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Annexin A1 expression and its prognostic significance in human breast cancer

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Annexin A1 (ANXA1) is a calcium- and phospholipid-binding protein and is considered to play an important role in tumorigenesis. However, the correlation between ANXA1 expression and tumor clinicopathological features in patients with breast cancer remains unclear. This study investigated the prognostic value of ANXA1 protein as breast cancer marker. Tissue microarray blocks, containing 20 cases of non-tumor breast tissue, 20 cases of benign breast lesion and 135 cases of breast cancer (107 with lymph node metastasis), were constructed. Expression of ANXA1 in these specimens was analyzed using immunohistochemistry. In non-tumor tissue and benign breast lesions, myoepithelial cells showed strong expression of ANXA1. Negative ANXA1 expression was significantly associated with advanced disease stage ($P<0.05$), especially pathological-N stage ($P<0.01$). The patients with loss of ANXA1 expression in tumor tissues showed a significantly worse overall survival compared with positive ones ($P<0.05$). ANXA1 did not correlate well with estrogen receptor (ER), progesterone receptor (PR) and HER2/neu status. Moreover, the level of ANXA1 expression in lymph node metastases was higher than corresponding primary breast cancer. These results suggest that ANXA1 may play a multifaceted role in breast cancer development, progression, and metastases.

Key words: Breast cancer; annexin A1; tissue microarray; metastasis

Breast cancer is one of the leading cause of cancer death in women throughout the world [1], largely due to the recurrence or disseminated disease. Therefore, a better understanding of carcinogenesis is crucial to improve diagnosis and treatment.

Annexin A1(ANXA1) belongs to the annexin superfamily composed of 13 proteins sharing a feature of calcium-dependent binding to phospholipids. ANXA1 is an important mediator in glucocorticoid-regulated inflammatory response and was found to be associated with cell proliferation, differentiation and apoptosis, signal transduction, as well as tumor progression and metastasis.

ANXA1 is considered to play an important role in carcinogenesis and/or progression by participating in cell proliferation and differentiation, cell signaling and metastasis [2, 3]. Although, ANXA1 has been detected in a variety of tumors,, the level of ANXA1 expression was discrepant. ANXA1 has been shown to be upregulated in pancreatic [4], hepatic [5] , head and neck [6] carcinomas but markedly downregulated in esophageal [7], prostate [8] and gastric [9] carcinomas. Likewise, *in vitro* studies showed ANXA1 played various roles in tumor cell lines. ANXA1 upregulated in colorectal carcinoma cell lines with poorly metastatic potentiality using two-dimensional gel electrophoresis and peptide mass fingerprinting analysis [10] In contrast, ANXA1 inhibited the proliferation of the Hep-2 human larynx epidermoid carcinoma cell line [11]. Interestedly, some researches demonstrated that ANXA1 expression was associated with tumor metastases. For examples, stronger staining of ANXA1 expression in lymph node metastases than primary tumor was observed in hepatocellular carcinoma [5] and lung cancer [12]. Lower ANXA1 expressions were observed in the metastases than the primary tumors for gastric cancer [13]. However, it is unclear how ANXA1 is expressed in breast primary tumors and metastases, and whether there is any association with malignancy and survival.

In this study, we evaluated the expression of ANXA1 in primary breast tumor and corresponding lymph node metastasis and determined its clinical and prognostic significance.

Materials and methods

Study design. A total of 175 cases, including primary breast cancer (n=135), fibroadenoma (n=20) and non-tumor breast

(*n*=20), were enrolled in this study. Non-tumor breast tissue were from normal parts of resected breast lobule hyperplasia.

A total of 135 patients with breast cancer, including 107 patients with lymph node metastases, had undergone radical mastectomy or modified radical mastectomy in the First Affiliated Hospital of Sun Yat-sen University from July 1999 to July 2003. The patient group ranged in age from 29 to 82 (median 50) years. None of these patients received preoperative chemotherapy or radiotherapy. Postoperatively, in patients aged less than 50 years, with node-positive, or estrogen receptor (ER) negative and/or tumor size >3cm, adjuvant chemotherapy regimen CMF (cyclophosphamide, methotrexate and 5-fluorouracil), CAF (cyclophosphamide, adriamycin and 5-fluorouracil) or taxanes-based chemotherapeutics was performed. While patients aged over 50 years, with ER negative, node-positive tumor also received CMF, CAF or taxanes-based chemotherapeutics, regardless of tumor size. Disease stage was classified according to the pTNM staging of American Joint Commission on Cancer criteria (AJCC, 6th ed, 2002) for breast cancer. In our study, disease stage was from I to III stage. Histopathologic classification was based on WHO (2003) Classification of Breast Tumor Histology. Repeated follow-up was performed on patients with breast cancer every 3 months for the first 18 months and then annually, and disease-free survival and overall survival were recorded from the date of surgery. The median follow-up was 59 months; (range 1 month – 7.4 years), and the end of the follow-up was in December 2006. A total of 130 patients were followed up, and 107 cases were still alive at the end of the follow-up. Samples used in this stydy were approved by the Committees for Ethical Review of Research involving human subjects at the First Affliated Hospital, Sun Yat-Sen University. The clinical and pathological features of these 135 breast cancer patients were summarized in Table 1

Tissue microarray (TMA). TMAs containing tissue samples of 20 non-tumor breast, 20 breast fibroadenoma and 135 primary breast cancer were establishedd using a manual arrayer (Beecher Instruments, Silver Spring, MD, USA). Haematoxylin-eosin-stained slides were examined to determine representative areas of the tumors from which core biopsies were taken. At least, 2 samples of different areas were taken from donor tissue blocks to a recipient paraffin wax block using techniques originally developed by Kononen et al.[14] The diameter of each core was 0.6 mm and the distance between of adjacent cores was 1 mm.

Immunohistochemistry. Consecutive 4-μm sections of paraffin-embedded tissue microarrays blocks were prepared and processed for ANXA1 protein staining. Sections were dewaxed in the 60˚C oven, then deparaffinized in xylene and rehydrated through a series of grade alcohols before all administration. The sections were routinely stained with hematoxylin-eosin for histological diagnosis and additional sequential sections served for immunohistochemical analysis. The slides were immersed in sodium citrate buffer (PH 6.0), heated on a microwave oven for 5 min and allowed to cool to room temperature. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 min. Nonspecific binding was blocked by incubating with normal goat serum for 10 min at room temperature. Afterward, slides were incubated with primary mouse monoclonal anti-ANXA1 antibody (dilution, 1:100; BD Transduction Lab, San Jose, CA) overnight at 4˚C, with secondary biotinylated antibody for 20 min at 37˚C, with peroxidase-conjugated streptavidin for 20 min at 37˚C, and in DAB peroxidase substrates solution until desired stain intensity developed. Between all steps we washed the slides twice with PBS (NaCl 80mg/ml, Na_2HPO_4 14.4mg/ml, KH_2PO_4 2.4mg/ml; PH 7.4) for 15 min each. Then, the TMA sections were counterstained with hematoxylin, dehydrated, and coverslipped. For the negative controls, bitinylated normal goat IgG was substituted for the anti-ANXA1 antibody. ER (mouse anti-human monoclonal antibodies; dilution, 1:50; DAKO), PR (mouse anti-human monoclonal antibodies; dilution, 1:50; DAKO) and HER-2/neu (rabbit anti-human polyclonal antibodies; dilution, 1:300; DAKO) were assessed respectively, in an overnight incubation, following standard immunohistochemistry procedures. The sections were photographed with a Spot RT color camera coupled to Nikon microscope.

*Evaluation of immunostaining.*The immunoreactivity was evaluated by determining the percentage of positive cells in each core and then taking the average of 2 cores. For ANXA1, positive result was defined as staining of 5% to 100% of tumor cells; negative result was defined as staining of < 5% tumor cells [15]. For ER, PR and HER-2/neu, positiveness was defined as 10% to 100% of moderate or strong nuclei stained positively; negativeness was defined as < 10% of tumor cells.

Statistical analysis. The correlations between the expression of ANXA1 and clinicopathological variables, ER, PR and HER-2/neu were analyzed for statistical significance by the chi-square test and Fisher's exact test. The Kaplan-Meier method was used to estimate survival rates, and the log-rank was used to assess survival-duration differences among groups. Statistical significance was considered as $P< 0.05$.

Results

ANXA1 localization in non-tumor, benign and malignant breast tissues. In this study, ANXA1 localization in non-tumor breast tissue was observed in cytoplasma in all myoepithelial cells, (Fig.1.a) as previously reported [16]. For breast fibroadenoma, ANXA1 expression also almost located in cytoplasma in myoepithelial cells (Fig.1.b)., In breast cancer tissues, immunoreactivity was observed mainly in tumor cells (Fig.1.d). A total of 135 cases with breast cancer, 59 cases expressed positively and 43 presented strong and moderate stainging.

Correlation between the expression of ANXA1 primary breast tumor and clinicopathological features of breast cancer patients.. In this study, microarray analysis revealed that expression of ANXA1 was lost in 76 cases out of 135 (56.3%), which is lower than previous observation [15]. ANXA1 expression negatively correlated with pTNM stage (P=0.019). Meanwhile, there was

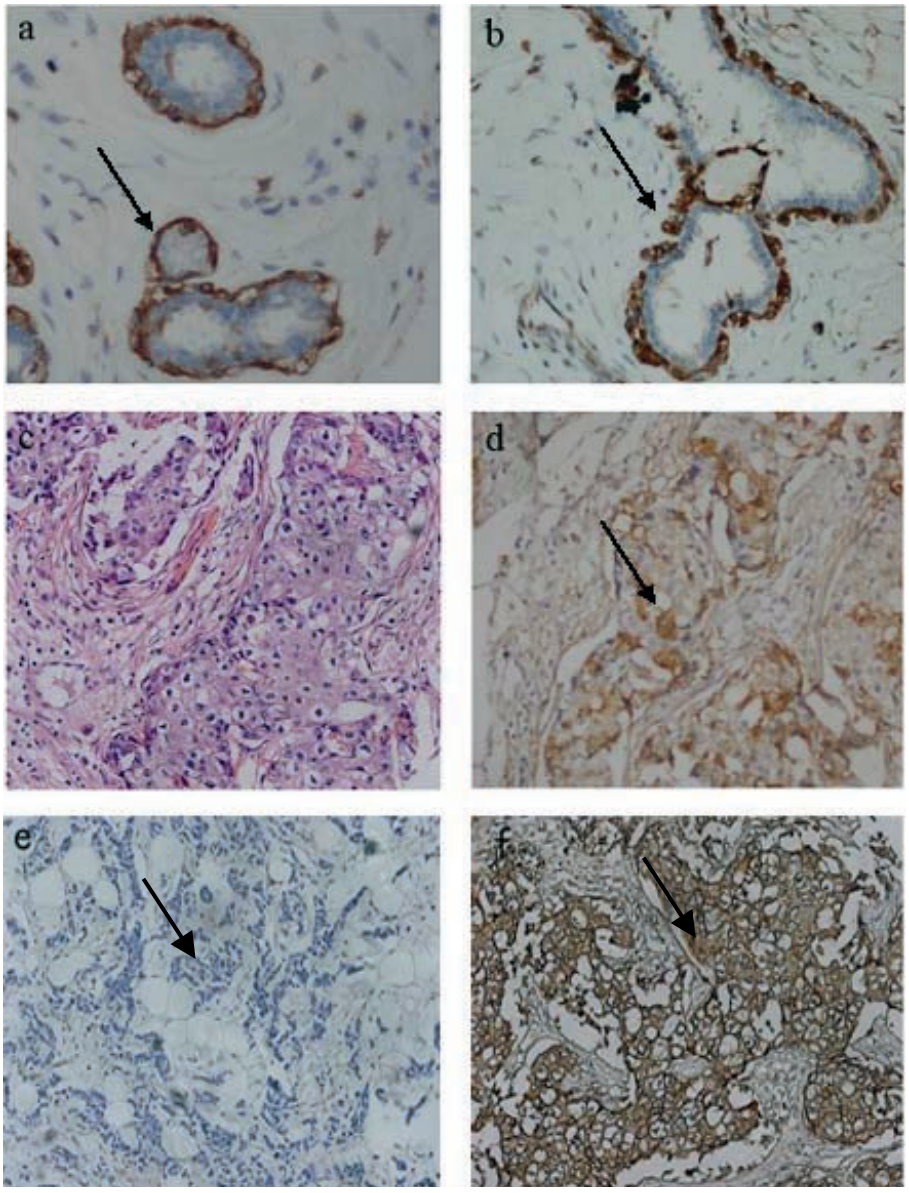

Fig. 1 ANXA1 expression in breast tissue samples. (a) ANXA1 stained mainly myoepithelial cells in normal breast tissue.×200. (b) ANXA1 stained mainly myoepithelial cells in breast fibroadenoma.×200. (c) HE staining of breast cancer tissue.×200. (d)ANXA1 stained mainly ductal cells and acini cells in breast cancer tissues.×200. (e, f)Negative ANXA1 expression in primary tissue and positive ANXA1 expression in metastasis tissue. ×200.

a significant correlation between negative ANXA1 expression and lymph node involved status (P<0.001). Moreover, there were no significant differences between ANXA1 expression and age and tumor size. In addition, we found that ANXA1 expression was not associated with ER, PR or HER-2/neu expression. (Table 1)

ANXA1 Expression in lymph node metastases. We further examined the level of ANXA1 in regional lymph node metastases. There were a total of 107 pairs of primary tumor and matched lymph node metastasis. Of them, 29 cases expressed positively both in lymph node metastasis and primary tumor, and 42 cases expressed positively only in lymph node metastasis. ANXA1-positive metastases Expressed stronger staining, in more advanced stages and poorer survival in our study. Interestingly, we found that lymph node metastases showed significantly higher ANXA1 expression than corresponding primary breast cancer (P<0.001) (Table 2) (Fig.1.e,f)

Correlation between negative ANXA1 expression and poor outcome in patients with breast cancer. Analysis of overall

Table 1 Patients grouping and the correlation between ANXA1 and clinicopathological features

Clinicopathological features	Total	ANXA1 expression		X^2	*P*
		Positive	Negative		
Age					
<50y	64	29	35	0.124	0.72
≥50y	71	30	41		
pTNM stage					
I	10	7	3	7.978	0.019*
II	55	29	26		
III	70	23	47		
pT stage					
pT I/II	89	40	49	0.163	0.686
pT III/IV	46	19	27		
pN stage					
pN 0	28	22	6	18.295	<0.001*
pN I	55	21	34		
pN II	33	11	22		
pN III	19	5	14		
histology					
Invasive ductal	124	53	71	–	–
Invasive lobular	1	1	0		
Mixed ductal/lobular	3	0	3		
Other	7	5	2		
Estrogen receptor					
Positive	69	25	44	3.203	0.074
Negative	66	34	32		
Progesterone receptor					
Positive	65	24	41	2.343	0.126
Negative	70	35	35		
HER-2					
Positive	53	23	30	0.003	0.954
Negative	82	36	46		

** P< 0.05 by chi-square test and Fisher's exact test.*

Table 2 The difference of ANXA1 between primary breast cancer and corresponding lymph node metastases

		Primary		total	χ^2	*P*
		positive	negative			
metastases	positive	29	42	71	13.593	<0.001*
	negative	8	56	64		
total		37	98	135		

** P< 0.01 by chi-square test*

survival by log-rank test demonstrated that ANXA1 negative group had a significantly worse overall survival than ANXA1 positive group (*P*=0.035) (Fig.2).The mean survival time for ANXA1 negative and positive groups was 73.7 months and 81.6 months, respectively. There were no significant associations between expression of ANXA1 and disease-free survival of breast cancer patients (*P*=0.551) (Fig.3).

Discussion

Published reports stated ANXA1 implicated in a broad range of molecular and cellular processes of physiology. It is a mediator of the anti-inflammatory actions of glucocorticoids in the host defense system [17]. It also contributes to the regulation of a variety of inflammatory pathways, cell proliferation, cell death signaling, differentiation and apoptosis. It is also reported in various tumors, but the findings are highly controversial and the underlying mechanism remains unknown. Moreover, the mechanism of how this protein participates in carcinogenesis and progression of tumor is not quite clear. Based on published reports and our work, we considered that alterations of ANXA1 expression are associated with histological origin and tumor stage.

we found that low ANXA1 expression was significantly associated with the advanced disease stage and worse overall survival compared with patients with high ANXA1 expression. Shen et al [18] reported down-regulation of ANXA1 expression in breast cancer. Cao et al. [15] revealed that ANXA1 expression was lost in 79% of breast carcinomas. This percentage is higher than 56.3% in this study. At the same time, we observed that ANXA1 expression was significantly negatively associated with patients axillary lymph node involvement. (*P*=0.004, this outcome didn't show in results), This result seems to be in agreement with recent studies [19, 20] which found ANXA1 expression up-regulated in non-metastatic breast cancer cell lines. Other similar studies showed that ANXA1 expression decreased in advanced head and neck cancer especially with lymph node metastases [9], and down-regulated in high-metastatic colorectal carcinoma cell lines [10]. These results indicate that ANXA1 expression was related to progression of breast cancer.

The current study suggests that ANXA1 have pro-apoptotic and antiproliferative functions. Hsieh et al. [21] illustrated that ANXA1 was involved in the calcium-mediated pathway after UV irradiation, showing pro-apoptotic function. Moreover, Alldridge LC and Bryant CE [22] found that ANXA1 has a cell-type independent, anti-proliferative function through sustained activation of the ERK signaling cascade. It indicates that loss of ANXA1 in breast cancer contributes to invasion and progression of breast cancer

On the other hand, ANXA1 was considered that it has anti-apoptotic function because of up-regulation in metastasis and anticancerogen-resistant tumor. In our study, a higher level of ANXA1 was observed in axillary lymph node metastases when compared with primary breast cancer (Table 2). A similar finding was observed by Shen [18] in human breast cancer and by Pencil et al [23] in rat metastatic mammary cancer. Shen et al[18] demonstrated that ANXA1 expression in breast cancer metastasis was higher in 13 cases but lower in 6 case. Pencil et al [23] found that ANXA1 protein levels was higher when the metastatic potential of rat adenocarcinoma cell lines

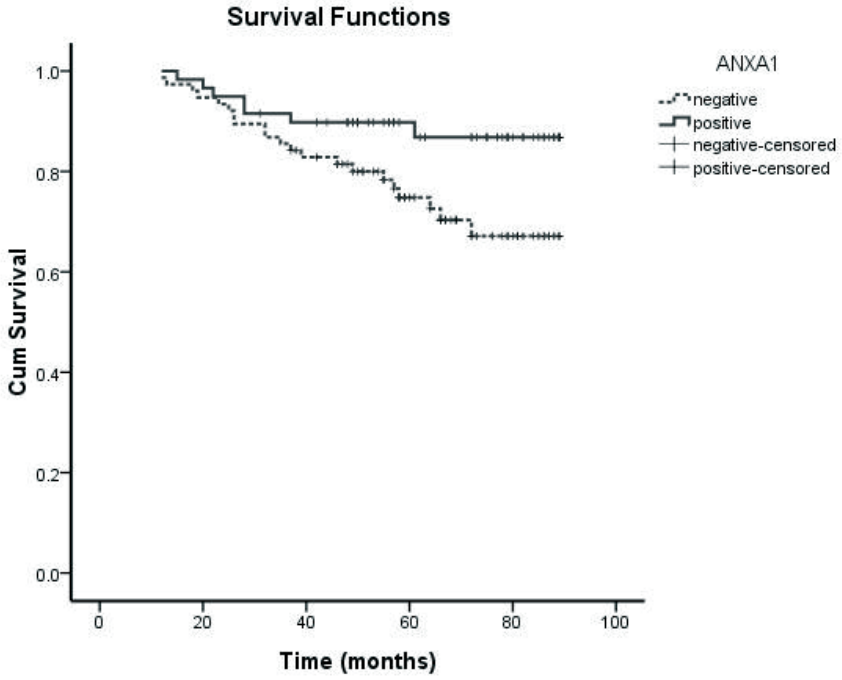

Fig. 2 Kaplan-Meier survival curves illustrating overall survival for 135 patients who had breast cancer with negative expression levels of ANXA1 versus positive expression levels of ANXA1 (*P*=0.035,Log-rank test).

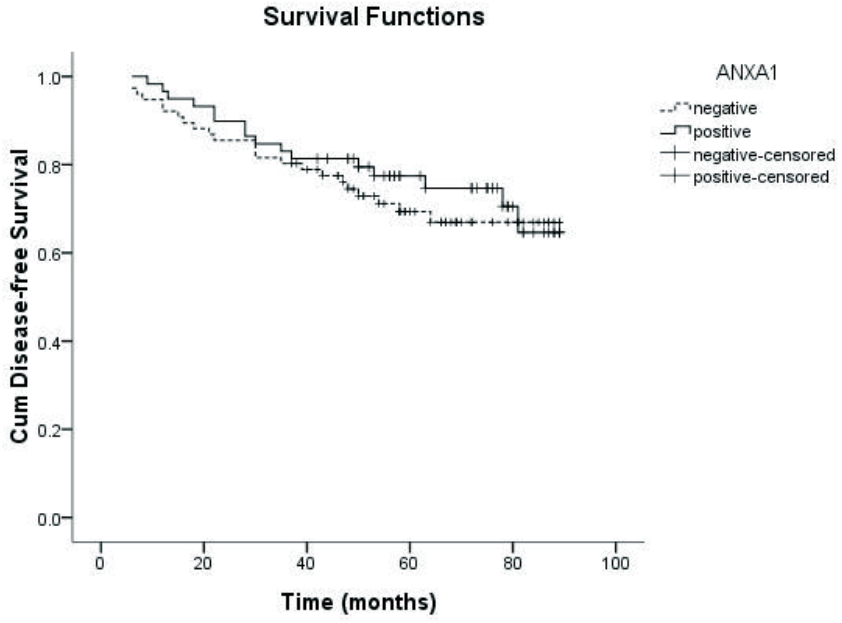

Fig. 3 Kaplan-Meier survival curves illustrating disease-free survival for 135 patients who had breast cancer with negative expression levels of ANXA1 versus positive expression levels of ANXA1 (*P*=0.551, Log-rank test).

(MTLn3, MTLn2, MTC.4: highest to lowest, respectively) via western blotting and flow cytometry. And ANXA1 expression of MTLn3 cells in lung metastases and primary tumor were 8-fold and 3-fold higher than that of cells in the MTC.4 primary tumor, respectively. This shows ANXA1 plays a critical role in human axillary lymph node metastasis of breast cancer.

There are some researches about mechanism. Yi and Schnitzer [24] found ANXA1 expressed on sprouting endothelial cells of normal mice whereas AnxA1-KO mice aortas exhibit impaired endothelial cell sprouting that is rescued by adenoviral expression of ANXA1, which showed that ANXA1 has pro-angiogenic function in vascular endothelial cell sprouting,and tumor growth and metastasis. Luthra et al. [25] observed that miRNA-196a correlated significantly inversely with ANXA1 mRNA levels in breast cancer cell lines, and miR-196a promoted cell proliferation, anchorage-independent growth and suppressed apoptosis, suggesting its oncogenic potential. It suggests that ANXA1 may have fuction of promoting tumor metastasis.

In summary, our results showed that the level of ANXA1 expression significantly decreased as primary breast tumor progressed, and increased in axillary lymph node metastasis. This interesting finding was similar with some published reports. Hao et al. [26] found differential gene and protein expression in primary breast malignancies and their lymph node metastases, they considered that in terms of expression levels, primary tumors were tightly clustered, whereas metastases exhibited a greater spread; this finding points to the more heterogeneous nature of metastases. Feng et al. [27] reported that 79 genes expressed differentially between primary cancers and metastasis, including matrix metalloproteinase 2 (MMP-2), fibronectin, osteoblast specific factor 2. Thus, we think that ANXA1 may carry out different mechanisms in primary versus metastatic breast cancer.

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