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ORIGINAL ARTICLE

The expression of *ANXA3* and its relationship with the occurrence and development of breast cancer

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Summary

Purpose: *To investigate the expression of ANXA3 in breast cancer tissues and normal breast tissues and its relationship with the clinicopathological features.*

Methods: *The expression levels of ANXA3 mRNA and protein were determined by fluorescent quantitative RT-PCR and Western blot, respectively, in 81 samples from breast cancer tissues and normal breast tissues. Flow cytometry, used to detect the cell cycle, further analyzed the differences in the cell proliferation indexes between cancer and the normal tissues. In addition, the correlation between the gene and protein expression levels of ANXA3 and the cell proliferation indexes in breast cancer tissues was calculated.*

Results: *The expression levels of ANXA3 mRNA and protein were significantly higher in breast cancer tissues than in control samples. Furthermore, their expression patterns were significantly different in patients with different axillary lymph node metastasis states, with or without vascular tumor thrombus, and with different molecular classifications. The expression of ANXA3 mRNA and protein in patients with triple-negative breast cancer was significantly higher than in Luminal A and B types. However, no significant differences were detected in the expression levels in other subtypes. Compared to normal tissues, the cell proliferation indexes of breast cancer tissues were significantly higher, and presented significant positive correlations with the ANXA3 gene and protein expression levels.*

Conclusion: *The expression of ANXA3 might play a crucial role in promoting the occurrence, development, and metastasis of breast cancer. It could serve as an important marker involved in the invasion and metastasis states, and the prognosis of breast cancer.*

Key words: *annexin A3, breast tumor, cell cycle, cell proliferation, histological grading, lymph node metastasis*

Introduction

Annexin A3, or *ANXA3*, plays an important role in the formation, proliferation, apoptosis, and signaling of tumor cells. Changes in the expression level of *ANXA3* can greatly affect the occurrence, development, drug-resistance, and metastasis of tumors [1-3].

However, the effects and roles of *ANXA3* in breast cancer are largely unclear. Breast cancer is one of the most common cancers in females around the world and its incidence is increasing every year. It is therefore of considerable importance to investigate the mechanisms involoved in the development, progression, invasion and metastasis of breast cancer for the improvement of the therapeutic approaches and patient survival.

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Methods

Sample collection

Samples were collected from the Breast Disease Center in the Fourth Hospital of Hebei Medical University, Hebei, China. Eighty-one samples from breast cancer tissues with complete information were collected from April 2015 to October 2015. All the patients were female, aged 29 to 80 years (median 50). Based on the third edition of WHO pathological classification of breast tumors, the 81 breast cancer samples were categorized into 66 cases of invasive ductal carcinoma, 4 cases of invasive lobular carcinoma, 2 cases of invasive carcinoma with mixed ductal and lobular features, 1 case of cribriform carcinoma, 1 case of secretory carcinoma, and 7 cases of ductal carcinoma *in situ*. According to the TNM tumor staging classification (Version 6) of AJCC (2003), the 81 cases were classified as 11 stage I cases, 50 stage II, 19 stage III, and 1 stage IV case. In order to obtain the normal (control) samples, 0.5 cm×0.5 cm sized breast tissues were randomly collected at the time of routine sampling, from regions located >7 cm away from the cancer tissues. If these samples were confirmed to be normal breast tissues through pathological examination, they were used in the study as the control group.

Main reagents

RNA isolator total RNA extraction reagent, HiScript II 1st Strand cDNA Synthesis Kit, and qPCR SYBR Green Master mix were purchased from Vazyme, China. Primers were synthesized by Shanghai Sheng Gong Company (China). PVDF membranes were purchased from Bio-Rad (Hercules, CA, USA). Rabbit anti-human antibodies for *ANXA3* were purchased by Abcam (Cambridge, UK), and mouse anti-human antibodies for β-actin were purchased from Proteintech (Rosemont, IL, USA).

Detection of ANXA3 mRNA expression in samples using fluorescent quantitative RT-PCR

The fresh breast cancer tissues and normal tissues were rinsed twice with cold phosphate buffered saline (PBS), and 1 ml RNA isolator was added into each sample. The routine one-step method was adopted to extract total RNAs which were then reverse-transcribed into cDNAs following the manufacturer's instructions. The cDNAs were used as templates to perform the PCR amplification (Eppendorf, Hamburg, Germany). Human *GAPDH* was used as an internal reference for normalization. Standard RT-PCR procedures were carried out with SYBR-Green I as the fluorescent dye. Three replicates for each sample were tested by RT-PCR. The upstream primer sequence for *ANXA3* was 5'-TCCGAAACATCTGGTGAC-3', while the downstream primer sequence was 3'-TCAAGTTCTTCGTAATACCGAT-5'. The upstream primer sequence for the internal reference *GAPDH* was 3'-CACTACCGTACCTGACACCA-5', and the downstream sequence was 3'-ATGTCGTTGTCCCACCACCT-5'. Quantitative PCR was performed using the Mx3000P PCR machine (Agilent, Santa Clara, CA, USA). The formula $2^{-\Delta Ct}$ was used to calculate the relative expression levels of *ANXA3* mRNA, where $\Delta Ct=CT_{ANXA3}-CT_{GAPDH}$. (Ct=cycle threshold).

Detection and quantification of ANXA3 proteins using Western blot

The breast cancer as well as the normal tissues were washed twice with cold PBS. Cell lysates were used to extract total cellular proteins and the protein concentration was detected using the bicinchonininc acid (BCA) method. 10% SDS-PAGE was employed to isolate the proteins, with a loading volume of 50 μg/well. The proteins were then transferred onto the PVDF membrane and sealed with 5% non-fat milk powder at 4°C overnight. Mouse anti-human *ANXA3* monoclonal antibodies (1:500 diluted) or 1:4000 diluted rabbit anti-human β-actin monoclonal antibodies were further added to the system, and incubated together at room temperature for 1 hr. The membrane was subsequently washed three times, and fluorescent-labeled secondary antibodies (1:20000 dilution) were added. The reaction system was incubated at room temperature for 2 hrs. The membrane was then examined using infrared imaging. β-actin was used as the internal reference to calculate the relative expression levels of *ANXA3* protein. The experiments were repeated three times.

Analysis of cell cycle using flow cytometry

The samples from cancer tissues and normal tissues, fixed by 70% alcohol, were washed twice with cold PBS. The tissues were then made into single cell suspensions by the net-twist method. The cell concentration was adjusted to 1×10^7 /ml. For each sample, 100 μl of cell suspension was added into the test tube, and 1 ml of propidium iodide (PI) dye solution was also added. The cells were stained in the dark at 4°C for 30 min and then washed once with cold PBS. Afterwards, 1 ml of PBS was added into the cells, and the DNA quantity of the cells was assessed using an Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). Multicycle advanced version (AV) was subsequently used to analyze the cell cycle. The formula for calculating the cell proliferation index (PI) was $PI=(S+G_2/M)/(G_{0/1}+S+G_2/M)\times100\%$.

Statistics

Statistical analyses were performed using the SPSS11.5 statistical software package. The data were presented as mean±standard deviation. One-way ANOVA was used to compare the means of multiple groups of samples, among which each two groups were compared by the least significant difference (LSD) test. Paired *t*-test was employed to compare two groups of samples. Correlation analysis was performed using Pearson correlation analysis, where $p<0.05$ was considered statistically significant.

Results

The expression level of ANXA3 mRNA as detected by fluorescent quantitative RT-PCR

Based on the results of fluorescent quantitative RT-PCR, *ANXA3* mRNA expression in breast cancer tissues was significantly higher than in normal breast tissues ($p<0.01$; Figure 1).

The expression level of ANXA3 proteins as detected by Western blot

The level of *ANXA3* protein was found to be significantly higher in samples from breast cancer tissues than in normal breast tissue samples ($p<0.01$). The Western blot results have been illustrated in Figure 2.

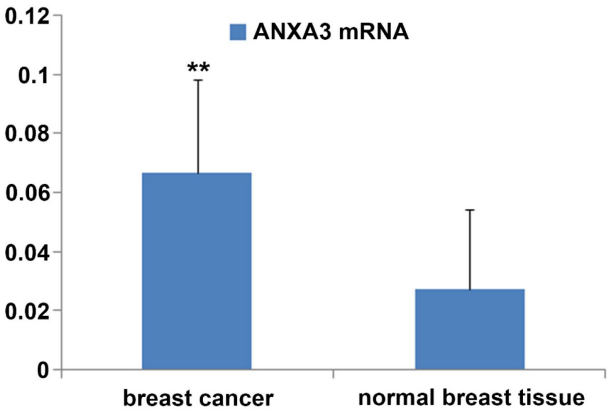

Figure 1. The expression level of *ANXA3* mRNA in breast cancer tissues and normal breast tissues detected by fluorescent quantitative RT-PCR. **p<0.01 *vs* normal breast tissue group.

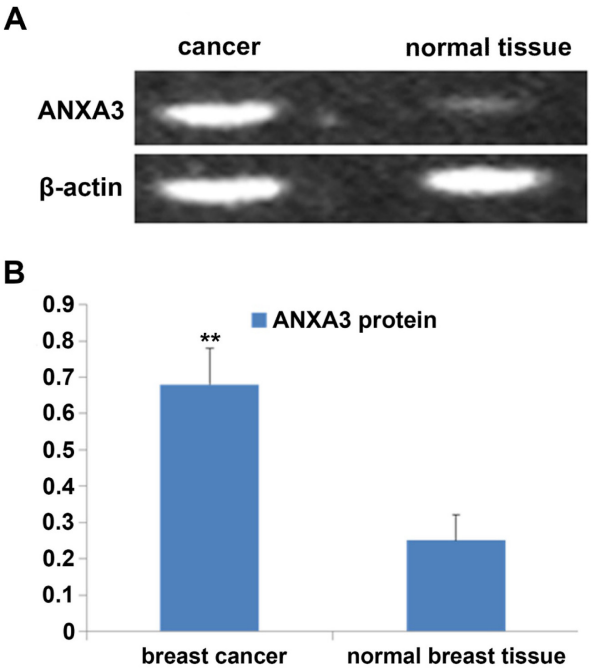

Figure 2. Level of *ANXA3* protein in breast cancer tissues and normal breast tissues, as detected by Western blot. **A:** a representative Western blot for *ANXA3* protein expression levels in different samples. **B:** The mean expression level of *ANXA3* protein in breast cancer tissues and normal breast tissues. **p<0.01 *vs* normal breast tissue group.

Table 1. Relationship between the expression level of *ANXA3* mRNA in breast cancer tissues and patients' clinicopathological features

Clinicopathological features	*n*	*ANXA3 mRNA expression Mean±SD*	*F*	*p value*
Lymph nodes			4.505	0.014
0	43	0.0577±0.0363		
1-3	21	0.0733±0.0030		
≥4	17	0.0815±0.0306		
Tumor size, cm			1.773	0.177
≤2	24	0.0750±0.0284		
>2, ≤5	54	0.0622±0.0326		
>5	3	0.0814±0.0116		
Histological grade			0.393	0.758
I	2	0.0581±0.0112		
II	40	0.0679±0.0211		
III	21	0.0705±0.0454		
Unclear	18	0.0605±0.0331		
Vascular tumor thrombus			3.879	0.025
Visible	18	0.0841±0.0321		
Invisible	36	0.0607±0.0232		
Unclear	27	0.0631±0.0368		
Molecular classification			2.790	0.046
Luminal A	13	0.0627±0.0185		
Luminal B	49	0.0631±0.0301		
HER2+	10	0.0647±0.0434		
Triple-negative	9	0.0943±0.0281		

Relationship between ANXA3 mRNA and protein expression levels and patients' clinicopathological features

The expression level of *ANXA3* mRNA in samples from breast cancer tissues were related to the axillary lymph node metastasis, vascular tumor thrombus, and molecular classification of tumors (p<0.05; Table 1). However, the *ANXA3* mRNA expression was not related to the tumor size or the histological grading (p>0.05; Table 1). More specifically, the level of *ANXA3* mRNA expression was found to be statistically higher in patients with lymph node metastasis than in patients without such metastasis (p<0.05); in patients with vascular tumor thrombus than in patients without thrombus (p<0.05); and in triple-negative patients than in patients with other molecular classifications (p<0.05). Concerning the relationship with lymph node metastasis, vascular tumor thrombus, molecular classification, and tumor size and histological grading, the expression of *ANXA3* protein followed the same pattern as the expression of *ANXA3* mRNA in breast cancer samples (Table 2).

Cell cycle of samples as detected by flow cytometry

The results of flow cytometry revealed that the percentage of $G_{0/1}$ cells in samples from breast cancer tissues was significantly lower than the value observed for samples from normal breast tissues (58.85±9.32% vs 82.52±3.86% ; p<0.01). However, the cell proliferation indexes of breast cancer tissues were significantly higher than the indexes estimated for normal breast samples (41.15±9.32% vs 17.48±3.86% ; p<0.01; Figure 3).

Correlation between ANXA3 expression levels and cell proliferation indexes

The *ANXA3* mRNA and protein expression levels in breast cancer tissues were positively correlated with the cell proliferation indexes (Pearson correlation coefficient 0.304 for mRNA; p= 0.006 and 0.341 for proteins; p=0.002

Discussion

Annexins are proteins of a structurally related calcium-dependent phospholipid-binding protein superfamily, accounting for about 2% of the total

Table 2. Relationship between the level of *ANXA3* protein in breast cancer tissues and patients' clinicopathological features

Clinicopathological features	*n*	*ANXA3 protein expression Mean±SD*	*F*	*p value*
Lymph nodes			7.008	0.002
0	43	0.6549±0.0836		
1-3	21	0.6783±0.1186		
≥4	17	0.7572±0.0929		
Tumor size, cm			0.729	0.486
≤2	24	0.6756±0.1005		
>2, ≤5	54	0.6816±0.1031		
>5	3	0.7514±0.1208		
Histological grade			1.008	0.394
I	2	0.6063±0.0991		
II	40	0.6723±0.0943		
III	21	0.7101±0.1055		
Unclear	18	0.6810±0.1162		
Vascular tumor thrombus			3.288	0.043
Visible	18	0.6514±0.0921		
Invisible	36	0.7139±0.0950		
Unclear	27	0.6611±0.1101		
Molecular classification			6.345	0.001
Luminal A	13	0.6446±0.0968		
Luminal B	49	0.6688±0.0906		
HER2+	10	0.6863±0.1260		
Triple-negative	9	0.8066±0.0557		

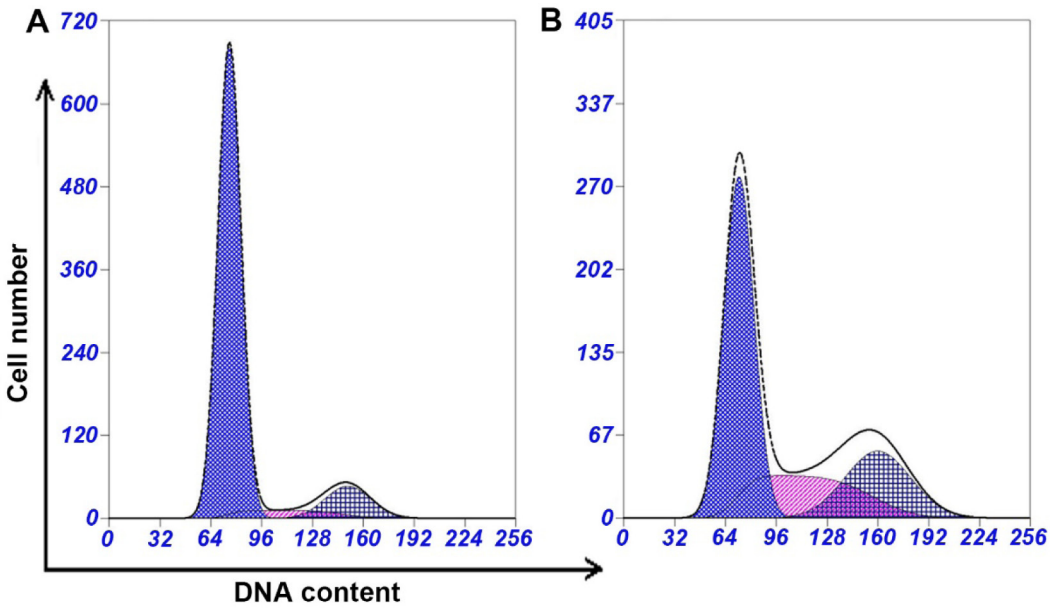

Figure 3. Flow cytometry results of **(A)** normal breast tissues and **(B)** breast cancer tissues. The cell proliferation indexes of breast cancer tissues were significantly higher compared with the indexes estimated for normal breast tissues.

cellular protein [5,6]. They are involved in membrane transport and a series of calmodulin-dependent activities happening at the membrane surfaces, including the transport of vesicles, membrane fusion in exocytosis, signal transduction, and the formation of calcium channel. They can regulate the inflammatory response, cell differentiation, and the interactions among cytoskeleton proteins. In the occurrence and development of tumors, ANXA can mediate cell signaling pathways, cell movement, tumor invasion and metastasis, angiogenesis, cell apoptosis, and drug resistance [7].

ANXA3 (Annexin A3) is a member of the Annexin superfamily. Studies have shown that the *ANXA3* gene is located in the chromosome 4q13-q22, and the protein consists of 323 amino acid residues [8,9]. The changes in *ANXA3* expression levels may have an important influence on tumor occurrence and development, drug resistance, and metastasis. Studies have shown that *ANXA3* expression is significantly higher in pancreatic and colorectal cancer samples than in benign samples, and significantly lower in prostate cancer samples than in benign prostate tissues [10]. Furthermore, several studies have revealed that the negative staining of *ANXA3* is an independent prognostic factor for prostate cancer [11-14]. These findings indicate that *ANXA3* can be used as a potential biomarker for the diagnosis, treatment, and prognosis of tumors.

In the current study, the expression level of *ANXA3* mRNA and protein were detected by fluorescent quantitative RT-PCR and Western blot, respectively. The expression of *ANXA3* mRNA and protein levels were significantly higher in the breast cancer tissues than in normal breast samples. This implied that the expression of *ANXA3* could be tissue-specific. The upregulated expression of *ANXA3* mRNA and protein in samples from breast cancer tissues further suggested that *ANXA3* might play a role in promoting the development and progression of breast cancer. Combining the data on the expression patterns of *ANXA3* mRNA and protein with the clinicopathological information, the expression levels were found to be related to axillary lymph node metastasis. Further analysis showed that the *ANXA3* mRNA and protein expression levels in samples from patients with ≥4 metastatic lymph nodes were significantly higher than in samples from patients without lymph node metastasis. These observations were in agreement with the results of Liu et al. [15] that *ANXA3* expression was significantly higher in lung cancer tissues with lymph node metastasis than in tissues without lymph node metastasis. Moreover, Chao Zeng et al. [16] reported that *ANXA3* expression was positively correlated with axillary lymph node metastasis. Taken together, these findings suggested that *ANXA3* might promote tumor metastasis.

Our study also revealed a significant relationship of *ANXA3* mRNA and protein expression levels with vascular tumor thrombus and molecular classification of tumors, while they were not found to be related to tumor size or histological grading. Further evaluation of the molecular classification

revealed significantly higher *ANXA3* mRNA and protein expression levels in triple-negative breast cancer patients than in Luminal A and Luminal B patients. However, no significant differences in *ANXA3* expression among other subtypes were observed. A large number of studies [17-20] has demonstrated that the prognosis of triple-negative breast cancer is worse than other subtypes, indicating that the upregulation of *ANXA3* expression might be associated with poor prognosis. These results implied that *ANXA3* might be involved in the occurrence, development, and metastasis of breast cancer, and can possibly be related with prognosis. Therefore, *ANXA3* can potentially serve as a marker of high value for determining the invasion, metastasis, and prognosis of breast cancer.

Data from flow cytometry established that the proliferation indexes of samples from breast cancer tissues were significantly higher than those of normal breast tissues; this result is in line with previous observations that malignant tumors have strong proliferative abilities [21]. Based on the statistical analysis, the expression levels of *ANXA3* mRNA and protein in breast cancer tissues were positively correlated with cell proliferation indexes. This suggested that the abnormally high expression of *ANXA3* in breast cancer tissues might promote the proliferation of breast cancer cells, and thus support the occurrence, development, and metastasis of breast cancer. Our study, therefore, offers an experimental basis to investigate and identify biological indicators that are essential for the early diagnosis and prognosis of breast cancer.

Conflict of interests

The authors declare no conflict of interests.

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