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Original Article

Correlations of contrast-enhanced ultrasound parameters with CerbB-2 gene expression in breast cancer lesions

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Abstract: Objective: To investigate CerbB-2 gene expression in breast cancer lesions and discuss its associations with contrast-enhanced ultrasound parameters, so as to provide guidance for subsequent studies. Methods: A total of 118 patients with breast cancer admitted to and treated in our hospital were enrolled. Breast lesions and surrounding tissues were observed by the contrast-enhanced ultrasonography. The protein and messenger ribonucleic acid (mRNA) expression of CerbB-2 and Akt in relevant tissue specimens obtained after operation were detected using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot, respectively, and the surgical specimens were tested by virtue of pathological examination and immunohistochemistry. Correlation analysis was determined between CerbB-2 protein expression in breast cancer with ultrasonographic features. Results: The relative expression of protein and mRNA of CerbB-2 and Akt in para-carcinoma tissues were remarkably lower than those in carcinoma tissues, displaying significant differences ($P<0.05$). The immunohistochemistry results indicated that there was a significant difference in the relative expression of CerbB-2 protein between carcinoma tissues and para-carcinoma tissues ($P<0.05$). The contrast-enhanced ultrasound parameters revealed that CerbB-2 protein expression had no significant associations with tumor size ($P>0.05$), but had significant differences in tumor number, enhancement boundary and degree of vascular invasion ($P<0.05$). Conclusion: The results of this research indicate that the CerbB-2 gene is highly expressed in the breast cancer lesions in a relatively high proportion of patients. Moreover, among the contrast-enhanced ultrasound parameters, the tumor number, enhancement boundary and degree of vascular invasion can predict the CerbB-2 expression level in the patient's lesions to some degree.

Keywords: Contrast-enhanced ultrasound, breast cancer, CerbB-2

Introduction

Breast cancer is the most prevalent tumor among women [1], and patients with the disease are becoming younger and younger. According to the prediction of World Health Organization (WHO), the current incidence rate of breast cancer is rising rapidly at a rate of nearly 2% every year. It is estimated that by 2020, the annual number of newly diagnosed breast cancer patients will reach 1.5-2 million, so that the detection, prevention and treatment of this disease is a great challenge facing humans. The occurrence and development of breast cancer are not only correlated with gene expression or dysregulation at lesion sites but is also related to local neovascularization. Appropriate living habits are important factors for preventing the occurrence of breast cancer, but currently the screening for the disease is the only means of improving therapeutic effects and reducing mortality rate. Early detection and timely treatment of breast cancer can result in good prognosis [2].

Ultrasound plays a vital role in the diagnosis of breast lesions [3]. New imaging techniques, including three-dimensional ultrasound, elastography and contrast-enhanced ultrasound, can analyze the shape, elasticity and flow of the breast lesions. Contrast-enhanced ultrasound, which was first applied to examine the breast lesions in the early 1990s, has been improving quickly over the past few years [4, 5]. However,

the micro-vessels of breast lesions cannot be detected by color Doppler ultrasound due to the low flow rate and patient's breathing or heart beat artifacts [6]. A majority of existing studies involve the application of contrast media to enhance the signal of color Doppler ultrasound. Various studies have demonstrated that the contrast media used for contrast-enhanced ultrasound are limited to the vessel lumen, which are still capable of improving the signal of color Doppler ultrasound [7-9].

The dynamic characteristics of contrast-enhanced ultrasound provide favorable methods for differential diagnosis [10]. In benign breast lesions, the blood vessels are distributed unilaterally and circumferentially and tapering regularly; while in malignant breast lesions, vascular tortuosity can be detected by ligating the blood vessels [11]. In addition, the malignant breast masses have more peripheral vessels than benign breast masses after baseline or contrast agent administration [12].

Through analysis of vascular morphology and physical properties, the contrast-enhanced ultrasound is able to identify the characteristic of breast neoplasms to some extent. The existence and expression changes of genes and cytokines are involved in the occurrence of breast cancer, and such changes may induce alterations of the local state of tissues. Whether correlations in the existence and expression of genes or cytokines with imaging parameters can be found and utilized via imaging methods is worth studying.

Materials and methods

Research samples

A total of 118 female patients with breast cancer treated in our hospital from August 2012 to June 2015 were enrolled. They were aged between 26-68 years old, with an average age of (46.6±13.7) years old. Inclusion criteria: Atypical ductal hyperplasia diagnosed by vacuum assisted breast biopsy performed on a single group of calcifications; absence of residual post-procedure, checked with mammogram performed immediately after the biopsy. Exclusion criteria: the presence of a personal history of breast cancer or other high-risk lesions, the presence of breast cancer gene mutations; association with other synchronous lesions, both more and less advanced proliferative lesions; the presence of multiple foci of hyperplasia or high percentage of hyperplasia. Routine blood biochemistry, ultrasound and other examinations were performed after admission and treatment. This research was approved by the Ethics Committee of our hospital, and signed informed consent was obtained from every patient before operation.

Contrast-enhanced ultrasound

IU22 ultrasonic scanner was adopted for ultrasonic examination. After conventional ultrasonic examination of the mammary gland, the lesions with rich blood supply or those in the most irregular shapes were selected as the target planes of the contrast-enhanced ultrasound examination. The contrast media was prepared according to common methods. Briefly, 59 mg SonoVue powder was mixed with 5 mL salt water and shaken sufficiently, so as to produce a suspension. The contrast-enhanced ultrasound examination was conducted through venous cannula after intravenous injection of 4.8 mg contrast media, followed by injection of 5-10 mL salt water. Real-time images were utilized to record the whole process of the examination for further analysis. The planes selected remained unchanged during the examination. The contrast-enhanced ultrasound images also contained lesions and surrounding tissues in the enhanced mode evaluating the breast lesions. As for lesions with a maximum diameter over 40 mm, a 2-5 MHz transducer was selected. During the examination, the patients maintained normal breathing as much as possible to reduce motion artifacts. All the images were read by two ultrasound physicians who had at least 6 years of experience in breast ultrasonic examination. Both examiners had no knowledge of the patients' clinical data and final pathological findings. The ultrasound physicians could observe the changes of each frame in the images and the enhanced mode in detail, and they could determine the perfusion defects, enhanced lesion edges, lesion shape, and enhancement time, etc.

Ribonucleic acid (RNA) extraction and fluorescence quantitative polymerase chain reaction (PCR)

TRIzol reagent method was adopted for one-step lysis and extraction of total RNA in the

Table 1. Primers used in fluorescence quantitative PCR

Name	Primer pair
CerbB-2	F: TGCTTTGGTTTGGGTGATTGCAGTCTCT
	R: CTTTGCTTTTACTGTCCTCTGCTAATGAG
Akt	F: CTGAGGTTGGCTCTGACTGTACCACCATCCC
	R: CTCATTCAGCTCTCGGAACATCTCGAAGCG
β-actin	F: ATCGGTCTTCCTATCCTGGGCTATTGCTGC
	R: TGCTGTCTTTGCGGGATGTCTCACGCATT

breast tissues, and messenger RNA (mRNA) in the total RNA was reversely transcribed using the Moloney murine leukemia virus (M-MLV) reverse transcription system in the reverse transcription kit. The fluorescence quantitative PCR was performed in the StepOne Plus system in accordance with the kit, with β-actin as the internal control for relative quantification of detected genes. The primer sequences applied are shown in **Table 1**. The fluorescence quantitative PCR was performed in triplicate, and a non-template control was included, so as to verify the reliability of the trails. The relative expression was expressed as $2^{-\Delta\Delta Ct}$.

Western blot (WB) assay

The proteins in the breast tissues were extracted, whose total concentration was measured by means of the protein quantification kit. Then the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane, followed by antibody reaction with corresponding antibodies (all diluted at 1:1000, Abcam, Cambridge, MA, USA) for 3 h, binding reaction with rabbit anti-mouse secondary antibody (1:5000, Santa Cruz, Santa Cruz, CA, USA) for about 3 h and color development by virtue of enhanced chemiluminescence. The primary antibodies used in this research included anti-CerbB-2 protein, p-Akt protein and β-actin (internal control). The grey-analysis was performed using ImageJ software.

Pathological analysis

All the patients underwent operations after the ultrasonic examination. The specimens were fixed in formaldehyde, embedded in paraffin, conventionally sliced and stained, and the pathological findings were regarded as the final diagnostic criteria.

Immunohistochemical staining

In order to investigate the CerbB-2 expression in the breast carcinoma and para-carcinoma tissues, the expression was detected using immunohistochemical staining. The pathological samples were deparaffinized, and then the tissue sections were placed in a constant-temperature incubator for about 20 min. Next, the sections were hydrated, followed by antigen retrieval. The parts with tissues in the sections were blocked with buffer drops, and then the sections were sealed and placed in a wet box for nearly half an hour. After that, the primary antibodies was added and put into the wet box in a refrigerator at 2-8°C overnight. Then the sections were washed with 0.1% PBT several times, given the appropriate secondary antibody and placed in the wet box for incubation and reaction for a certain period, followed by washing, color development, staining at room temperature for about half an hour, termination of reaction, section counterstaining and examination under a light microscope.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 16 software package was adopted for t-test and χ^2 test of data. Logistic regression analysis was used for correlation analysis between CerbB-2 protein expression in breast cancer with ultrasonographic features. $P<0.05$ suggested that the difference was statistically significant.

Results

CerbB-2 mRNA and Akt mRNA expression in breast carcinoma and para-carcinoma tissues detected via fluorescence quantitative PCR

The expression of CerbB-2 mRNA in the 118 patients were detected via real-time fluorescence quantitative PCR, and the results showed that the relative expression was (0.82±0.24) in breast carcinoma tissues and (0.22±0.09) in para-carcinoma tissues, with a significant difference ($P<0.05$). Similarly, the expression of Akt mRNA between tissue typs also had a significant difference ($P<0.05$, **Table 2**).

CerbB-2 and Akt proteins in breast carcinoma and para-carcinoma tissues detected via WB

The expression of CerbB-2 and Akt proteins in the patients were determined through WB

Table 2. CerbB-2 and Akt mRNA expressions detected via fluorescence quantitative PCR

Index	Breast carcinoma tissue (n=118)	Para-carcinoma tissue (n=118)	*P* value
CerbB-2	0.82±0.24	0.22±0.09	<0.0001 (t=25.43, df=234)
Akt	0.56±0.18	0.15±0.07	<0.0001 (t=23.06, df=234)

Note: P<0.05, significant difference. t test was used for measurement data comparison between two groups of breast carcinoma tissue and para-carcinoma tissue.

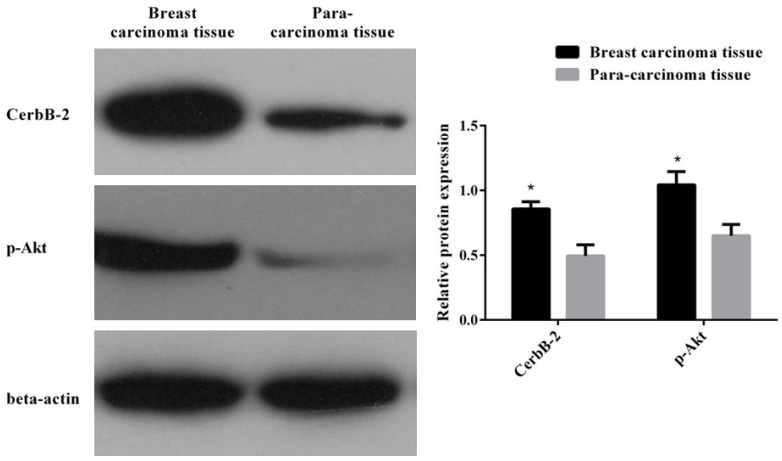

Figure 1. CerbB-2 and Akt protein expressions in tumor patients by western blot. *P<0.05: significant difference, compared to Para-carcinoma tissue.

(**Figure 1**). It was indicated that the relative expression of CerbB-2 protein was (0.57±0.28) in carcinoma tissues and (0.12±0.04) in para-carcinoma tissues, and the difference was statistically significant between the two groups (P<0.05). Meanwhile, the expression of p-Akt protein also had a significant difference between the two groups (P<0.05).

CerbB-2 protein in breast carcinoma and para-carcinoma tissues detected via immunohistochemistry

The expression of CerbB-2 protein in the 118 patients was further detected by means of immunohistochemistry. Typically, H&E staining indicated an increase in the endothelial cell size and an exudative infiltration of lymphocytes in breast carcinoma tissues compared to clear normal adipocytes and collagen in para-carcinoma tissues. The immunohistochemistry results revealed that the expression of CerbB-2 protein was markedly increased in the breast carcinoma tissues (**Figure 2**). CerbB-2 protein was highly expressed in breast carcinoma tissues of 66.90% (79/118) patients, but basically not expressed in para-carcinoma tissues.

Correlations of CerbB-2 protein expression in breast cancer with ultrasonographic features

As shown in **Table 3** and **Figure 3**, CerbB-2 protein expression had no significant associations with tumor diameter in breast carcinoma tissues (P>0.05), but it had significant differences in tumor number, enhancement boundary and degree, vascular invasion and lymphatic metastasis (P<0.05). In the ultrasonographic images, the proportion of unclear enhancement boundary, high enhancement, vascular invasion and lymphatic metastasis were 65.25% (77/118), 77.11% (91/118), 64.41% (76/118) and 67.80% (80/118), respectively.

Discussion

Proto-oncogene CerbB-2 has a close relation to the occurrence of breast cancer and exerts certain effects and functions in the growth of normal breast tissues. In a pathological state, however, tissue products can effectively promote the proliferation and differentiation of cancer cells. The breast cancer patients with overexpressed CerbB-2 often have very high malignancy of cancer and poor treatment effects.

The contrast-enhanced ultrasound can dynamically display the morphology and shape of microvessels in tumors, making it possible to identify early and differentially diagnose benign and malignant breast neoplasms [13]. In this research, the contrast-enhanced ultrasound for breast cancer manifested high enhancement and unclear boundary enhancement, accounting for 77.1% (91/118) and 65.3% (77/118), respectively; for which the reason

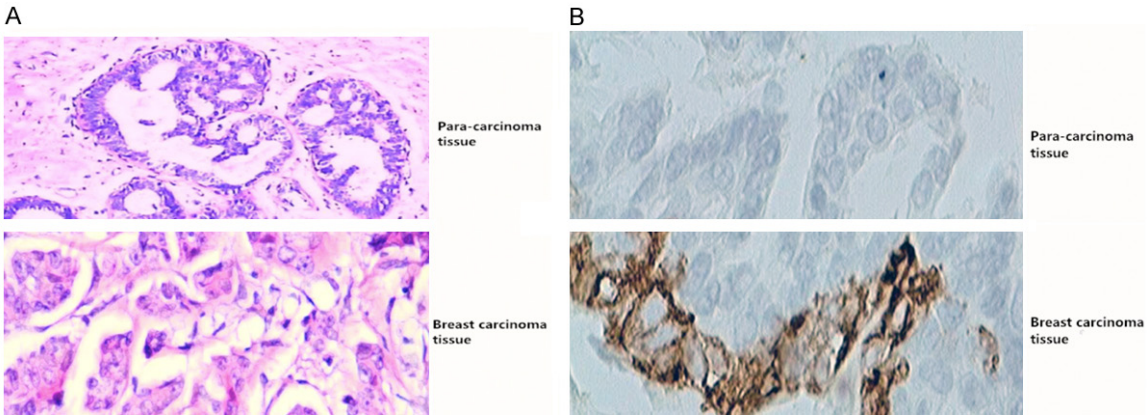

Figure 2. Pathological findings of patient with breast cancer. A. Pathology of carcinoma and para-carcinoma tissues. B. Immunohistochemical assay results of CerbB-2 protein in carcinoma and para-carcinoma tissues.

Table 3. Correlations of CerbB-2 protein expression in breast cancer with ultrasonographic features

Ultrasonic manifestation		n	CerbB-2 protein expression		χ^2	P
			Low expression	High expression		
Tumor diameter	<2 cm	45	26	19	0.015	0.94
	≥2 cm	73	53	20		
Tumor number	1	49	26	23	7.30	0.009
	>1	69	53	16		
Boundary	Clear	41	22	19	5.12	0.025
	Unclear	77	57	20		
Enhancement degree	High enhancement	91	66	25	5.59	0.018
	Low enhancement	27	13	14		
Vascular invasion	No	42	23	19	4.38	0.036
	Yes	76	56	20		
Lymphatic metastasis	No	80	59	21	5.71	0.017
	Yes	31	19	18		

Note: Enumeration data were presented as case (n), and chi-square test was adopted.

may be its association with the growth pattern of breast cancer. Proto-oncogene CerbB-2 is involved in neovascularization. Since the cancer cells need great amount of nutrient supply in the process of rapid growth, which cannot be satisfied by local nutrition, new blood vessels need to be generated through the regulation and signal transduction of oncogenes, thus increasing the number of blood vessels and enriching blood supply. The new blood vessels are not distributed evenly due to partial dysregulation of the signaling pathways, which are characterized by softness, thin walls, easy deformation and disordered structure in breast carcinoma tissues; thereby leading to good accumulation of ultrasound contrast agents in them. Angiography displays high enhancement. In breast carcinoma tissues, stronger enzyme activity of CerbB-2 indicates higher expression level of CerbB-2, more blood vessels and higher enhancement degree of the contrast-enhanced ultrasound, showing a positive correlation.

It is fairly hard to cure metastatic breast cancer, which requires intensive research on the molecules controlling or participating in the invasion and metastasis of breast carcinoma tissues. Receptor tyrosine kinase ErbB-2 is overexpressed in 30% breast carcinoma tissues, and tumors with ErbB-2 overexpression have high metastatic potential and poor prognosis [14, 15].

CerbB-2 is implicated in the proliferation of carcinoma tissues [16]. Some studies have dem-

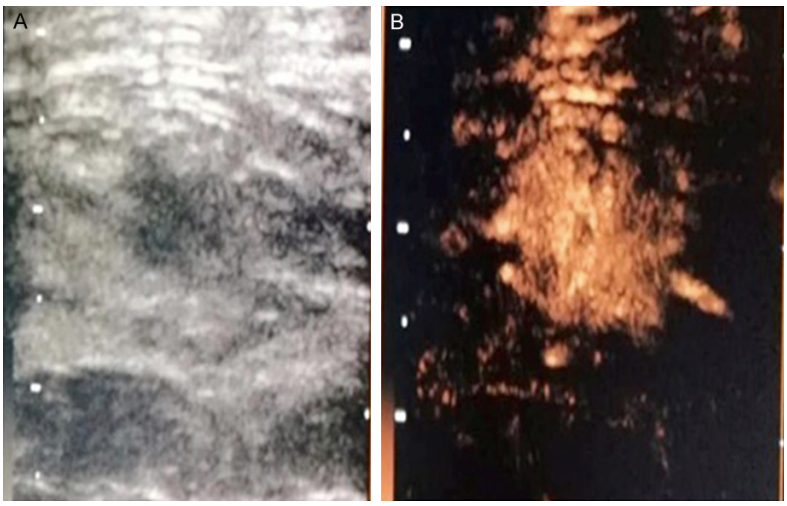

Figure 3. Ultrasonic examination of patient with breast cancer. A. Results of conventional ultrasound. Left breast had mass for 2 months. Conventional ultrasound showed irregular hypoechoic mass in the upper quadrant of the right breast with heterogeneous internal echo. B. Results of contrast-enhanced ultrasound. The lesion presented high enhancement, and the area of lesion was significantly larger than that of conventional ultrasound. The boundary was not clear, and the blood vessels showed "solar sign".

onstrated that CerbB-2 expression is positively correlated with the grade of tumor tissues and negatively correlated with the prognosis effect. Furthermore, the breast cancer patients with high CerbB-2 expression have relatively shorter survival time. Meanwhile, the CerbB-2 expression rate in intraductal papilloma with malignant tendency is lower than that in breast cancer but higher than that in benign breast diseases.

In this research, the results of contrast-enhanced ultrasound in high CerbB-2 expression group indicated more apparent characteristics of malignant tumors in the patients, including high enhancement, vascular invasion, lymphatic metastasis, unclear boundary enhancement and multiple lesions, suggesting that the expression intensity of CerbB-2 is prominently associated with the features of contrast-enhanced ultrasound. These results may be related to the overexpression of CerbB-2 in breast carcinoma tissues, which exerts signal regulating effects. It has been reported that some signaling proteins and pathways are conducive to enhancing the metastasis of breast cancer with CerbB-2 overexpression [17-19], which is related to transmembrane proteins, such as integrin [20, 21], and cytoplasmic signaling pathways, such as those mediated by Ras and Src or by PI3K/Akt pathway [22, 23]. All these findings are consistent with the result in this research that the expressions of Akt protein and mRNA were remarkably higher in carcinoma tissues than those in para-carcinoma tissues. However, in spite of considerable progress made in some basic studies, the signaling events mediating the invasion and metastasis of tumor cells with CerbB-2 overexpression have not been clarified yet, and there is still a lack of choices for treatment of breast cancer with CerbB-2 overexpression.

According to this research, the contrast-enhanced ultrasound parameters for breast cancer have certain correlations with CerbB-2 expression, which can partially reflect the malignancy and prognosis effects of breast cancer. In terms of the diagnosis, treatment and prognosis evaluation of breast cancer, the contrast-enhanced ultrasound results can provide technical guidance for clinicians in selecting treatment protocols and offer support for better clinical treatment.

Disclosure of conflict of interest

None.

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