression. In our study, previous result of annexin A1 expression in PDAC was confirmed, and on top of that we found the subcellular localization of annexin A1 was not only confined in cytoplasm but also existed in nuclei, which may mean a lot to the initiation and progression of PDAC.

Materials and Methods

Patients and tissue samples

In our study, there were 83 patients with PDAC who underwent surgery at the department of Surgery, The Tenth People's Hospital affiliated to Shanghai Tongji University and The First People's Hospital affiliated to Shanghai Jiao

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Tong University between 2004 and 2011.12. All patients were definitively identified as having PDAC based on morphological criteria, immunohistochemical staining, and clinicopathological findings according to the seventh edtion of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. Histological diagnosis of these samples was all PDAC. None of these patients have received preoperative chemotherapy or radiotherapy. Written permission to use human tumor tissues was obtained from the patients prior to surgery. Surgically removed tumors and matched non-cancerous or normal tissue samples were cut into two parts, one was snap-frozen in liquid nitrogen before storage at -80 °C, and the other was fixed with 10% formalin for histopathological diagnosis.

Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted from tissues using TRIzol reagent(Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and then was used to synthesise the first-strand complementary DNA (cDNA) with the SuperScript II preamplification kit (Fermentas, Maryland, USA). Then, 1ul of the reverse transcriptase (RT) product was used as the template to amplify specific annexin A1 fragments. The polymerase chain reaction (PCR) conditions were optimized individually for each gene studied, and the cycle number for PCR was adjusted to 30, so that the reactions fell within the linear range of product amplification. The expression of the housekeeping gene, β-actin, was used as an internal control. The RT-PCR product was analyzed by electrophoresis on a 2% agarose gel and confirmed by sequencing using an Automatic Sequencer. Signal intensities were quantified using densitometry. The annexin A1 messenger RNA (mRNA) level was quantified by the intensity ratio of the target signal to the β-actin control under the same PCR reaction conditions. The PCR primers used were as follows: for annexin A1, forward primer 5'-GCTGTGCATTGTTTCGCTTA-3', reverse primer 5'-GCAGGCCTGGTTTATTGAAA-3'; for β-actin, forward primer 5'-GGAGTCCTGTGGCATCCACG-3', reverse primer 5'-CTAGAAGCATTTGCGGTGGA-3'.

Immunohistochemistry

Immunostaining was done on paraffinembedded 4um sections of formalin-fixed tumor tissues, placed on chrome-alum gelatin-coated glass slides, and dried 30 min at 70°C. After rehydration, tissue blocks were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. Following citrate buffer antigen retrieval, the sections were blocked by incubation in 5% bovine serum albumin in phosphate-buffered saline. Expression of annexin A1 was assessed using rabbit anti-human annexin A1 polyclonal antibody (Santa Cruz, California, USA) at a 1:50 dilution followed by use of an EnvisionTM Detection Kit, Peroxidase/DAB, Rabbit/Mouse (Gene Tech, Shanghai, China) according to the manufacturer's instructions. Then the slides were stained with diaminobenzidine, washed, counterstained with hematoxylin, dehydrated, treated with xylene, and mounted. Two observers assessing immunostaining intensity were blind to the patients' information using a microscopy (CTR 6000; Leica, Wetzlar, Germany). The extent of staining was classified as follows: negative(-); weak staining(1+), less or equal to 25% of cells staining positive; moderate staining(2+), 25 to 50% of cells staining positive and strong staining(3+), greater than 50% of cells staining positive. Each observer estimated the percentage of cells stained and graded the intensity of immunostaining based on a visual assessment of the intensity of brown reaction product within the cell cytoplasm. The final immunostaining score reported was the average of two observers.

Western blotting

Total protein was extracted with lysis buffer. The protein concentration was determined by BCA protein assay (BCATM Protein Assay Kit, Pierce, USA). 40ug protein was separated using 12% SDS-PAGE for electrophoresis and then transferred onto PVDF membranes. The membranes were incubated with a 1:500 dilution of Rabbit anti-human annexin A1 polyclonal antibody (Santa Cruz, California, USA) and β-actin polyclonal antibody (Sigma) overnight at 4°C. The membranes were washed with washing buffer (PBS and 0.1% Tween 20) and then incubated with appropriate HRP-conjugated secondary antibody (1:2000) for one hour at 37°C. The blots were developed by using the ECL-detection system (Santa Cruz), quickly dried, and exposed to ECL film. All experiments were repeated at least three times and gave similar results.

Statistical analysis

SPSS 18.0 statistical software package (SPSS, Chicago, IL) was used for the statistical analysis. Student's t and Mann-Whitney U tests were used to analyze the relationship between annexin A1 expression and clinicopathological factors. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Cox's proportional hazards model was used to identify the prognostic factors that influenced survival. $P< 0.05$ values were deemed statistically significant.

Results

Clinicopathologic characteristics of the selected patients

A total of 83 patients that fitted the inclusion criteria were enrolled in this study. We compared the annexin A1 expression levels among PDAC patients with different clinicopathological characteristics (Table 1). The mean age of all patients was 59.0±12.0 years (range 34-85) The tumor of 50 patients located in the head of pancreas, while 33 in middle and distal. The patients were classified from three aspects, T, N and M, according to the seventh edtion of the tumor-node-metastasis (TNM) classification of the International Union against Cancer (UICC). T indicates the size of the tumor and whether it has invaded nearby tissue. T1 is a tumor with a diameter less than 2 cm; T2 is a tumor with a diameter of 2-4 cm; T3 is a tumor with a diameter of 4-6 cm; T4 is a tumor with a diameter of more than 6 cm. N indicates lymph node metastasis. N0 indicates no lymph node involvement; N1 is involvement

Table 1. Relationship of Annexin A1 Expression with Pathological Parameters of Tumor

Clinical parameters	High* Nuclear positive (n = 13)	Nuclear negative (n = 36)	sum (n = 49)	Low*	p-value
Age (y)					
≥ 60	7	13	20	17	0.655
< 60	6	19	25	17	
Gender					
male	5	15	20	19	0.189
female	8	21	29	15	
Location					
Proximal	9	22	31	19	0.649
Middle and Distal	4	14	18	15	
Tumor size (cm^3)					
≥ 50	4	14	18	9	0.655
< 50	9	22	31	25	
Stage					
0	0	0	0	0	0.001
I (IA, IB)	4	1	5	14	
II (IIA, IIB)	5	20	25	19	
III	4	5	9	1	
IV	0	0	0	0	
Tumor invasion					
T1	2	1	3	1	0.378
T2	7	29	36	29	
T3	2	6	8	4	
T4	2	0	2	0	
Lymph node metastasis					
N0	7	18	25	18	0.865
N1	6	18	24	16	
Distant metastasis					
M0	13	36	49	34	
M1	0	0	0	0	

High*, High annexin A1 expression in cytoplasm; Low*, Low annexin A1 expression in cytoplasm (n = 34)

of lymph nodes. M indicates distant metastasis. M0 indicates no distant metastasis; M1 is involvement of distant metastasis. Then, 7 stages are classified, including 0 (TisN0M0), IA (T1N0M0), IB (T2N0M0), IIA (T3N0M0), IIB (T1N1M0, T2N1M0, T3N1M0), III (T4, Any N, M0), IV (Any T, Any N, M1).

Annexin A1 expression in PDAC

Quantitative real-time RT-PCR was performed to detect the annexin A1 mRNA expression in 20 cases of PDAC and corresponding adjacent pancreatic tissues. We found that 12 of the 20 patients (60%) showed a higher expression level of annexin A1 mRNA in PDAC than in non-cancerous tissue. As demonstrated in Figure 1, our results revealed the average expression level of annexin A1 mRNA in PDAC tissues to be significantly higher than in corresponding non-tumor pancreas (P<0.05).

To further detect the expression of annexin A1 protein in PDAC, we performed western blot and immunochemistry. Twenty micrograms of protein extracts of PDAC tissues and their corresponding normal pancreas tissues from 20 different patients was prepared for Western blot analysis using annexin A1 antibody which could detect specific bands migrating at 37ku (Figure 2). Western blot showed that the expression of annexin A1 was low in normal pancreas tissues, while the level of annexin A1 expression

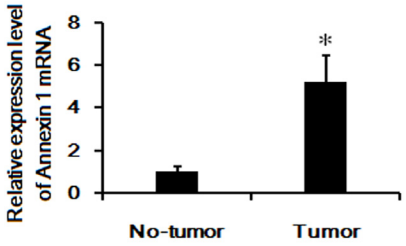

Figure 1. Annexin A1 Expression in PDAC and Matched Non-tumor Pancreatic Tissue Samples Detected by qRT-PCR and Normalized to Actin (n = 20). Annexin A1 expression in PDAC tissues and normal tissues were shown as the mean ± SD, p < 0.05

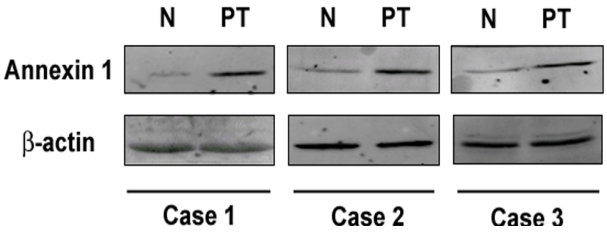

Figure 2. Western Blot Analysis of Annexin A1 in Pancreatic Ductal Adenocarcinoma (PDAC). Twenty micrograms of total protein extracts from pancreatic tissues were run on 12% SDS-PAGE, and annexin A1 protein expression was probed with rabbit anti-annexin A1 polyclonal antibody. N: normal pancreas tissues; PC: PDAC tissues. β-actin was used as an internal control

was markedly increased in PDAC. Overexpression of annexin A1 was found in 65% (13/20) PDAC tissues.

Immunochemistry was performed to analysis the 83 cases of PDAC tissue samples. The positive staining of annexin A1 protein was predominantly localized in the cytoplasm and of the epithelial cells. Only sporadic cells showed annexin A1 nuclear staining in a small subset (15.7%, 13/83) of PDAC specimens. In our study, as showed in Figure 3, 5 tumor tissues showed negative staining (-), 29 tumor tissues showed weak staining (1+), 31 showed moderate staining (2+) and 18 showed strong staining (3+). In addition, among the 18 strong staining tissues, 13 showed annexin A1 nuclear staining. According to the annexin A1 immunoreative intensity, 34 (41%) patients were classified as low-annexin A1 group and 49 (59%) patients were classified as high-annexin A1 group. Correlation between annexin A1 expression and clinicopathological parameters

To further investigate whether annexin A1 protein up-regulation is linked to the clinical parameters of pancreatic cancer patients, we detected the correlation of annexin A1 expression in PDAC tissues with various clinicopathological factors. As revealed in Table1, we found that the level of annexin A1 protein expression was significantly correlated with advanced stage (p=0.001), whereas there was no significant correlation between annexin A1 protein expression level and other clinicopathological factors including gender (p=0.189), age (p=0.655), tumor location (p=0.649), tumor invasion (p=0.378), tumor size (p=0.655), and lymph node metastasis (p=0.865).

Association of annexin A1 expression with survival

To further clarify the association of annexin A1

Table 2. Univariate and Multivariate Analysis of Clinicopathological Factors Affecting Overall Survival Rate of PDAC Patients

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p-value	HR (95%CI)	P-value
Age (≥ 60/< 60)	0.689 (0.391 – 1.215)	0.198	1.943 (0.257 – 20.082)	0.256
Gender (male / female)	1.437 (0.624 – 3.306)	0.394	1.657 (0.266 – 5.895)	0.934
Tumor location (proximal / middle and distal)	0.707 (0.392 – 1.272)	0.247	1.235 (0.149 – 4.067)	0.563
Tumor size (cm3) (≥ 50 / < 50)	0.927 (0.506 – 1.698)	0.807	0.576 (0.227 – 7.852)	0.925
Stage	1.437 (0.624 – 3.306)	0.394	1.667 (0.150 – 3.916)	0.506
Tumor invasion (T)	0.868 (0.497 – 1.515)	0.618	1.409 (0.157 – 3.57)	0.163
Lymph node metastasis (N)	1.239 (0.776 – 1.980)	0.37	0.448 (0.062 – 8.764)	0.655
Distant metastasis (M)	0.790 (0.459 – 1.358)	0.393	0.476 (0.062 – 7.712)	0.636
Annexin A1 expression in cytoplasm (low/high)	0.462 (0.201 – 1.062)	0.069	0.281 (0.059 – 1.128)	0.035

HR, hazard ratio; 95%CI, 95% confidence interval

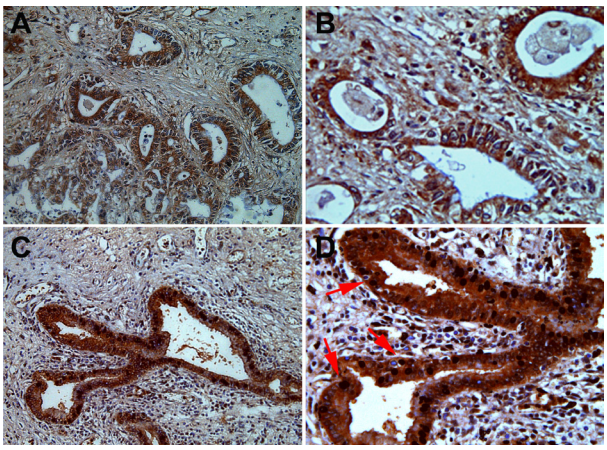

Figure 3. Immunohistochemical Detection of Annexin A1 Expression in PDAC Tissue Samples. The staining of annexin A1 protein was mostly located in the cytoplasm of the epithelial cells, and there were some weak nuclear staining of epithelial cells (arrows). (A) and (B): low expression; (C) and (D): high expression. (A) and (C) ×200; (B) and (D) ×400

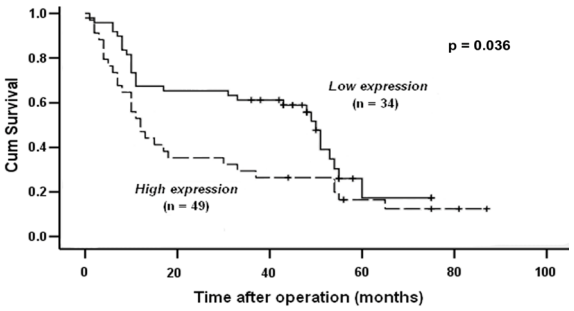

Figure 4. Kaplan-Meier Analysis of Overall Survival Rate of PDAC Patients with High Annexin A1 Expression (n = 49) and Low Annexin A1 Expression (n = 34), Respectively. The overall survival rate between the two groups showed significantly different (p = 0.036, log-rank test)

expression with prognosis of PDAC patients, we performed Kaplan-Meier method. Through the method, patients with low annexin A1 expression tissues had significantly decreased overall survival rate than those with high annexin A1 expression (p=0.036, Figure 4). Thus, the result revealed that the survival of PDAC patients could be affected by annexin A1 expression.

In order to nail down whether the prognostic significance of annexin A1 can be regarded as a predictor of overall survival in PDAC patients, we conducted univariate and multivariate analyses (Table 2). Through univariate analysis, no factor was significantly associated with overall survival of PDAC patients. Through multivariate analysis, annexin A1 expression was an independent prognostic factor for overall survival of PDAC patients (p=0.035).

Discussion

Annexin A1, initially as a strong inhibitor of glucocorticoid-induced eicosanoid synthesis and phospholipase A2 (PLA2), may have important regulatory roles in tumor development and progression. Evidence for this lies in the clear observations that its expression is in a tumor-specific manner. It has been reported that annexin A1 is up-regulated in hepatocellular carcinoma (de Coupade et al., 2000), bladder cancer (Cui et al., 2007), larynx cancer (Silistino-Souza et al., 2007), human breast cancer (Kang et al., 2012) and pituitary adenoma (Mulla et al., 2004), while it is absent or down-regulated in human esophageal squamous cell carcinoma (Liu et al., 2003), oral squamous-cell carcinoma (Nomura et al., 2009), prostate cancers (Silistino-Souza et al., 2007) head and neck cancers (Garcia Pedrero et al., 2004), intestinal-type sinonasal adenocarcinoma (Rodrigo et al., 2011), B-cell non-Hodgkin's lymphomas (Vishwanatha et al., 2004), thyroid cancers (Petrella et al., 2006) and endometrial carcinoma (Da et al., 2001). In our study, we found that annexin A1 was significantly overexpressed in PDAC tissues by real-time RT-PCR, Western blot and immunohistochemistry, which was consistent with the early study of annexin A1 expression in pancreatic cancer (Bai et al., 2004). Then, why did the expression of annexin A1 show prodigious difference between PDAC and normal tissues? There may be a lot of factors involved. For instance, the overexpression of epidermal growth factor-receptor (EGF-R) is one of the most frequently detected genetic changes in the initial stages of pancreatic cancer and greatly correlates with the progression, invasion and metastasis of pancreatic cancer (Liu et al., 2012), and annexin A1 is happened to be a substrate for EGF-stimulated tyrosine kinase and can be phosphorylated by EGF-R tyrosine kinase to mediate proliferation. Therefore, when pancreatic cancer initiated and developed, EGFR expression increased, consequently annexin A1 was phosphorylated to play its part, and then the annexin A1 expression increased as a feedback.

Survival analysis indicated that the level of annexin A1 expression was significantly correlated with advanced stage, which suggests that annexin A1 could act as a novel biomarker to identify aggressive PDAC. It is worth to mention that we observed the nuclear staining of annexin A1 in PDAC tissues for the first time, which may contribute to the better understanding of the mechanism that annexin A1 was involved in. Interestingly, 13 out of 18 strong staining tissues showed nuclear staining, which has not been detected and reported in PDAC yet. However, similar observation was detected in esophageal squamous cell carcinoma (ESCC), oral squamous cell carcinoma (OSCC), and gastric cancer. In 2003, Yu Liu et al first detected the translocation of annexin A1 from cellular membrane to nuclear membrane in ESCC cells, and proposed that translocation of annexin A1 protein in ESCC may correlate with the tumorigenesis of esophageal cancer (Liu et al., 2003). Likewise, in 2008, Lin et al found the nuclear localization of annexin A1 protein in OSCC. Moreover, prognostic significance of annexin A1 nuclear staining showed a lower overall survival (Lin et al., 2008). Afterwards, Fengjia ZHU et al also found that positive nuclear staining of annexin A1 in gastric cancer tissue was connected with advanced disease stage and peritoneal dissemination (Zhu et al., 2010). As a result, alteration of annexin A1 subcellular distribution may also be involved in monitoring the progress of cancer and predicting the prognosis of patients. It is a pity that we have not analysis the correlation between subcellular localization of annexin A1 expression and clinicopathological parameter and survival analysis due to the sample size, we hope to fine down it in the following studies. Not withstanding its limitation, this study does suggest that annexin A1 could express in both cytoplasm and nuclei, which may provide new evidence to better understand the mechanism how annexin A1 played its role.

It is generally known that subcellular localization of a protein probably provides significant clues to its function (Horton et al., 2007). As cytosolic proteins with pools of both a soluble and stable form or reversibly connected with components of the cytoskeleton or proteins, annexins mediate interactions between cell and extracellular matrix (Moss & Morgan., 2004). As a result, annexin A1 can translocate from the cytosol to the cell surface or the nuclei. Evidences have been showed that annexin A1 mediates various important physiologic processes depending on its subcellular localization in vitro and in vivo in a variety of human malignancies. For instance, at intracellular level, annexin A1 can interact with the cytosolic form of phospholipase A2 (cPLA2) and cyclooxygenase 2 (COX-2) to block enzyme activity (Kim et al., 1994; Hannon et al., 2003), and annexin A1 can mediate apoptosis by inducing the dephosphorylation of BAD, allowing BAD to translocate to the mitochondria, whereas annexin A1 itself translocates to the nucleus (Solito et al., 2003a), however, the mechanism and functional relevance of annexin A1 nuclear localization is still unknown, and annexin A1 also can be phosphorylated by EGF-R tyrosine kinase, protein kinase C (PKC), platelet-derived growth factor receptor tyrosine kinase (PDGFR-TK), and hepatocyte growth factor receptor tyrosine kinase (HGFR-TK) to mediate proliferation (Lim & Pervaiz., 2007); at the external membrane level, annexin A1 acts as a negative regulator of inflammatory process, including blocking the rolling of polymorphonuclear leukocyte on endothelial cells (Perretti et al., 1996), and also acts on its receptor, identified as the fomyl peptide receptor (FPR) and the formyl peptide receptor like-1 (FPRL1), to inhibit cell adhesion and migration, as well as inducing detachment of adherent cells (Rescher et al., 2002). Moreover, it can be phosphorylated and translocated to membrane by glucocorticosteroid through PKC (protein kinase C), PI3K, MAP kinase (mitogen activated protein kinase) and Ca2+ dependent pathways (Solito et al., 2003b), and binds to phosphatidylserine to mediate the engulfment of apoptotic cells when recruited to the cell surface (Arur et al., 2003).

There are several conditions that annexin A1 translocated to the nucleus region. When treated with heat, hydrogen peroxide, sodium arsenite or EGF, A549 and heLa cells induced annexin A1 expression and translocation from the cytoplasm to the nucleus and perinuclear region (Rhee et al., 2000; Radke et al., 2004), which may suggest that annexin A1 can protect the essential nuclear components from the stressors. Moreover, kim et al proposed that PMA-induced translocation of annexin A1 to the nucleus may participate in the regulation of cell proliferation and differentiation (Kim et al., 2003). In addition, several studied have approved that nuclear translocation of annexin A1 is involved in the regulation of cellular proliferation (Alldridge & Bryant., 2003; Alves et al., 2008). Similarly, in our study, as the tumor tissues of PDAC patients with high expression of annexin A1 and weak nuclear staining showed worse prognosis and outcome, we hypothesisd that annexin A1 translocated to the nuclei may be involved in the different processes in both cytoplasm and nuclei including specific cellular functions as well as signal transduction and the regulatory effects of annexin A1 may involve both transcriptional and translational levels. And also we can also come to a conclusion that regulation of annexin A1 activity and function may be achieved by not ony adjusting its expression and phosphorylation, but also by changes of its subcellular localization.

In summary, we found that annexin A1 expression was increased in PDAC tissues while the annexin A1 expression was low or absent in normal prancreatic tissues and the localization of annexin A1 was changeable in cytoplasm, nuclei and membrane of tumor cells. Compared with low expression of annexin A1, high expression especially together with weak nuclear staining in PDAC tissue showed worse prognosis and correlated with advanced stage. And the function of annexin A1 in different location may be totally different, but there must be some intrinsic connections between them which need more attention.

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