

Overexpression of annexin 1 in pancreatic cancer and its clinical significance

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Abstract

AIM: To investigate the expression of annexin I in pancreatic cancer and its relationship with the clinicopathologic factors, and to evaluate its potential clinical significance.

METHODS: Annexin I expression was analyzed by Western blot and immunohistochemical staining in pancreatic adenocarcinoma and multi-tissue microarrays (MTAs).

RESULTS: Western blot analysis showed that annexin I was overexpressed in 84.6% (11/13) pancreatic ductal adenocarcinomas. Immunohistochemistry analysis of pancreatic cancer in MTAs showed that annexin I protein was 71.4%(30/42) positive which was markedly increased compared with that in the tumor matched normal pancreas tissues 18.4%(7/38) ($P < 0.01$). In the meantime, the high expression of annexin 1 was correlated with the poor differentiation of pancreatic adenocarcinoma.

CONCLUSION: Annexin 1 overexpression is a frequent biological marker and correlates with the differentiation of pancreatic cancer during tumorigenesis.

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INTRODUCTION

Pancreatic cancer is one of the most lethal malignancies with less than 3-5% of the overall five-year survival rate, and the patients normally die within six months after diagnosis^[1]. There

are some indications that the incidence of pancreatic cancer following an upward increase, in recent years it has reached a plateau and in some countries there is even a slight decrease. But in China the incidence and mortality rates of this disease have taken an upward trend countrywide. Based on the data of demography and death collected through Chinese Disease Surveillance Point System (DSPS) over the period of 1991-2000, the age-standardized mortality rate due to pancreatic cancer increased from 2.18 in 1991 to 3.26 in 2000 per 1000 000 populations and the peak mortality of pancreatic cancer might arrive in China in the next few decades^[2]. In the United States, more than >30 000 people were diagnosed and died of pancreatic cancer in 2003, representing the fourth leading cause of cancer death^[1]. The significant factor for the poor prognosis of pancreatic cancer may be attributed to its biological aggressiveness, the difficulty of early diagnosis, and poor response to conventional therapeutics, those reflect a fact that pancreatic cancer is a poorly understood disease and the etiologic factors and the molecular basis for these characteristics are unknown. Comparisons of global gene and protein expression profiles between pancreatic cancer and normal pancreas using high-throughput methods could provide important information about the molecular characteristics and reveal some new specific or associated biomarkers of pancreatic cancer with promise for development into novel diagnostic or therapeutic targets^[3-7].

Annexin 1, a member of annexin family, was found with expression alterations in different kinds of malignant tumors. The molecular mechanisms and the clinical significance of annexin I altered expression still remain a debate. In this study, we investigated annexin I expression and distributions in a large number of pancreatic cancer specimens via Western blot and immunohistochemistry analysis based on multi-tissue microarrays (MTAs).

MATERIALS AND METHODS

Patients and specimens

Fresh tissue samples of 13 pancreatic cancers and their corresponding normal counterparts were obtained at the time of resection with informed consent from Cancer Institute and Hospital (CIH), Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC) during November 2001 and November 2003. The 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. Histological diagnosis of these samples was all pancreatic ductal adenocarcinomas. This group consisted of 8 males and 5 females with a median age of 64 years (range, 39-75 years). None of them received preoperative radiotherapy or chemotherapy.

Formalin-fixed paraffin-embedded tissue blocks of pancreatic cancer and normal pancreatic tissue were collected from the archives of the Department of Surgery at CIH, CAMS and Mudanjiang Tumor Hospital between January 1991 and August 2002 and subjected to tissue microarray construction. There were 32 pancreatic ductal adenocarcinomas, 6 mucinous

adenocarcinomas, 4 acinar cell carcinomas, 7 islet cell carcinomas, 8 ampulla of Vater carcinomas. The median age of these patients (37 males and 20 females) at the diagnosis was 60 years (range, 19-71 years).

Human pancreatic cancer cell lines BxPC-3 and PANC-1 were purchased from American Type Culture Collection (Manassas, VA). BxPC-3 and PANC-1 cells were cultured in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, and supplemented with 100 g/L heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Western blot analysis

Total tissue and cell lysate were prepared in extraction buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10 g/L Triton-100, 1 g/L SDS, 1 mmol/L EDTA, 1 mmol/L AEBSF, 20 µg/mL aprotinin, and 20 µg/mL leupeptin. After centrifugation at 12 000 g for 15 min at 4 °C, the supernatant was collected, and protein concentration was determined by Bradford method^[8]. Equal amounts of total protein (10 µg) from each sample were loaded and separated by 120 g/L SDS-polyacrylamide gel electrophoresis, and then transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After blocked with 50 g/L nonfat dry milk in PBS (pH 7.4) with 1 g/L Tween-20, membranes were probed with a mouse anti-annexin I monoclonal antibody (1:1 000 dilution, BD Biosciences Pharmingen, Chicago, IL), followed by subsequent incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:3 000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). Visualization of the protein bands was performed by the enhanced chemiluminescence kit (Santa Cruz Biotechnology). Parallel Western blot was probed with an anti- α -tubulin monoclonal antibody (Santa Cruz Biotechnology) as a loading control.

MTAs construction and immunohistochemistry analysis

Formalin-fixed paraffin-embedded tissue blocks containing pancreatic adenocarcinoma and normal pancreatic tissues were identified on the hematoxylin and eosin stained slide and marked. The marked areas in the corresponding paraffin block (donor block) were used for tissue microarray construction. From these defined areas of each specimen, triplicate tissue cores with a diameter of 0.6 mm were taken from donor block and arrayed into a recipient paraffin block using a tissue puncher/arrayer (Beecher Instruments, Silver Spring, MD) as previously described^[9]. Five micrometer sections of the tissue array block were cut and placed on Fisherbrand Colorfrost/Plus microscope slides (Fisher scientific, Pittsburgh, PA) for immunohistochemical staining.

The streptavidin-peroxidase method was used for the immunostaining of annexin I. Briefly, after deparaffinization in xylene and rehydration in grade ethanol, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 min. Tissue sections were then heated at 100 °C in 10 mmol/L citrate buffer (pH 6.0) for 10 min to retrieve antigens and pre-incubated with normal horse serum for 20 min at room temperature. Mouse anti-annexin I monoclonal antibody (BD Biosciences Pharmingen) diluted 1:100 was used as the primary antibody, and the specimens were incubated with it overnight at 4 °C, followed by addition of biotinylated anti-mouse secondary antibody and streptavidin-horseradish peroxidase (Zymed Laboratories, South San Francisco, CA). 3,3'-diaminobenzidine was used as a chromogen, and hematoxylin was used for counterstaining. For negative control purposes, the same procedure was followed except that the primary antibody was replaced by PBS. Known immunostaining-positive slides were used as positive controls.

The level of annexin I expression was calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score) as follows. No staining was scored as 0, 1-10% of cells with positive staining were scored as 1, 10-50% as 2, 50-70% as 3, and 70-100% as 4. Staining intensity was rated on a scale of 0 to 3 as follows: 0=negative (no color); 1=weak (weak yellow), 2=moderate (yellow), and 3=strong (brown). The raw data were converted to the immunohistochemical score (IHS) by multiplying the quantity and staining intensity scores. Therefore, the score could range from 0 to 12. The IHS score >3 was considered as positive expression^[10].

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 software package (SPSS, Chicago, IL). The annexin I expression in different groups was analyzed using Mann-Whitney *U* test. The correlation between annexin I and each clinicopathologic factor was assessed with the Spearman rank correlation test. *P* value of less than 0.05 was considered statistically significant.

RESULTS

Western blot analysis of annexin I expression

Ten micrograms of protein extracts of pancreatic ductal adenocarcinoma tissues and their corresponding normal pancreas tissues from 13 different patients was prepared for Western blot analysis using monoclonal anti-annexin I antibody. This antibody could detect specific bands migrating at 37 ku (Figure 1). Western blot revealed that the expression of annexin I was low or undetectable in normal pancreas tissues. The level of annexin I expression was markedly increased in pancreatic ductal adenocarcinoma. Annexin I overexpression was found in 84.6% (11/13) pancreatic ductal adenocarcinoma tissues. There was also a strong expression of annexin I in pancreatic cancer cell lines.

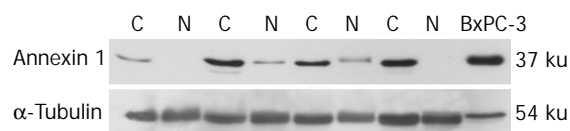


Figure 1 Western blot analysis of annexin I in pancreatic ductal adenocarcinoma. Ten micrograms of total protein extracts from pancreatic tissues and cell lines were run on 120 g/L SDS-PAGE, and annexin I protein expression was probed with mouse anti-annexin I monoclonal antibody and visualized by chemiluminescence (*top panel*). C: pancreatic ductal adenocarcinoma tissues; N: normal pancreas tissues; BxPC-3: human pancreatic adenocarcinoma cell line. α -Tubulin was used as an internal control (*bottom panel*).

Immunohistochemistry analysis of annexin I using pancreatic cancer MTAs

We carried out immunohistochemical studies for annexin I on the paraffin-embedded pancreatic cancer tissue microarray (Figure 2. A, B). This tissue microarray contained a total of 256 tissue spots consisting of 32 pancreatic ductal adenocarcinomas, 6 mucinous adenocarcinomas, 4 acinar cell carcinomas, 7 islet cell carcinomas, 8 ampulla of Vater carcinomas, and 38 normal pancreas tissues. In the normal pancreas, the positive rate of annexin I expression was 18.4% (7/38). It was found that most of normal pancreatic acinar and ductal cells did not express annexin I. There were only a small number of acinar cells that were observed. Annexin I cytoplasmic positive and the scattered positive cells were mainly located on the outside of acinar lumen (Figure 2. C, D). In contrast, positive expression of annexin I in

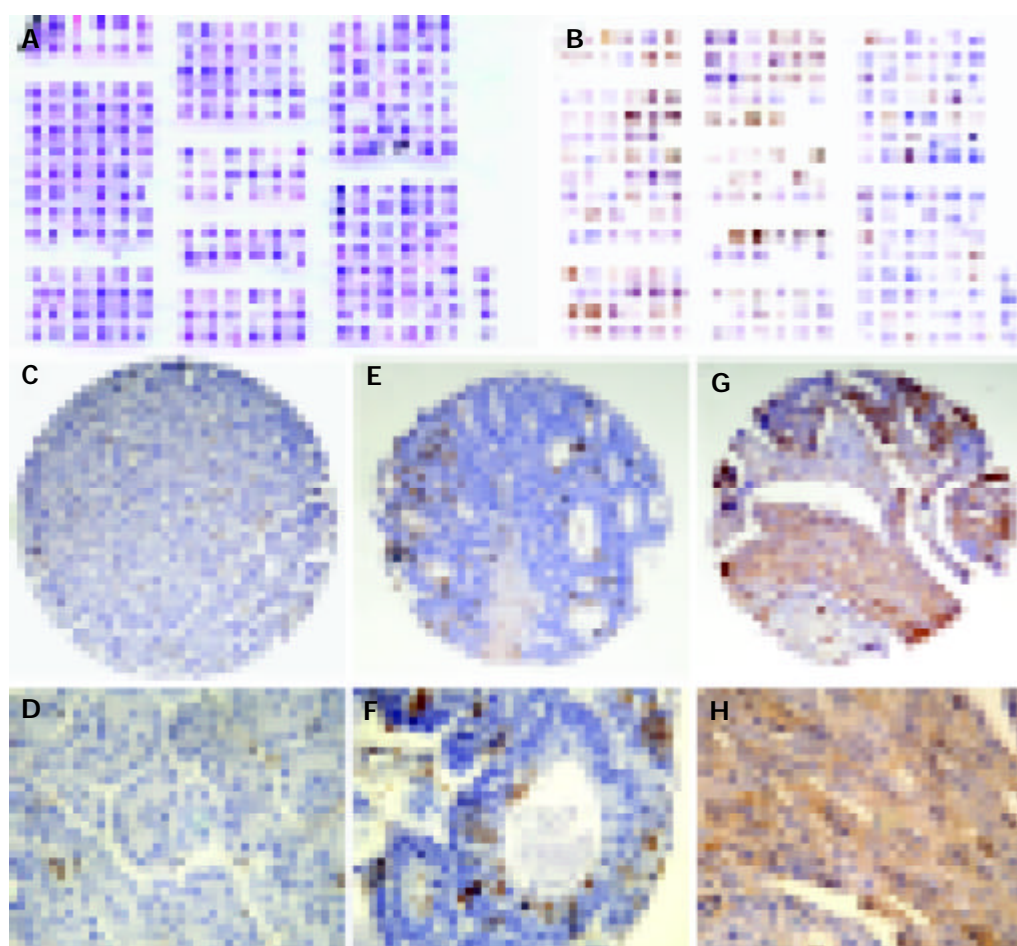


Figure 2 Immunohistochemical analysis of annexin I expression on pancreatic cancer MTAs. A and B: Overview of the H & E staining and immunohistochemical staining of annexin I on pancreatic cancer MTAs; C ($\times 200$) and D ($\times 400$): normal pancreas; E ($\times 200$) and F ($\times 400$): well differentiated ductal adenocarcinoma; G ($\times 200$) and H ($\times 400$): poorly differentiated ductal adenocarcinoma.

pancreatic cancer (71.4%, 30/42) was up-regulated significantly ($P < 0.01$). The different subtypes of the tumor were observed partially positive (Table 1). The possible relationships between annexin I expression with some clinicopathologic factors were additionally analyzed. It was found that the positive expression rate of annexin I in poorly differentiated pancreatic ductal adenocarcinomas (81.8%, 9/11) increased markedly compared with the well and moderately differentiated types (71.4%, 15/21) ($P < 0.05$). According to the progress of tumorigenesis, the distributions of annexin I were found altered from the outside of normal acinar lumen to the inside cancerous acinar lumen in the well differentiated ductal adenocarcinomas, and then to the most of poorly differentiated ductal adenocarcinomas separately (Figure 2. C, D, E, F, G, H). There were no statistically significant correlations between annexin I expression and lymph node metastasis and TNM stages (Table 2).

Table 1 Annexin I expression in normal pancreas and cancer tissues

Group	<i>n</i>	Positive rate of annexin I expression (%)	<i>P</i> value
Normal pancreas	38	18.4(7/38)	<0.0001
Pancreatic cancer	42	71.4(30/42)	
Ductal adenocarcinoma	32	75(24/32)	
Mucinous adenocarcinoma	6	83.3(5/6)	
Acinar cell carcinoma	4	25(1/4)	
Islet cell carcinoma	7	57.1(4/7)	
Ampulla of Vater carcinoma	7	57.1(4/7)	

Table 2 Correlation of annexin I expression with clinicopathologic factors of pancreatic adenocarcinoma on tissue microarray

Clinicopathologic factors	<i>n</i>	Positive expression rate of annexin I (%)	<i>P</i> value
Histological differentiation			0.012
High and moderate	21	71.4(15/21)	
Poor	11	81.8(9/11)	
Lymph node metastasis			0.810
Yes	8	75(6/8)	
No	34	73.5(25/3)	
TNM stage			0.551
I	15	66.7(10/15)	
II	21	81(17/21)	
III	5	60(3/5)	

DISCUSSION

Annexin I belongs to the family of the calcium and phospholipid-binding proteins, called annexins. Annexins are cytosolic or associated with the membrane or the cytoskeleton in a calcium-dependent manner. Annexin I is one of the more extensively studied annexins, which was initially cloned as phospholipase A2 (PLA2) inhibitor^[11]. Annexin I is a steroid-regulated protein and thus implicated in some actions of glucocorticoids, including inhibition of cell proliferation, anti-inflammatory effects, the regulation of cell migration, differentiation, death and the hypothalamic-pituitary axis^[12-16]. To date, there are some contradictory descriptions on annexin I expression in human

cancers. It has been reported that annexin I is up-regulated in human breast cancer^[17], hepatocellular carcinoma^[18], and pituitary adenoma^[19] and down-regulated in human esophageal squamous cell carcinoma^[20], prostate cancer^[21], and endometrial carcinoma^[22]. In this study, we found that annexin I was significantly overexpressed in pancreatic cancer by Western blot and immunohistochemistry, which was consistent with the results of gene expression profile analysis.

Annexin I is expressed in a tissue-specific manner in rodents. The highest annexin 1 expression level was found in lung and placenta; moderate in spleen, thymus, prostate, and submaxillary gland; and low (or absent) in muscle, brain, and liver^[23]. However, it was found that annexin I expression and phosphorylation were not only up-regulated during liver regeneration and transformation in antithrombin III SV40 T large antigen transgenic mice, but also overexpressed at both the transcriptional and translational levels in tumorous and nontumorous regions of hepatocellular carcinoma (HCC)^[18,24]. Annexin I up-regulation has been found to be correlated with increased synthesis of epidermal growth factor (EGF) and consequently with increased phosphorylation of EGF receptor (EGFR). Annexin I is a substrate for tyrosine kinases such as EGFR^[23,25] and for serine/threonine kinases such as protein kinase C^[26]. Annexin I can specifically modulate the extracellular signal-regulated kinase (ERK) signal cascade at an upstream site probably by associating with key signal components including the adaptor protein Grb2. Increased expression of annexin I could lead to constitutive activation of ERK1/2 kinase in macrophages^[27]. These findings implicated that annexin I might involve in mitogenic signal transduction and regulate cell growth. It was found that the level of annexin I expression increased three to four fold when quiescent human diploid foreskin fibroblasts (HFF) cells were stimulated to proliferate^[28]. This observation suggested that annexin I might be directly or indirectly involved in cellular proliferation. Pancreatic cancer demonstrated abnormally high expression of a number of important tyrosine kinase growth factors and receptors, particularly of the EGF family, which may contribute to the neoplasm's growth by autocrine and paracrine effects^[29]. Because annexin I is a substrate protein of EGFR, we can postulate that activated EGFR pathway promotes the annexin I up-regulation and then might associate with pancreas malignant transformation. We evaluated the relationship between annexin I and the clinicopathological factors of pancreatic cancer, and found that higher annexin I expression was correlated with the poorly differentiated type of pancreatic cancer, which was similar to the finding in HCC^[18]. These results suggest that annexin I is also involved in histological differentiation. It is interesting that the location of annexin I expression in different histologically differentiated types was changed, the reason for this change is not clear, which might be due to the role of annexin I in different places^[30].

On the other hand, enhanced expression of annexin I could reduce *in vitro* peripheral blood lymphocyte response to mitogens and might involve in the immunosuppressive mechanism of tumor-bearing hosts^[31]. Annexin I-derived peptides could inhibit antigen-driven human T cell proliferation and cytokine production^[32]. High constitutive levels of annexin I in leukaemic cells might protect them against immune-mediated killing^[33]. These evidences suggested that elevated annexin I might influence the immune defence system of body and might serve as a poor prognostic marker.

In conclusion, the present results show that overexpression of annexin I is a frequent event in pancreatic cancer, which may be one of the factors that link with the malignant transformation, lower differentiation and poor prognosis of pancreatic cancer. Detection of annexin I expression may be assistant to clinical diagnosis and can assess the prognosis of

pancreatic cancer. However, more efforts need to address the molecular mechanisms.

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