Expression of *ERβ* gene in breast carcinoma and the relevance in neoadjuvant therapy

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Abstract. In the present study, we examined the expression of the estrogen receptor β (*ERβ*) gene in breast cancer and its relevance in neoadjuvant therapy. In total, 120 breast cancer patients who were hospitalized in the Departments of Breast Disease and Medical Oncology served as the subjects of this study. The subjects were diagnosed with breast cancer phase II to phase IIIA, as confirmed by aspiration biopsy and iconography. The patients were divided into two groups in a randomized control manner, with 60 patients in each group. The experimental group was administered the taxotere + epirubicin + cyclophosphamide (TEC) plan for 3-4 cycles of chemotherapy before the modified radical operation of breast cancer. In the control group, no TEC chemotherapy was carried out prior to operation. Instead, the breast lesion was removed directly by operation. After the operation, the IHC method was used to stain the ERβ protein in the lesion tissue. The patients were classified according to whether the basement membrane was broken through; 5 cases had non-infiltrative carcinoma and 115 cases had infiltrative carcinoma. According to the pathology of the lesion, 114 cases had breast ductal carcinoma, 2 cases had mucinous breast carcinoma (of which there were 2 cases combined with ductal carcinoma), and 4 cases had breast lobular carcinoma. The *ERβ* gene was found to be expressed in normal and breast cancer tissues. When *ERβ* gene expression was compared before and after the chemotherapy, its expression was significantly increased in breast cancer tissues, which shows a significant statistical difference (P<0.05). In the experimental group, the expression of *ERβ* gene in carcinoma tissue was significantly lower than that in the control group, and differences were statistically significant (P<0.05). Therefore, expression of the *ERβ* gene in breast carcinoma tissues was high. The application of adjuvant chemotherapy before the modified radical operation for breast carcinoma can significantly lower the level of ERβ expression. The expression levels of ERβ gene in the carcinoma tissue of the patients can be treated as the evaluation index for neoadjuvant chemotherapy. Regarding targeted therapy and corresponding drug development for breast carcinoma, ERβ can act as one of the specific drug targets.

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Introduction

Breast carcinoma refers to the malignant tumor of breast epithelial cells and accounts for over 20% of all malignant tumors diagnosed in women. Thus, it is a major cause of mortality that threatens the health of women (1). To improve the quality of life of breast carcinoma patients, various studies have been carried out combining surgical operation and post-operation adjuvant therapy (2). The etiological agents that lead to breast carcinoma and the mechanisms behind these causative agents are relatively complicated. Factors that increase the likelihood of breast cancer include family history, contact with radioactive rays, certain chemical substances, proto-oncogene activation and stimulation of biotic factors, which can all lead to the malignant proliferation or oncogenic mutation of breast epithelial tissue (3).

The formation of an adjuvant therapeutic schedule for breast carcinoma patients requires judgment which takes into account the combination of clinical classification and stage of the patients. Currently, we administer post-operation chemotherapy to treat breast carcinoma patients, the purpose of which is to reduce the postoperative recurrence as much as possible and prolong the disease-free survival and five-year survival rate. In terms of chemotherapy cocktails, we primarily administer taxotere + adriamycin + cyclophosphamide (TAC) and the taxotere + epirubicin + cyclophosphamide (TEC) plans (4-8), adriamycin amycin + cyclophosphamide + paclitaxel (AC-P) plan, adriamycin amycin + cyclophosphamide + taxol (AC-T)

plan and adriamycin amycin + cyclophosphamide (AC) plan. The mechanism of action of chemotherapy drugs is to curb DNA proliferation, mitosis or other biological processes through interference into the different phases of the cell cycle. Therefore, in most cases, these drugs lack specificity and damage normal tissues and cells of the human body (3,4). Previous findings showed that the combination of chemotherapy drugs and targeted therapeutic drugs can lead to good outcome for the treatment of breast carcinoma (4-6).

Estrogen receptor β (*ERβ*) gene encodes estrogen receptor β. Estrogen is of great significance in normal physiology, aging and the treatment of many diseases (9). The main function of the estrogen nuclear receptor is the transcription factor for the activation of ligand, which can mediate the genetic transcription of tissues and organs regulated and controlled by this hormone (10).

Since Mufudza *et al* (11) cloned the gene of estrogen receptor α (ERα), there have been numerous studies conducted on this estrogen receptor showing that, in normal breast tissue, there is only 7-10% of ERα expression and it is present mostly in the breast cells that are in their non-proliferation phase (11). However, the expression levels of ERα vary based on the menstrual cycle. Clinical studies have shown that the expression levels of ERα are closely associated with the degree of differentiation and grade of malignancy and location of the transfer of breast cancer cells (11-14). Therefore, some scholars consider that ERα can be treated as one of the factors in the evaluation of prognosis of breast carcinoma. However, due to the fact that ERα is only expressed in a small quantity of breast epithelial cells, the sensitivity and specificity of determining the prognosis of breast carcinoma by ERα is poor. ERβ is the new member of the super family of steroid hormone receptors. It has six functional zones: A-F (5,15). Leygue *et al* found ERβ mRNA expression in breast (16). Additionally, the ERβ mRNA was detected in breast carcinoma samples and the mammary epithelial cells of several types of individuals. The study by Lanfranchi and Brind showed that the ratio of ERα and ERβ in breast carcinoma patients varies as breast carcinoma develops (17). However, whether ERβ can be treated as the evaluation index for breast carcinoma treatment and prognosis remains to be determined.

Clinical data and pathological samples of 120 breast carcinoma patients were collected to study the expression levels of the *ERβ* gene in breast carcinoma and to examine its clinical relevance in neoadjuvant therapy.

Materials and methods

Sample selection. In the present study, we retrospectively analyzed the clinical data of 120 female patients with breast carcinoma who were hospitalized at the Department of Breast Surgery of the Affiliated Hospital of Shandong Academy of Medical Sciences. We collected and categorized clinical data including factors such as age, disease time, childbearing history, and menstrual history. According to the clinical performance, imagological examination, and pathological examination of patients, we classified and identified the stages of the breast carcinoma in patients and performed corresponding neoadjuvant chemotherapy TEC plans as well as an operation plan. Inclusion criteria were: Patients that were diagnosed with breast carcinoma pathologically. Exclusion criteria were: i) Presence of malignant tumors in other systems, excluding those with the breast carcinoma transferred to niduses; ii) patients without definite diagnosis; iii) patients with cognitive disorder or mental problems; iv) failure in getting the samples of the patients due to certain reasons; v) uncooperative patients or family members; vi) patients that quit from this study; and vii) patients with poor general condition, and thus unsuitable for the examination and treatment.

Neoadjuvant therapeutic schedule. The neoadjuvant therapeutic schedule, i.e., TEC plan, refers to the chemotherapeutic schedule of taxotere + epirubicin + cyclophosphamide cocktail. We carried out the TEC plan for 3-4 cycles before the operation with 3 weeks for each cycle. After chemotherapy, we performed a modified radical operation for breast carcinoma. Taxotere (Rhône-Poulenc Rorer, Paris, France) 75 mg/m^2 d1, (Famaxin; Pfizer, Inc., New York, NY, USA): 100 mg/m^2 d1, and cyclophosphamide (Baxter, Deerfield, IL, USA) 500 mg/m^2 d1 were administered.

Immunohistochemical methods. Breast carcinoma paraffin sections were dewaxed in water and 3% H_2O_2 then incubated for 10 min at 20˚C. The section was washed with distilled water and soaked in PBS for 5 min twice. The tissue was incubated with 5% PBS diluted goat serum (Life Technologies, Carlsbad, CA, USA) and sealed, and then incubated for 10 min at 20˚C. The goat serum was discarded and the primary rabbit polyclonal ERβ antibody (dilution, 1:100; cat. no. ab60709; Abcam, Cambridge, MA, USA) was added. The tissue was incubated for 1-2 h at 37˚C or stored at 4˚C overnight. We added the proper amount of the working goat monoclonal biotin-labeled anti-human IgG secondary antibody (dilution, 1:1,000; cat. no. NEF803001EA; PerkinElmer, Inc., Waltham, MA, USA) to the sample and incubated it for 10 min at 37˚C. HRP was added (S7571; Sigma-Aldrich, St. Louis, MO, USA), incubated for 10 min at 37˚C and 1-2 drops of DAB Plus Chromogen (TA-125-HD) was added to 1 ml DAB Plus Substrate (TL-125-HDX) (both from Thermo Fisher Scientific, Waltham, MA, USA). We blended and added drops to the section, incubated the section for 10 min at 37˚C, and washed and sealed the section.

Statistical analysis. SPSS 20.0 software (Chicago, IL, USA) was used to conduct statistical analysis. We described the continuous variable of the normal distribution using mean and standard deviation. When the continuous variables show skewed distribution, we described it using the median and quartile range. The absolute percentage was used for the classified variables. The Student's t-test was applied for the continuous variables. For a comparison of classified variables, the Pearson's χ^2 test was applied. The univariate analysis was applied to analyze the correlation between ERβ and the other factors.

Results

Clinical description and general conditions. We collected the data including age, weight, menstrual period, disease course and other information regarding general clinical conditions

Table I. Collection and analysis of the general clinical data of patients in the control and disease group (mean ± SD).

Groups	Cases (n)	Age (years)	Weight (kg)	Menstrual period (day)	Disease course (months)
Control	60	53.8±8.4	69.7±12.4	28.4±2.5	5.2±1.1
Disease	60	55.2±9.2	68.2±3.8	31.2±1.1	6.8±2.3
T-value	-	0.78	0.32	0.85	0.44
P-value	-	0.25	0.75	0.45	0.58

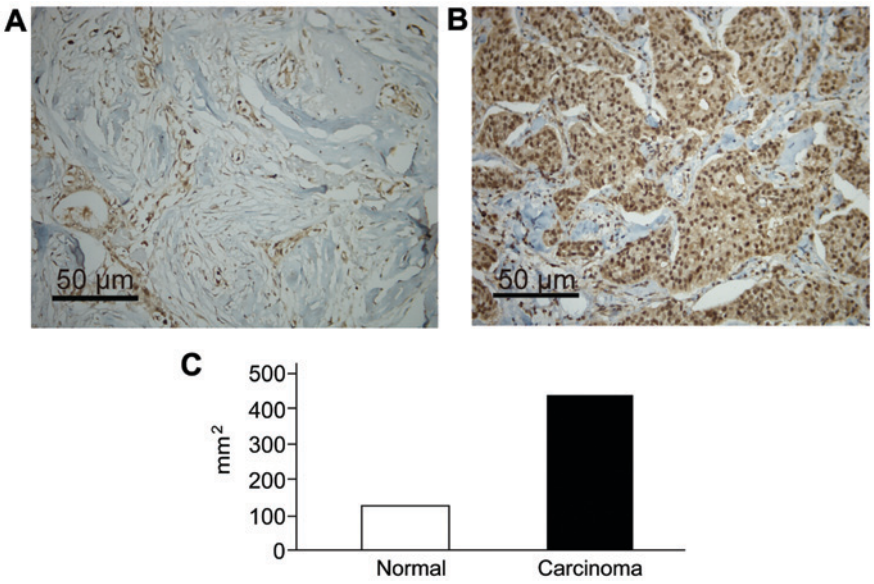

Figure 1. Immunohistochemical staining of estrogen receptor β (ERβ) in normal breast and carcinoma tissues; magnification, x200. (A) Normal breast tissue; (B) breast carcinoma tissues of the same patient; (C) ERβ expression level is higher in the carcinoma tissues than that in the normal tissues, showing statistical significance (P<0.05).

for analysis. Most of the patients were aged 50-60 years. The age, weight, menstrual period and disease course of patients with breast carcinoma showed no significant statistical differences (P>0.05) (Table I). We also statistically analyzed the childbirth history, contact with radioactive rays and family history (Table I). Results of statistical analysis of the pathological classification of breast carcinoma are shown in Table I.

Expression of ERβ in normal and breast carcinoma tissues. There were different degrees of ERβ expression in normal and breast carcinoma tissues. Immunohistochemical results revealed that *ERβ* gene was significantly increased in the lesion tissues, and that the results were statistically significant (P<0.05) (Fig. 1).

ERβ level before and after neoadjuvant chemotherapy. Prior to the operation, we carried out neoadjuvant chemotherapy for 3-4 cycles for patients in the experimental group. After the operation, we stained the breast carcinoma tissue immunohistochemically. We found that, after the administration of neoadjuvant chemotherapy, the ERβ expression level in the breast carcinoma tissues of the patients was greatly decreased, which showed statistical significance (Fig. 2) (P<0.05).

ERβ level of the patients after the recurrence of breast carcinoma or before and after metastasis. We collected tissue samples of patients that had recurrence of, or had metastasis after the operation, and stained for ERβ immunohistochemically. The results revealed that the ERβ levels for patients who have recurrence after the operation were significantly higher than that after the operation (P<0.05). The ERβ expression level was significantly increased in lymphatic metastasis, and the results were statistically significant (Fig. 3) (P<0.05).

Discussion

The ever increasing detection rate of patients with breast carcinoma is mainly due to the upgrade of the testing apparatus and awareness of breast cancer in women. In terms of the treatment of breast carcinoma, in the past, surgical operation combined with post-operation chemotherapy were often applied to curb the growth and proliferation of cancer cells to the most optimal degree. However, the medical field is constantly evolving and has new opinions regarding current treatment methods about breast carcinoma. Investigators are examining whether it is possible to apply targeted chemotherapy prior to surgery to markedly decrease the size of the tumor to avoid physical and mental damage to the women. In addition, targeted treatment based on targeted gene specificity can significantly improve prognosis and survival of the patients. Targeted drugs that were used in the past are mainly from the HERB (12) receptor family at the surface of cancer cells. However, as the disease develops, the drug resistance of the targeted therapy drugs increase, which

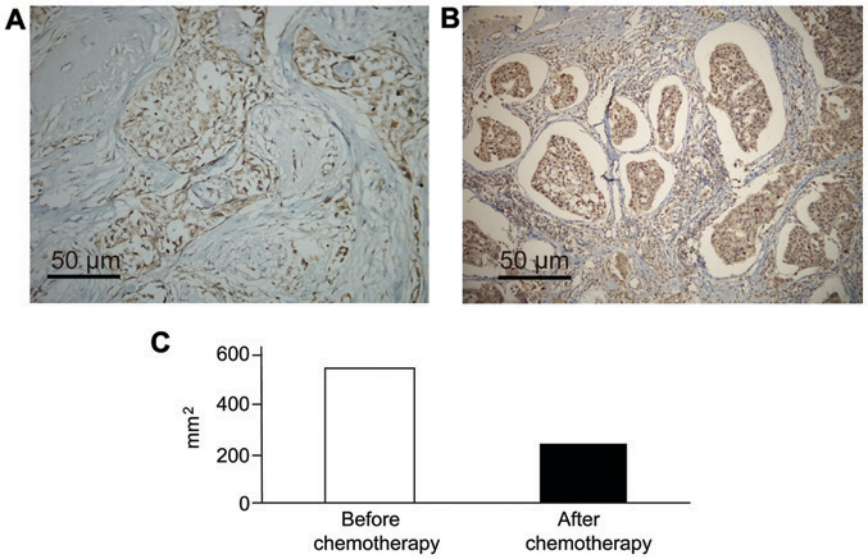

Figure 2. Immunohistochemical staining of estrogen receptor β (ERβ) before and after chemotherapy in breast carcinoma tissues. (A) Before chemotherapy; (B) after chemotherapy of the same patient, staining of the breast carcinoma (ductal carcinoma) tissues immunohistochemically (magnification, x200) after the neoadjuvant chemotherapy; (C) the ERβ expression level in the breast carcinoma tissues of the patient is significantly decreased, showing statistical significance (P<0.05).

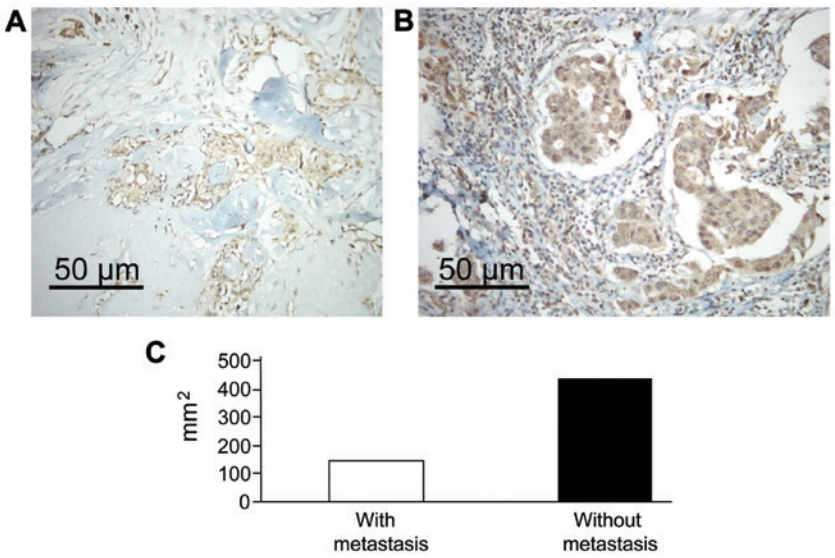

Figure 3. Immunohistochemical staining of estrogen receptor β (ERβ) with or without recurrence or metastasis after the operation. (A) Without recurrence or metastasis; (B) with recurrence or metastasis; (C) for patients with recurrence and metastasis, the ERβ expression level in the breast tissues of the patient is significantly higher than that after the operation, showing statistical significance (P<0.05).

is one of the major challenges in the clinical treatment of breast carcinoma. Based on this, we designed and carried out the present study. For breast carcinoma patients who have a definite diagnosis, we arranged for three to four cycles of pre-operation 'adjuvant chemotherapy' (TEC plan) (13) before the surgery. Additionally, we measured the expression levels of *ERβ* gene before and after the operation to provide a theoretical basis for the development of new types of targeted drugs for treatment.

In the present study, we found that, among the 120 breast carcinoma patients, there were different degrees of expression of the *ERβ* gene when looking at the healthy and breast carcinoma tissues of the patients. However, the expression in the breast carcinoma tissues significantly increases, showing significant statistical difference (P<0.05). The high expression of ERβ also indicates that the cell proliferation conditions are exuberant in the body of breast carcinoma patients (15). Past studies, such as that conducted by Leygue *et al* (16), found the expression of ERβ mRNA in the breast carcinoma tumor samples and the mammary epithelial cells of several types of people. In addition, Leygue *et al* (16) showed that the ratio of ERα and ERβ in the breast carcinoma patients varies as breast cancer develops. Compared to surrounding normal breast tissues, the ERα mRNA/ERβ mRNA ratio in ERα-positive breast carcinoma tissues significantly increases. This discovery indicates that ERβ may play a part in the occurrence and development of the breast carcinoma (17-19). However, the ability to discern the occurrence and development of

breast carcinoma and prognosis by analyzing the expression of ERβ is still not thoroughly researched and lacks the absolute specificity of the cells in the tissue (20). Whether the expression levels of ERβ are related to the differentiation level of cells in tissue requires further research and verification (21). Even so, this study has provided reasonable theoretical support for the diagnosis, treatment, prognosis evaluation, recurrence, transfer and other adverse events of breast carcinoma. It has also suggested a reasonable research direction for the development of targeted treatment drugs. To establish ERβ as a biomarker, the ability to check ERβ expression level by serology can significantly increase the detection rate of ERβ. This allows physicians to reasonably evaluate the regression and prognosis of tumors in clinical application according to the specific expression levels.

In the present study, we designed the pre-operation neoadjuvant chemotherapy (TEC plan) for 3-4 cycles to test the expression levels of *ERβ* gene in cancer tissues of patients and identified a correlation between the ERβ level and the clinical stages of breast carcinoma and lymphatic metastasis, and the results were statistically significant (P<0.05). We hypothesized that among patients who have lymphatic metastasis and poor pathological analysis, the number of cancer cells of executant proliferation greatly increase. If we reverse this process, we can come to the conclusion that the higher the ERβ level is, the higher the stage of cancer is; however, we lack experimental proof. Our results indicate that the expression levels of ERβ are not related to the pathological classification of breast carcinoma. After the patients received neoadjuvant chemotherapy, the expression levels of the ERβ gene significantly decrease compared to levels before the neoadjuvant chemotherapy and the results were statistically significant (P<0.05). This indicates that there may be some value of using ERβ levels for clinical reference in evaluating the effect of chemotherapeutic drugs. However, we only focused on the *in vitro* study of ERβ in clinical breast carcinoma patients. There is no support of our findings in animal experiments. The related targeted therapeutic drugs remain to be developed. Theoretical support is necessary for issues concerning whether the targeted therapeutic drugs can improve the survival rate and disease-free survival period in animal models of breast carcinoma.

Additionally, our results did not show any correlation between the size of cancer and ERβ expression levels. This is different from the results of past studies. Zu *et al* (19) studied this issue and suggested that in gastric adenocarcinoma, there is a correlation between the size and prognosis of the cancer and the expression of tumor markers. However, Yang *et al* (20) indicated that there was no significant correlation between the size of the tumor and the proliferation and metabolism degree of the cancer cells. Combined with our study results, we think that, in breast carcinoma, the size of tumor in the breasts do not have significant correlation with the metabolism of the tumor. In malignant breast tumors, there are differences in differentiation degree of cancer cells. We found that, in the undifferentiated or low-differentiated carcinoma patients, often, the size of the tumor is not large and in most cases, there have already had distant metastasis or lymphatic metastasis as previously reported (11).

Through the collection and study of the samples before and after the operation, we recognize the limitations of our study. For example, in our early studies, we found that the expression of ERβ gene varies according to the dynamic change and the meaning of such changes during the treatment process of patients. However, further *in vitro* and *in vivo* experiments are needed for verification. Furthermore, during the occurrence and development of breast carcinoma, the function of the ERβ gene, i.e., the working mechanism, still remains to be explored. Even though there has been no theoretical support for these study results in the study of breast carcinoma, it has provided good insights for our future study.

In conclusion, the *ERβ* gene is of important clinical value for the evaluation of recurrence, transfer and death of breast carcinoma patients. We can treat ERβ as the evaluation index for the neoadjuvant chemotherapy when observing its clinical effects. In the targeted therapy and corresponding drug development for breast carcinoma, ERβ can act as one of the specific drug targets.

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