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Original Article

Relationship between the expression of TRβ1 and the molecular typing and clinicopathological features of breast cancer

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Abstract: Objective: To study the relationship between the expression of TRβ1 and the molecular typing and clinicopathological features of breast cancer. Methods: The expression of TRβ1, ER, PR and HER-2 proteins in 208 cases of invasive breast cancer, 52 intraductal carcinoma and 22 normal breast tissue was detected by immunohistochemistry in order to analyze the relationship between the expression of TRβ1 protein and clinicopathological parameters of breast cancer. Western blot was performed to detect the effect of TRβ1 silencing on the expression of Notch signaling pathway proteins and Epithelial-mesenchymal transition (EMT)-related proteins in MCF-7 cells. Results: In the 208 cases of invasive breast cancer tissues, over expression of TRβ1 protein was found in 88 cases while low expression in 120 cases, and the immunohistochemical score was (3.9±3.1). TRβ1 protein was found over expressed in all the 52 cases of intraductal carcinoma and 22 cases of normal breast tissue, with the immunohistochemical score of (9.7±2.1) and 12.0, respectively, and there was no significant difference between the two groups (P>0.05) while both of them were significantly lower than the invasive breast cancer group (P<0.05). The expression of TRβ1 protein in the invasive breast cancer tissues was significantly correlated with lymph node metastasis (P=0.041), molecular typing (P=0.037) and histological grade (P<0.001) while it was negatively correlated with HER-2 expression (r=0.926; P<0.001) and irrelevant with age (P=1.024), ER expression (P=0.834), PR expression (P=0.351) or TNM staging (P=1.032). Compared to normal MCF-7 cells, the expression of Notch1, Dell 1, Jagged-1 and vimentin proteins increased by 1.44 times, 1.53 times, 1.50 times and 1.45 times respectively in the TRβ1 expression silenced MCF-7 cell. Conclusions: The expression of TRβ1 protein in breast cancer tissues decreased with the increase of HER-2 expression and histological grade. The depletion of TRβ1 protein may activate the Notch signaling pathway and enhance the EMT ability of breast cancer cells thus promoting the cancer cell migration.

Keywords: TRβ1, breast cancer, molecular typing, clinical pathology

Introduction

Nowadays, breast cancer has become the malignant tumor with the highest incidence among women in the world as well as in China. Statistics show that in 2015, the number of new cases of breast cancer in China was estimated to be 272,000, while 71,000 of patients died of breast cancer [1]. Breast cancer not only seriously threatens the life and health of the patients, but also brings heavy burdens to the society and the families. Because breast cancer is highly heterogeneous and has multiple tissue subtypes, the patients often exhibit different clinical manifestations, treatment responses and prognosis.

In recent years many studies [2-4] showed that the thyroid function is closely related to tumorigenesis, invasion and migration of breast cancer, and that the risk of breast cancer in women following menopause is positively related to the three iodine thyroid levels in the body. Thyroid hormone receptor β1 (TRβ1) is a thyroid hormone receptor protein expressed in both the thyroid gland and the breast. The research of Park JW et al. [5] showed that the mutant TRβ1 had carcinogenic activity, which could continuously bind with p85α and lead to the activation of PI3K-AKT-ERK/STAT3 signaling pathway, thus promoting cell proliferation and invasion as well as inhibiting cell apoptosis. Meanwhile, the research of Gu et al. [6] found that TRβ

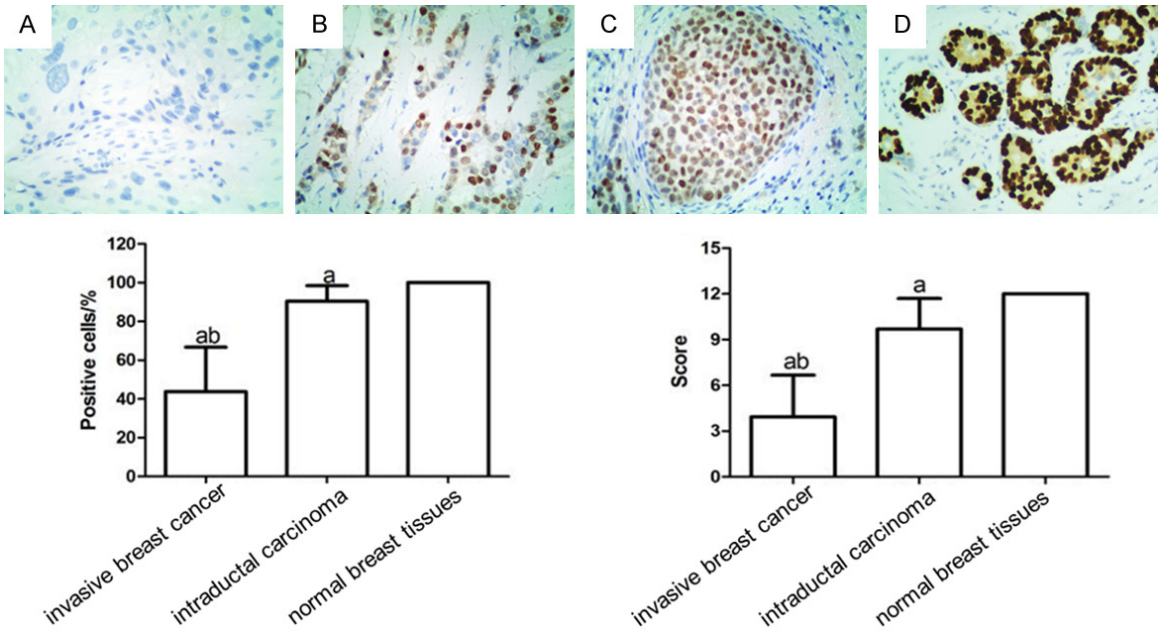

Figure 1. Immunohistochemical detection of TRβ1 protein expression. A: The breast tissues of one of the invasive breast cancer patients showed negative expression of TRβ1 protein; B: The breast tissues of one of the invasive breast cancer patients showed 47% positive cells with TRβ1 expression; C: The breast tissues of one of the intraductal carcinoma patients showed 90% positive cells with TRβ1 expression; D: One of the normal breast tissues showed 100% positive cells with TRβ1 expression; a represented statistically significant difference compare with normal breast tissues, $P<0.05$; b represented statistically significant difference compare with intraductal carcinoma tissues, $P<0.05$.

showed low expression in triple negative breast cancers, and that enhanced expression of TRβ could improve the susceptibility of triple negative breast cancer cells to docetaxel and doxorubicin, two kinds of chemotherapeutic drugs. Therefore, we can conclude that the TRβ1 expression in breast cancer tissues is related to not only the tumorigenesis of breast cancer, but also the proliferation, apoptosis, invasion, migration and drug resistance of breast cancer cells. Furthermore, it may be closely related to the pathological features, molecular typing and prognosis of breast cancer. However, there are few reports about the relationship between the expression of TRβ1 and the molecular typing/clinicopathological features of breast cancer both in China and abroad. Therefore, in this study we detected the expression of TRβ1 in breast cancer tissues and normal breast tissues by immunohistochemistry and analyzed its relationship between the molecular typing and clinical pathological features of breast cancer, which provided theoretical basis for further studies on the pathogenesis of breast cancer and the development of targeted therapeutic drugs.

Materials and methods

Clinical data and specimens

260 cases of breast cancer tissue samples were collected from patients confirmed by pathology and underwent radical surgery in the Department of Pathology, Cangzhou Central Hospital, from January, 2014 to December, 2015. Among them, 208 cases were invasive breast cancer specimens and 52 cases were intraductal carcinoma specimens. All the patients were not treated with radiotherapy or chemotherapy before surgery, and all were females aged 24-72 years old, with the average age of (51.6±8.2). Meanwhile, 50 specimens of normal breast tissues around benign breast lesions from 22 cases of female patients aged 24-68 years old (with the average age of (52.3±7.5)) were selected as controls contemporaneously, among which 10 cases were hyperplasia of mammary glands, 8 cases were breast cysts and 4 cases were breast fibromas. The study was approved by the ethics committee of Cangzhou Central Hospital.

Table 1. Correlation between TRβ1 expression and breast cancer pathological data

Clinical parameters	N/cases	TRβ1		X^2	P
		Low expression/ cases	High expression/ cases		
Age					
≤50 year	86	54	32	0.021	1.204
>50 year	122	66	56		
Tumor diameter					
≤2 cm	42	24	18	0.316	0.972
>2 cm	166	96	70		
Lymph node metastasis					
Yes	84	66	18	12.583	0.041
No	124	54	70		
ER					
Positive	144	82	62	0.160	0.834
Negative	64	38	26		
PR					
Positive	104	54	50	1.418	0.351
Negative	104	66	38		
HER-2					
Positive	100	68	32	4.235	0.029
Negative	108	52	56		
TNM stage					
I	36	20	16	0.046	1.032
II	70	42	28		
III	82	48	34		
IV	20	12	8		
Molecular types					
Luminal A	77	34	43	3.267	0.037
Luminal B	72	46	26		
HER-2 (+)	28	22	6		
Basal-like	31	18	13		
Histological grade					
I	42	14	28	12.027	<0.001
II	94	50	44		
III	72	56	16		

Cell line and reagents

Human MCF-7 cell line (ATCC, USA); TRβ1, HER-2, PR, ER, Notch1, Dell 1, Jagged-1, E-cadherin, vimentin and β-actin primary antibody, goat anti-rabbit, goat anti-mouse secondary antibody (Abcam, US); TRβ1 knockout lentivirus particles (Santa Cruz, USA); Cell total-protein extraction kit, BCA protein quantification kit and immunohistochemistry kit (Abcam, USA).

Immunohistochemistry

All clinical specimens were rinsed by sterile water to remove blood and tissue fluids after surgical resection, and then made into 4 um tissue sections after paraffin embedding. Immunohistochemistry of TRβ1, HER-2, PR and ER was performed according to the instructions of the purchased kit, and PBS was used as the negative control of primary antibodies. 5 different visual fields were selected for each section and were blindly read under 200 × magnification lenses by two pathologists with Associate Senior or above titles. The positive cell rate was determined as the proportion of positive cells to total cells. TRβ1 could be expressed in the nucleus, cytoplasm as well as cell membranes, and the dyeing conditions were scored according to the scoring criteria. The final score of each specimen was shown as A= positive cell rate score × dyeing score, and A=0-2 was determine as negative expression, A=3-5 was determined as weakly positive expression, A=6-8 was determined as positive expression and A=9-12 was determined as strongly positive expression. Negative expression and weakly positive expression were determined as low expressions, while positive expression and strongly positive expression were determined as high expressions.

In addition, PR and ER were located in the nucleus while HER-2 was located on the cell membrane. Positive was determined as no less

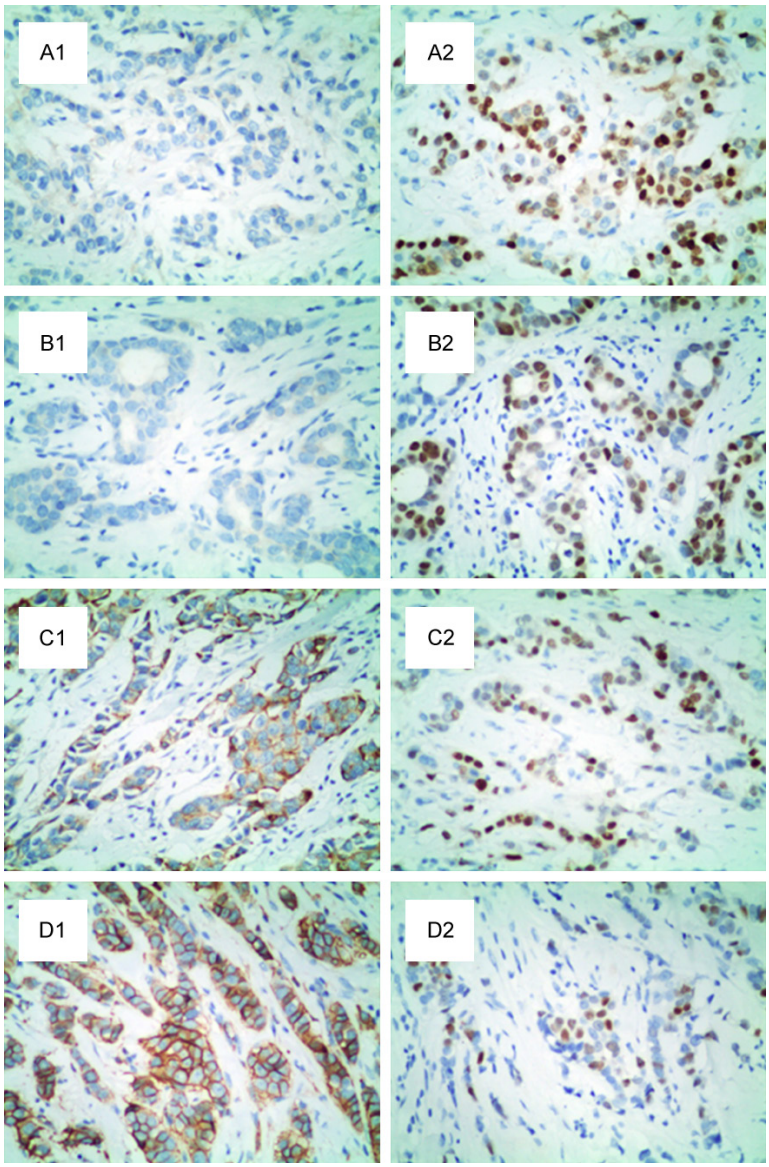

Figure 2. Immunohistochemical detection of TRβ1 expression in different molecular types of breast cancer. Left panel A1-D1: Immunohistochemistry of TRβ1 expression in Luminal A, Basal-like, Luminal B or HER-2 overexpression subtypes of breast cancer tissues; right panel A2-D2: Immunohistochemistry of TRβ1 expression in Luminal A, Basal-like, Luminal B or HER-2 overexpression subtypes of breast cancer tissues; 200×.

than 10% cells were positively stained while negative was determined as less than 10% cells were positively stained.

Western blot analysis

Total proteins were extracted by tissue total protein extraction kit, and the BCA kit was used to determine the protein concentrations. 75 μg of total proteins in each group was loaded for SDS-PAGE and transferred to a wet NC membrane. The membrane was blocked with 5% no-fat milk, incubated with primary antibodies and secondary antibodies before image development. The band density was analyzed by Image-J software and normalized against β-actin levels to be presented as the final results.

Cell culture and treatment

MCF-7 cells were cultured in the DMEM medium + 10% FBS, and were subjected to passage at the ratio of 1:3 one day before experiments. After 24 h of regular culture, the TRβ1 knockout lentivirus particles was added for 48 h before changing to fresh medium (10% FBS + 1.0 μg/ml puromycin) and continue to culture regularly for 96 h. Cells were lysed and the protein expressions were detected by Western blots.

Statistical analysis

Statistical analysis was performed using the SPSS19.0 statistical program, measurement data was measured by (mean ± standard deviation), and t test was used to analyze the differences between groups. Enumeration data was measured in percentage, and chi-square test was used to compare the differences between groups. Pearson analysis was used to examine the correlation between HER-2 expression and TRβ1 expression.

Results

TRβ1 expressions in breast cancer tissues and normal tissues

The TRβ1 protein expressions in 208 cases of invasive breast cancer specimens, 52 cases of intraductal carcinoma specimens and 50 cases

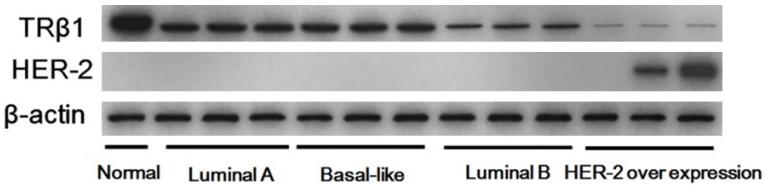

Figure 3. Western blot detection of TRβ1 expression in different molecular types of breast cancer. Western blot detection of TRβ1 protein expression in one case of normal breast tissues and 3 cases of each of the following subtypes of breast cancer tissues, Luminal A, Basal-like, Luminal B and HER-2 overexpression.

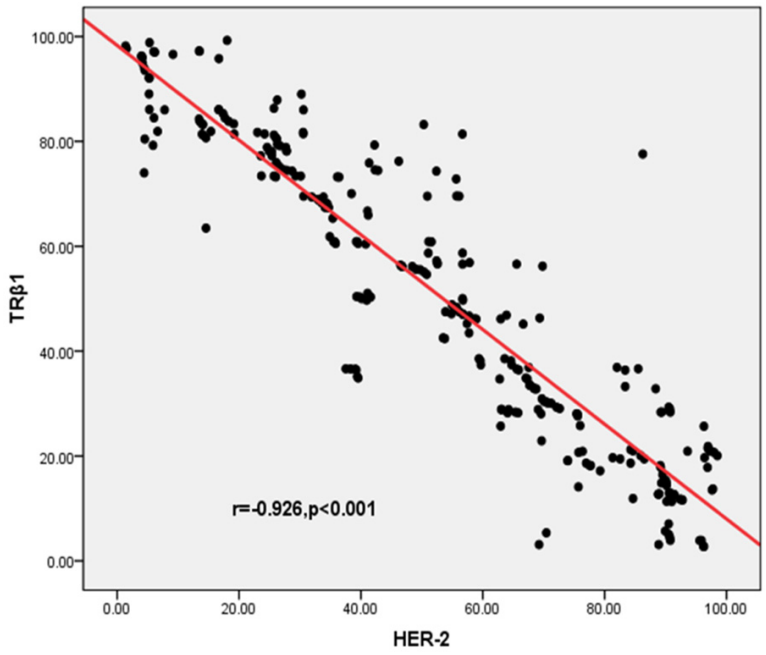

Figure 4. Relationship of HER-2 expression with TRβ1 expression in HER-2 positive breast cancer tissues.

of normal breast tissue specimens were detected according to the procedures described in 1.3. The results were as follows: the proportions of TRβ1 protein positive cells in invasive breast cancer, intraductal carcinoma and normal breast tissues were (43.8±35.4)%, (90.3±10.2)% and 100%, respectively, and the immunohistochemistry scores were (3.9±3.1), (9.7±2.1) and 12, respectively (**Figure 1**).

Correlation of TRβ1 expression in invasive breast cancer with the pathological data

The expression of TRβ1 in 208 cases of invasive breast cancer specimens was scored according to **Table 1**. 3, and the results showed there were 88 cased with high expressions while 120 cases with low expressions of TRβ1. The expression of TRβ1 in invasive breast cancer tissues was unrelated to age (P=1.024), tumor diameters (P=0.972), ER expression (P=0.834), PR expression (P=0.351) or TNM staging (P=1.032). The expression of TRβ1 protein in patients with lymph node metastasis was significantly lower than that in patients without lymph node metastasis (P=0.041) while its expression in HER-2 positive patients was significantly lower than that in the HER-2 negative patients (P=0.029). Meanwhile, there were significant differences in the expression of TRβ1 protein in breast cancer patients with different molecular subtypes (P=0.037), while the higher the histological grade of breast cancer was, the lower the expression of TRβ1 protein (**Table 1**).

Correlation of TRβ1 expression with HER-2 expression in breast cancer tissues

According to the molecular typing of breast cancer, the 208 cases of invasive breast cancer patients were divided into 77 cases of Luminal A type of breast cancer, 31 cases of Basal-like type of breast cancer, 72 cases of Luminal B type of breast cancer and 28 cases of HER-2 overexpression type of breast cancer. The expressions of HER-2 and TRβ1 proteins in different molecular types of breast cancer were examined by immunohistochemistry (**Figure 2**). Western blot was performed to detect the expression of TRβ1 protein in one case of normal breast tissues and 3 cases of each of the following subtypes of breast cancer tissues, Luminal A, Basal-like, Luminal B and HER-2 overexpression (**Figure 3**). The results showed that the TRβ1 protein expression decreased with the increase of HER-2 protein expression in breast cancer tissues, and that the expression of these two proteins was negatively correlated (r=-0.926, P<0.001) (**Figure 4**).

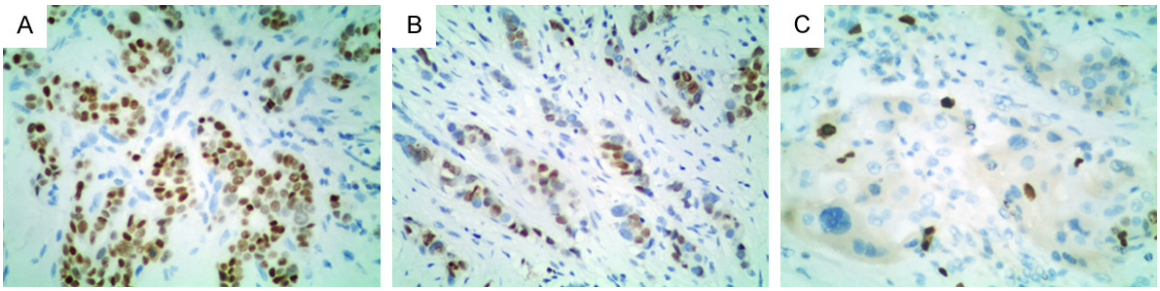

Figure 5. Immunohistochemical detection of TRβ1 expression in breast cancer tissues with different histological grades. Immunohistochemical detection of TRβ1 protein expression in the breast cancer tissues graded as histological grade I (A), II (B) and III (C); 200 ×.

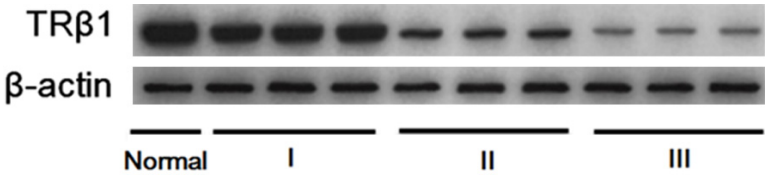

Figure 6. TRβ1 expression in breast cancer tissues of different histological grades. Western blot detection of TRβ1 expressions in 1 case of normal breast tissue and 3 cases of each of histological grade I, II or III breast cancer tissues.

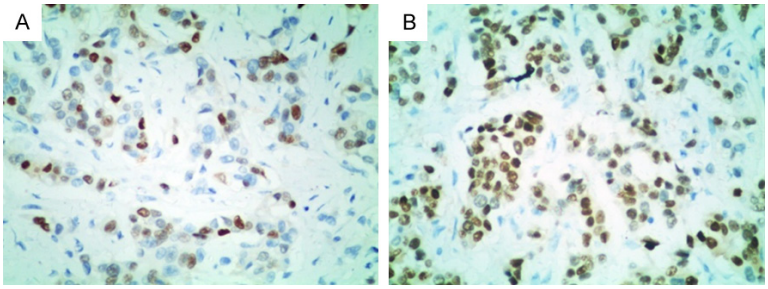

Figure 7. Immunohistochemical detection of TRβ1 expression in lymph node metastasis and non-lymph node metastasis breast cancer tissues. A: Lymph node metastasis breast cancer tissues; B: Non-lymph node metastasis breast cancer tissues; 200 ×.

Correlation of TRβ1 expression with histological grading of breast cancer

The 208 cases of breast cancer tissues were dived into 42 cases of histological grade I, 94 cases of histological grade II and 72 cases of histological grade III according to the histological grading of breast cancer. Immunohistochemistry was performed to detect the expression of TRβ1 protein in different histological grades of breast cancer tissues. 1 case of normal breast tissue and 3 cases of each of histological grade I, II or III breast cancer tissues were randomly selected, and Western blot was performed to detect the expression of TRβ1 protein. The results showed that the expression of TRβ1 in breast cancer tissues decreased with the increase of the histological grade (**Figures 5** and **6**).

Effect of TRβ1 on the expression of EMT-related proteins in breast cancer

The 208 case of breast cancer samples were divided into 84 cases of lymph node metastasis breast cancer and 124 cases of non-lymph node metastasis breast cancer according to the metastasis states, and the expression of TRβ1 was detected by immunohistochemistry. 1 case of normal breast tissue and 3 cases of either of lymph node metastasis or non-lymph node metastasis breast cancer tissues were randomly selected, and Western blot was performed to detect the expression of TRβ1 protein. The results showed that the expression of TRβ1 protein in lymph node metastasis breast cancer tissues was significantly lower than that in non-lymph node metastasis breast cancer tissues (**Figures 7** and **8A**).

Compared to normal MCF7 cells, the TRβ1 silencing MCF-7 cells exhibited increased expression of Notch1, Dell 1, Jagged-1 and vimentin proteins by 1.44-fold, 1.53-fold, 1.50-fold and 1.45-fold, respectively, while the expression of E-cadherin protein was 78.95% lower (**Figure 8B**).

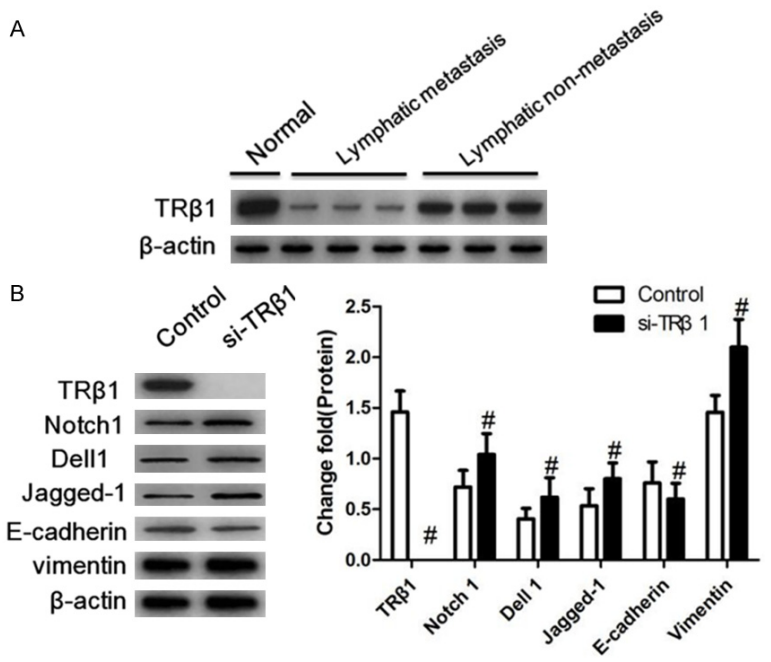

Figure 8. Depletion of TRβ1 promoted the metastasis ability of breast cancer cells. A: Western blot of 1 case of normal breast tissue and 3 cases of either of lymph node metastasis or non-lymph node metastasis breast cancer tissues; B: After TRβ1 protein expression was inhibited in MCF-7 cells, the expression of the indicated proteins were detected by Western blot; #represented statistically significant difference compared with the control group, $P<0.05$.

Discussions

Thyroid hormone regulates the development of organs and normal physiological activities in the vertebrate by targeting the thyroid hormone receptors (TRs) in the nucleus. The TR is a member of the ligand-dependent nuclear receptor transcription factor superfamily that is consists of three main subtypes. The most well studied TR is TRβ1, which is expressed in the mammary gland and thyroid gland. Since Weinberger et al. cloned TRβ1 from chromosome 3p24.1 in 1989, more and more studies have shown that TRβ1 is abnormally expressed in a variety of tumor tissues, and is closely related to the development and progression of cancer [7, 8]. In this study, we detected the expression of TRβ1 protein by immunohistochemistry in 208 cases of invasive breast cancer, 52 cases of intraductal carcinoma and 22 cases of normal breast tissue, and found that the expression of TRβ1 protein in intraductal carcinoma was significantly lower than that in normal breast tissue and significantly higher than that in invasive breast cancer. Ling et al. [8] detected the expression of TRβ1 mRNA in breast cancer tissues by PCR, and found significantly lower expression of TRβ1 mRNA in all the 105 cases of breast cancer specimens. Gene expression refers to the transformation of genetic information into a biologically active protein molecule in the process of cell life by transcription and translation of the genetic information stored in the DNA sequence. Transcription is the process that by using DNA single strands as the template which pairs with the ribonucleotide, mRNA was synthesized by the catalytic action of the RNA polymerase. Translation is the pairing of mRNA with tRNA, and the amino acids on tRNA are linked together to complete the synthesis. Therefore, our study discovered the low expression of TRβ1 in breast cancer tissues at the translation level while the studies of Ling et al. [8] showed low expression of TRβ1 in breast cancer tissues at the transcription level, both of which indicated the low expression of *TRβ1* gene in breast cancer tissues.

Further analysis of the relationship between TRβ1 expression in breast cancer tissues and the clinical pathological parameters showed that the expression of TRβ1 in invasive breast cancer tissues was unrelated with age, tumor size, ER expression, PR expression or TNM staging, but was significantly correlated with lymph node metastasis, HER-2 expression, histological grade and molecular typing. HER-2 is the short description for proto-oncogene human epidermal growth factor receptor 2, which is located on chromosome 17q12-21.32 and encodes a transmembrane receptor-like protein with a molecular weight of 185,000. It has the tyrosine kinase activity and can be used as an independent prognostic factor for breast cancer [9]. In this study, we found that the expression of TRβ1 in HER-2 positive patients was significantly lower than that in HER-2 negative patients, and that in the 100 cases of HER-2 positive breast cancer tissues, the expression of TRβ1 protein was negatively cor-

related with HER-2 protein expression. For breast cancer cells [10], when the *HER-2* gene is overexpressed in tumor cells, the cell membrane will possess too much HER-2 protein, which stimulates the outrageous growth and invasion of cancer cells. Therefore, HER-2 positive breast cancer patients are associated with more dangerous conditions, more likelihood of recurrence and metastasis, and shorter survival time. Meanwhile, oncology researches showed that [11, 12] the tumor histological grade was positively associated with the risk of distal metastasis and local recurrence in cancer patients. In this study, we found that the higher the histological grade of breast cancer, the lower the expression of TRβ1 protein, which suggested that TRβ1 may be a new target for breast cancer targeted therapy, and that its expression might help to inhibit the recurrence and invasion of breast cancer cells thus improving the survival time of patients.

Tumor molecular typing was first proposed in 1999 by the US National Cancer Institute, and with the exploration in the molecular field of breast cancer in recent years, the breast cancer molecular classification is also constantly changing. On the Stagen International Early Breast Cancer Therapy Expert consensus in 2013, the following breast cancer molecular subtypes were listed based on the clinical-pathologic definition: Luminia A type, Luminia B type, HER-2 overexpression type and Basal-like type (equivalent to the three negative breast cancers). At present, the classification of breast cancer molecular types can be delineated according to the protein expression characteristics detected by the widely used immunohistochemical methods, which guides the formulation of targeted clinical treatments for different molecular subtypes of breast cancer [13]. In this study, we found that the expression characteristics of TRβ1 protein in Luminia A type, Basal-like type, Luminia B type and HER-2 overexpressing type of breast cancer tissues were significantly different.

Invasion and migration are among the most significant features of malignancy as well as the most important causes of death in patients with malignant tumors. In this study, we found that the expression of TRβ1 protein in breast cancer patients with lymph node metastasis was significantly lower than that in patients without lymph node metastasis, suggesting that the abnormal expression of TRβ1 protein may be involved in the regulation of invasion and migration of breast cancer cells. The studies of Xu et al. [14] and Andrieu et al. [15] have shown that Notch receptors are highly expressed in breast cancer tissues and that multiple oncogenes can increase the invasion/migration ability of breast cancer cells by activating Notch signaling pathway thus further promoting the progression of breast cancer.

Therefore, in order to further explore the mechanism of TRβ1 protein on the invasion/migration ability of breast cancer cells, we compared the expressions of Notch signal pathway proteins such as Notch1, Dell 1 and Jagged-1 in *TRβ1* gene silencing MCF cells and normal MCF cells. The results showed the expression of Notch1, Dell 1, Jagged-1 and vimentin proteins in TRβ1 silencing MCF-7 cells were significantly increased, while the expression of E-cadherin protein was significantly decreased.

EMT refers to the transformation of epithelia cells into mesenchymal cells, which empowers the cells with the ability to metastasize and invade, including gain of stem cell characteristics, reduced apoptosis and senescence, and increased immunosuppression. The EMT process not only plays a vital role during development, but is also involved in tissue healing, organ fibrosis and tumorigenesis. As for tumor cells, the increased EMT can inhibit the expression of intercellular junction proteins, which results in decreased intercellular connections and promotes the invasion and migration of tumor cells into the surrounding healthy tissues [16]. The vimentin protein is a marker for mesenchymal features in the EMT process, and its high expression is an important indication of EMT [17, 18]. E-cadherin protein plays an important role in the mutual adsorption of cell-cell or cell-basal lamina materials. As a marker for epithelial features in the EMT progress, E-cadherin inhibits the migration of tumor cells. When the expression of E-cadherin protein decreases, it suggests that the migration/invasion ability of tumor cells is probably enhanced [19, 20].

In summary, the expression of TRβ1 protein in breast cancer tissues decreased as the HER-2 expression and the histological grade increased. In addition, TRβ1 protein depletion may enhance the EMT ability of breast cancer cells by activating Notch signaling pathway,

thus promoting migration of breast cancer cells.

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Disclosure of conflict of interest

None.

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