High BTBD7 Expression Positive is Correlated with SLUG- Predicted Poor Prognosis in Hormone Receptor- Negative Breast Cancer

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Abstract

BACKGROUND: Hormone receptor-negative breast cancer (HRNBC), including triple-negative breast cancer (TNBC) and human epidermal growth factor receptor 2 (HER-2) overexpressing breast cancer, is prone to metastasis and has a poor prognosis. BTB/POZ domain-containing protein 7 (Btbd7) is considered to regulate Slug and the epithelial-mesenchymal transition (EMT) process. However, the role of Btbd7 in HRNBC is unclear. This study aimed to investigate the localization and function of Btbd7 in HRNBC as well as its relationship with Slug and the EMT process.

METHODS: Bioinformatics analysis was performed to evaluate the effect of the \textit{BTBD7} and \textit{SLUG} genes expression on the prognosis in HRNBC patients. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to identify differentially expressed genes (DEGs) and possible mechanism in breast cancer. Expression of Btbd7 and Slug in 144 HRNBC and 30 benign lesion tissues obtained from the patients was evaluated using immunohistochemistry and immunofluorescence staining. MDA-MB-231 cells were transfected with \textit{BTBD7} siRNA and the expression levels of Btbd7, Slug, and key EMT proteins were analyzed. CCK-8 and cell invasion assays were performed to evaluate cell proliferation and invasion in response to \textit{BTBD7} silencing.

RESULTS: The total positive rate of Btbd7 expression in HRNBC tumor tissue was 66.7% (96/144), which was higher than that in normal adjacent tissue (NAT) (52.1% 75/144 P=0.001) and benign breast lesion tissues (20%, 6/30 P<0.001). TCGA and immunohistochemistry staining both indicated that higher Btbd7 and Slug expression in HRNBC was associated with poor clinicopathologic features and prognosis. Co-expression of Slug and Btbd7 proteins could be found in HRNBC tissue and MDA-MA-231 cells. \textit{BTBD7} silencing significantly up-regulated the epithelial marker E-cadherin (P<0.05), down-regulated the mesenchymal markers α-SMA and Slug (P<0.05), and suppressed the proliferation and invasion abilities of cells. GO and KEGG analyses based on 322 DEGs showed that \textit{BTBD7} may be associated with generic transcription in breast cancer.

CONCLUSION: Higher expression of \textit{BTBD7} was a poor prognostic factor in HRNBC patients and \textit{BTBD7} silencing inhibited EMT through regulation of Slug expression. \textit{BTBD7} might act as a potential molecular target for gene therapy in HRNBC patients.

Background

According to the International Agency for Research on Cancer, breast cancer is the most common malignant tumor and the leading cause of cancer-related deaths among women worldwide.[1] Breast cancer is clinically categorized into four therapeutic groups according to the hormone receptor and HER-2 status, and both the HER-2 overexpression and TNBC are hormone receptor-negative breast cancers. [2-4]

HER-2 overexpression, which is defined as the lack of the expression of hormonal receptor (HR) and overexpression of HER-2 in breast cancer cells, accounts in 10-25% of the breast cancer cases. [5] The overexpression of the HER-2 gene is a poor prognostic factor, and the development of HER-2
targeted therapy has revolutionized the treatment of these cancers. However, drug resistance to trastuzumab may lead to only the transient benefits of targeted therapy in these breast cancer patients. TNBC, which is defined by the lack of expression of HR and HER-2 in breast cancer cells[6-8], accounts for approximately 10% of all breast cancer cases worldwide.[9] TNBC is characterized by a younger onset age (age < 50 years), larger tumor size, and higher rate of lymphatic and distant metastases, especially in the lungs, liver, and bone. [10, 11] Existing therapeutic options for TNBC, however, are surgery, chemotherapy, and radiotherapy, which often have limited effects.

EMT is generally accepted as a hallmark of tumor invasion and metastasis in breast cancer; during this process, the polarity, adhesion ability, and differentiation characteristics of mesenchymal tissues are altered due to biochemical changes in normal mammary epithelial cells.[12, 13] Slug, also known as snail2, is known to be a regulatory factor for the induction of the EMT process acting by repressing E-cadherin gene transcription via E-box elements, specifically EboxA and EboxC.[14-16]

Btbd7 [BTB (POZ) domain-containing 7] is a member of the BTB (bric-a-brac tramtrack broad complex) protein family. It has a conserved BTB/POZ protein-protein interaction motif[17], which is identified as a critical regulatory factor in epithelial cell dynamics and branching morphology.[18] Research on embryonic development found that high focal expression of Btbd7 leads to the local regulation of Slug, E-cadherin, and epithelial cell motility.[19] Btbd7 has been proven to promote metastasis and be an adverse prognostic factor in non-small cell lung cancer (NSCLC)[20], hepatocellular carcinoma[21] and human salivary adenoid cystic carcinoma (SACC)[22]. However, whether Btbd7 participates in the development of metastasis and affects the prognosis of HRNBC is unclear. In this study, we aimed to investigate the role of Btbd7 in HRNBC prognosis. To this end, we analyzed $BTBD7$ expression in breast cancer using The Cancer Genome Atlas (TCGA), immunohistochemistry (IHC), and conducted in vitro experiments to investigate the localization and function of Btbd7 in cancer cells.

Methods

Tissue samples

This study was performed on 30 paraffin-embedded tissues isolated from benign breast lesions (15 were identified as fibrocystic mastopathy, while the other 15 were breast fibroadenoma), 144 paraffin-embedded tissues isolated from the patients with hormone receptor-negative breast cancer, including tumor tissue and NAT, which were selected 3–5 cm away from the site at which the primary tumor was obtained. All materials were obtained from The Third Affiliated Hospital of Sun Yat-sen University and The First Affiliated Hospital, Shantou University Medical College, between 2007 and 2016. The inclusion criteria were that all patients were treated surgically with radical mastectomy or conservative quadrantectomy in these two hospitals, the pathological pattern was invasive ductal carcinoma (IDC), and estrogen receptor (ER) and progesterone receptor (PR) were negative. The clinicopathological data, including age, sex, type of surgery, HER-2 receptor, tumor size, location, tumor node metastasis (TNM) staging, histology, and lymphatic invasion, were collected retrospectively from the inpatient record and
pathology department. All patients completed a telephone follow-up interview after the initial surgery. The exclusion criteria were that the patients had additional cancers or other life-threatening diseases, or the patients had distant metastasis before the operation. TNBC and HER-2 overexpression breast cancer patients were categorized according to the tumor-node-metastasis staging system classification (American Joint Committee on Cancer, AJCC). Tumor cells exhibiting greater than or equal to 10% positivity for ER or PR at any staining intensity were considered hormone receptor-positive. The HER-2 staining score was evaluated from 0 to 3+. A HER-2 score of 0–1+ was considered negative, and when the HER-2 score was 2+ and 3+, further examination of fluorescence in situ hybridization (FISH) was performed (Supplementary Figure 1). Of the 144 hormone receptor-negative breast cancer tissues, 94 (62.3%) were TNBC tissues, and 50 (34.7%) were HER-2 overexpressing breast cancer tissues.

**Immunohistochemical staining and scoring for Btbd7 and Slug expression**

Each tissue section (5 µm × 5 µm) was dewaxed, rehydrated, and treated with 0.3% hydrogen peroxide to block endogenous peroxidase, followed by antigen retrieval. The sections were incubated with rabbit anti-Btbd7 (1:100, ab204362; Abcam) and mouse anti-Slug (1:20, sc-166476, Santa Cruz Biotechnology) overnight at 4°C. After washing, the bound antibodies were detected using horseradish peroxidase-conjugated secondary antibody (Maxin, Fujian, China) and diaminobenzidine (Xilong Scientific, China), followed by counterstaining with hematoxylin (Keygen Biotech, Nanjing, China). The primary antibody was omitted from the negative control samples.

The evaluation and scoring of Btbd7 and Slug expression were conducted by two independent investigators with pathological training following a blind protocol. The IHC results of Btbd7 and Slug staining were evaluated by multiplying the scores of proportions of positively stained cells by their staining intensity scores. For Btbd7 and Slug, the staining intensity was visually scored as 0 (no staining at all), 1 (weak), 2 (medium) or 3 (strong). The staining extent was also scored as 0 (0% – 10%), 1 (10% – 24%), 2 (25% – 50%), 3 (51% – 75%) or 4 (> = 75%). A multiplicative score of 2 or more was considered as a positive staining.

**Cell Lines and Cell Culture**

Human breast cancer cells MDA-MB-231, SKBR-3, and MCF-10A were obtained from the American Type Culture Collection (ATCC) and were cultured at 37°C and 5% CO2 in DMEM, (high glucose) supplemented with 10% fetal bovine serum.

**Reverse transcription-quantitative polymerase chain reaction**

The forward primer for \textit{BTBD7} was 5'-AAAGGAGCTTTCTCTACAAGCC-3' and the reverse primer was 5'-GCCCCATACTCTGGTGAGGA-3'. Relative mRNA expression levels were calculated using the \(2^{-\Delta Ct}\) method, based on threshold cycle values, and were normalized to the internal control.

**siRNA Transfections**
Silencer Select siRNA targeting *BTBD7* was obtained from GenePharma Shanghai. The siRNA1 sequence was *CAAGTATTGTACTGATGTA*, and the siRNA2 sequence was *CCCGGACATTGCAGAAAGA*. For transient transfection, the cells were cultured in a 24-well plate for 24 h before the experiment. The cells were then transfected with Lipofectamine 2000 according to the manufacturer’s instructions. Following the transfection, the cells were harvested at 24–48 h to measure protein and gene expression levels.

**Western blot analysis**

Total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. Blocked membranes were incubated with the primary antibodies, including Btbd7 (1:1000 ab204362, Abcam), Slug (1:100, sc-166476, Santa Cruz Biotechnology), E-cadherin (1:2000 ab40772, Abcam), α-SMA (1:1000, 19245, Cell Signaling Technology), followed by horseradish peroxidase-conjugated secondary antibodies (AS005 Asbio). Finally, protein expression was examined using an ECL kit. Densitometry measurements were performed using ImageJ software.

**Cell proliferation assays**

For the cell counting kit-8 (CCK-8) assays, the cells were seeded in a 96-well plate, approximately $2 \times 10^3$ cells per well. The proliferation capacities of the cells cultured for 0, 24, 48, and 72 h were tested using the CCK-8 assay (Dojindo). The cell growth curves were plotted using the absorbance value at each time point. Each experiment was performed three times independently.

**Matrigel invasion assay**

The invasive abilities of the cells were examined using a 24-well transwell with 8-μm pore polycarbonate membrane inserts (Corning, NY, USA) and Matrigel (BD Bioscience) according to the manufacturer’s instructions. Matrigel 20 μL (1:3 dilution) was added to each insert and 100 μL of cell suspension containing $3 \times 10^5$ cells were transferred to the upper chamber and incubated for 36–48 h. The filters were stained with hematoxylin. Cells that appeared on the lower surface of the filter were counted in five random high-magnification microscopes. Each experiment was performed three times independently.

**Immunofluorescent staining**

The cells were fixed with 4% paraformaldehyde, blocked with 1% BSA, and the tissue sections were dewaxed and debenzolized. They were then incubated with rabbit anti-Btbd7 (1:400 dilution, ab204362; Abcam, Cambridge, MA) and mouse anti-Slug (1:100 dilution, sc-166476, Santa Cruz Biotechnology) antibodies overnight at 4°C. The primary antibodies were followed by incubation with secondary antibodies conjugated to goat anti-mouse Alexa Fluor 546 (Invitrogen A-11030) and goat anti-rabbit Alexa Fluor 488 (Invitrogen A-11008). Nuclei were counterstained with DAPI. The cells and tissue sections were examined using a Zeiss LSM880 confocal fluorescent microscope (Zeiss, Germany).

**Bioinformatics Analysis**
Gene expression data of 1082 breast cancer samples[23] were obtained from Affymetrix HGU133A and HGU133+2 microarrays and divided into $BTBD7$ low- and high-expression groups according to the median expression value of $BTBD7$. R package ‘edgeR’ (R version 3.5.2) was used to identify DEGs. Genes with correlation coefficients greater than 0.55 or less than -0.55 and false discovery rate (FDR) < 0.05 were considered as DEGs. To explore the functional roles of $BTBD7$, GO was performed on the DEGs using DAVID (https://david.ncifcrf.gov/).[24] Terms with FDR< 0.05 were selected and visualized using R language. KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/kobas3/?t=1)[25] was used to confirm the GO and KEGG terms in DAVID. GO terms with corrected P<0.05, and terms of KEGG pathway with P<0.05 are listed.

**Statistical analysis**

SPSS for Windows version 21.0 was used for data analyses. All experiments were performed in triplicates and the data were expressed as means ± SD and analysed using Student’s t-test. For overall survival (OS) and disease free survival (DFS), the Kaplan–Meier method was used. The un-paired t-test was performed to identify the DEGs, and the paired t-test was used to compare the IHC finding of protein expression between NAT and tumor tissue. Univariate and multivariate Cox regression models were used to determine the relationship between multiple variables and OS and DFS. A P-value of < 0.05 was considered statistically significant.

**Results**

**The demographic and clinicopathological characteristics of HRNBC patients**

Overall, 144 HRNBC patients were studied, and the demographic and clinicopathological characteristics are shown in the Table 1. All biopsies were collected from women aged from 24 to 86 years at the time of surgery, with an average age of 50.4 years. The follow-up period ranged from 27 to 130 months, with an average of 75 months. A total of 113 (78.5%) and 31 (21.5%) patients had tumors with TNM stages I–II and III, respectively. Thirty (20.8%) and 114 (79.2%) patients had T classifications of T1 and T2-T4, respectively. In total, 57 (39.6%) patients were devoid of lymph node invasion. Forty-eight (33.3%) and 96 (66.7%) patients had well + moderate cell differentiation and poor cell differentiation of IDC, respectively. Fifty (34.7%) patients had HER-2 positivity. During the follow-up period, 29 (20.1%), 19 (10.5%), 10 (6.9%), 16 (11.1%), and 3 (2.1%) patients had lung, bone, liver, chest wall, and brain metastasis, respectively.

**Expression of Btbd7 in HRNBC and benign breast lesions and its relationships with clinicopathological parameters**

IHC analysis was performed to investigate Btbd7 expression in HRNBC and benign breast lesion tissues. Positive immunohistochemical staining of Btbd7 was mainly observed in the cytoplasm of the cells. Benign breast lesions and NAT cells exhibited negative or weaker staining when compared with that in HRNBC cells (Figure 1 A). The total positive rate of Btbd7 expression in HRNBC tumor tissue was 66.7% (96/144), which was higher than that in NAT (52.1% 75/144, P<0.001) and benign breast lesion tissues.
Increased Btbd7 expression in HRNBC was significantly associated with larger tumor volume and poorer TNM stage (Figure 1 B, P<0.05). However, no significant association was observed between the expression of Btbd7 and other clinicopathological factors in HRNBC.

Survival analysis

The data from TCGA showed that in HRNBC patients and in those in HER-2 overexpression breast cancer subgroup, the higher **BTBD7** mRNA expression tended to be associated with shorter DFS (P<0.001 Figure 1C-D). However, the DFS in patients with TNBC was insufficient to adequately analyze prognosis. As shown by the Kaplan-Meier survival curves in Figure 1E-F, the median estimated DFS (93.5 ± 5.0 vs. 72.7 ± 5.8 P=0.003) and OS (98.4±3.8 vs 92.2 ± 5.2 P=0.028) times in the patients with low Btbd7 expression were longer than those in patients with high Btbd7 expression.

In order to analyze the function of Btbd7 in TNBC and HER-2+ patients, we performed a subgroup analysis of these two molecular subtypes.

In the TNBC subgroup analysis, the patients with high Btbd7 expression had significantly shorter DFS (P=0.049) and OS (P=0.048) than those with low Btbd7 expression (Figure 1 G-H). As shown in Supplementary figure 2 A-J, among the patients aged <50 years and with TNM stages 1–2 and well + moderately differentiated IDC, a higher Btbd7 expression level was associated with shorter DFS time. As shown in Supplementary figure 2 K-T, among the patients aged >50 years and with lymph node metastasis, a higher Btbd7 expression level was associated with shorter OS time.

In the HER-2+ subgroup analysis, the patients with high Btbd7 expression had significantly shorter DFS (P=0.026) than those with low Btbd7 expression (P=0.026, Figure 1 I). However, no significant differences were observed in the median estimated survival time between HER-2+ patients with low and high Btbd7 expression (P=0.338 Figure 1 J). As shown in Supplementary figure 3 A-J, among the patients aged >50 years, tumor diameter >2cm, lymph node metastasis, and poorly differentiated IDC, a higher Btbd7 expression level was associated with shorter DFS time. However, as shown in Supplementary figure 3 K-T, a higher Btbd7 expression level was not associated with shorter OS time among subgroups.

Tumor recurrence and metastasis were the main causes of death in breast cancer patients. In our study, we found that high Btbd7 expression was significantly associated with a higher lung metastasis rate (28.1% 27/96) in comparison to patients with low Btbd7 expression (4.2% 2/48, P=0.001), while no association could be detected between Btbd7 expression and bone, liver, brain metastasis, and chest wall recurrence (P>0.05) (Table 2).

Correlation between Btbd7 and Slug expression in HRNBC patients

As Slug is recognized to be an important regulatory factor in EMT, in order to verify the level of influence of Btbd7 on EMT, correlation analyses on Btbd7 and Slug were performed. IHC (Figure 2A) and immunofluorescent (Figure 2F) staining in the HRNBC tumor tissue showed that Slug protein was
observed mainly in the cytoplasm and the cell nucleus, and co-expression of Btbd7 and Slug could be observed in the tumor tissue.

The data from TCGA revealed that in HRNBC patients, the patients with high SLUG mRNA expression tended to have shorter DFS than those with low expression (P=0.014, Figure 2B), but there was no significant difference in OS between these groups (Figure 2C). Our IHC results also indicated that high Slug protein expression in the HRNBC patient tissue was associated with shorter DFS (P=0.001) and OS (P=0.003) time (Figure 2D-E), which indicated that Slug was also a prognostic indicator in HRNBC patients. The rate of high Slug expression in patients with high Btbd7 was 72.9% (70/96), while the rate was 41.7% (20/48) in those with low Btbd7 (Supplementary Table 1). A scatter diagram was further performed to identify the correlation between these two markers. The linear correlation coefficient was calculated to be 0.304 with a P-value of less than 0.001. As such, the expression of Btbd7 was weakly positively correlated with Slug expression (Figure 2F).

Considering the prognostic significance of Btbd7 and Slug, we generated receiver operating characteristic (ROC) curves to assess the predictive value of the 3- and 5-year DFS rate. As showed in figure 2G-H, the area the curve (AUC) in both Btbd7 and Slug expression positive was 0.745 for 3 years, and 0.735 for 5 years, which was higher than that in pT, pN, and Btbd7 or Slug expression positive alone (P<0.05), which indicated that combination of Slug and Btbd7 proteins expression levels had the better prognostic value in evaluating HRNBC DFS rate.

Univariate and multivariate analyses

Furthermore, the factors potentially affecting HRNBC prognosis, including DFS (Table 3) and OS (Table 4), were analyzed using Cox regression model. Concerning DFS, the univariate Cox regression analysis showed that good prognostic factors were with no lymphatic metastasis (P=0.038), lower TNM stage (P=0.007), well or moderate IDC differentiation (P=0.026), lower Btbd7 (P=0.003), and lower Slug expression level (P=0.001). In multivariate Cox regression analysis, however, the age (P=0.014), Btbd7 (P=0.013), and Slug (P=0.011) were independent prognostic factors. It was found that following the univariate Cox regression analysis of the OS, only younger age (P=0.006), lower Btbd7 expression levels (P=0.035) and lower Slug expression levels (P=0.006) were good prognostic factors. In the multivariate Cox regression analysis, we found that age (P=0.001) the Btbd7 (P=0.036), and Slug (P=0.021) were independent prognostic factors.

BTBD7 promotes MDA-MB-231 cell proliferation and metastasis via EMT pathway

As the clinical data and IHC analyses demonstrated that high Btbd7 and Slug were poor prognostic factors in HRNBC patients, and the EMT pathway may be the key element for understanding the molecular mechanism of Btbd7 and Slug in breast cancer, it is important to identify the localization and function of these proteins within the cells.
MDA-MB-231 and SKBR-3—the cell lines with negative sex hormone receptor—had higher Btbd7 expression, than immortalized mammary cells did (MCF-10A) (P<0.01; Figure 3B). Immunofluorescence staining results (Figure 3A) demonstrated that MDA-MB-231 cells showed co-expression of Slug and Btbd7 protein, which was consistent with the results obtained from HRNBC tissues.

Subsequently, we knocked down the expression of Btbd7 using short interfering RNA in MDA-MB-231 cells. As shown in Figure 3C-D using qRT-PCR and western blot analysis, the Btbd7 expression was successfully knocked down. While Btbd7 expression was silenced in MDA-MB-231 cells, the epithelial marker E-cadherin was up-regulated (P<0.05), and the mesenchymal markers α-smooth muscle actin protein (α-SMA) and Slug (P<0.05) were down-regulated, indicating that the EMT process was inhibited (Figure 5D).

To corroborate the function of Btbd7 in MDA-MB-231 cells, the CCK-8 and cell invasion assays were used to analyze cell proliferation and invasion ability. The CCK-8 assay showed that relative cell growth was significantly decreased in BTBD7-siRNA groups at 48 and 72 h compared with those in the control group (Figure 3E). Cell invasive assay showed lower invasive properties in BTBD7-siRNA groups than in the control group (Figure 3F).

**BTBD7 is associated with generic transcription of the breast cancer.**

To explore the functional roles of BTBD7 in breast cancer, we screened out 332 DEGs (Supplementary Table 2) according to the expression level of BTBD7. The top three up-regulated genes were TRIP11, DICER1, and ATG2B, and the top 3 down-regulated genes were LSM4, PAM16, and SNRPA. DAVID was used to analyze the DEGs and enriched 30 GO terms (Figure 4A, Supplementary Table 3), such as DNA-templated transcription, regulation of transcription, and integral component of the membrane. To enlarge this observation, KOBAS 3.0 was performed, and 25 KEGG terms (Figure 4B Supplementary Table 4), such as signal transduction and generic transcription pathways, were obtained. These results revealed that BTBD7 was associated with generic transcription, which may be the mechanism by which BTBD7 promotes tumor invasion and metastasis.

**Discussion**

Cancer metastasis is the leading causes of death in patients with malignant tumors[26-29]. Previous research[30] has shown that different BRCA molecular subtypes had significantly different prognoses. Our study aimed to determine the predictive factors for hormone receptor-negative breast cancer, including that with HER-2 overexpression and TNBC.

In the immunohistochemical and different cell lines studies, Btbd7 protein was mainly expressed in the cytoplasm of tumor cells, with the significantly higher levels in HRNBC tumor tissue and in negative sex hormone receptor breast cancer cells than in the NAT, benign lesion tissue, and immortalized mammary cells. Our results were similar to that derived from Chuifeng Fan et al.[31], in which Btbd7 expression was elevated in NSCLC tissues compared with normal lung tissues. This suggests that Btbd7 is a probable
biomarker for malignant cancer. Fan et al. [31] further observed that increased Btbd7 expression in NSCLC was significantly associated with lymph node metastasis and advanced TNM stages. Liu et al. [32] found that in salivary adenoid cystic carcinoma, positive rates of Btbd7 expression were significantly associated with lymph node metastasis. In our study, the pathological analysis showed that the overexpression of Btbd7 in HRNBC was associated with larger tumor volume as well as with poorer TNM stages. As such, the results herein suggest that Btbd7 may be an important molecule promoting the malignant behavior of tumors.

TCGA database and immunohistochemical analysis demonstrated that HRNBC patients with high BTBD7 mRNA expression levels had a poor prognosis. A study conducted by Yi-Ming Tao et al.[21] showed that BTBD7 mRNA expression in hepatocarcinoma was capable of promoting cancer cell proliferation. In addition, Luo et al.[20] observed that NSCLC patients with negative Btbd7 expression had a longer OS time than those with positive Btbd7 expression.

TNBC subgroup analyses indicated that in patients aged <50 years and TNM stages 1–2 and well + moderately differentiated invasive ductal carcinoma, high BTBD7 expression indicated a shorter DFS, and in patients age >50 years and with lymph node metastasis, high BTBD7 expression indicated a shorter OS. HER-2 overexpression patients subgroup analyses indicated that among the patients aged >50 years, tumor diameter >2 cm, lymph node metastasis, poorly differentiated IDC, and a higher Btbd7 expression level were associated with shorter DFS time. However, we did not find any significant association with the OS time among the HER-2 + patients; thus, a larger sample size is required. This may suggest that clinical doctors should pay more attention to this patient population who exhibit Btbd7 expression.

A study by Tomohiro Onodera et al.[19] showed that Btbd7 was a dynamic regulator of branching morphogenesis and was required for the branching of embryonic mammalian salivary glands and lungs. As described in a previous study[20, 31], Btbd7 may contribute to lung cancer development and poor clinical outcome in patient with NSCLC. Our study has further proved that high Btbd7 expression can contribute to the development of metastatic lung cancer (P = 0.001). As a result, we suggest that annual chest radiography or chest computed tomography examination should be performed in HRNBC patients with high level of Btbd7 expression.

Slug is an important transcriptional factor regulating the expression of genes responsible for the EMT.[33, 34] Slug has been demonstrated to downregulate epithelial markers such as E-cadherin as well as the up-regulate the expression of mesenchymal markers such as N-cadherin and fibronectin. Moreover, Liu Yang et al.[22, 35] identified a positive correlation between Btbd7 and Slug expression in SACC tissues; TCGA data and IHC experiments in our article illustrated a common result showing that at both in mRNA and protein level, the high Slug expression was associated with poor prognosis in HRNBC. Immunohistochemical and immunofluorescent staining of the HRNBC tumor tissue and MDA-MB-231 cells verified the co-expression of Btbd7 and Slug in the cells. The ROC curves of the 3- and 5-year DFS showed that combining Btbd7 and Slug expressions had the best predictive value for HRNBC recurrence.
Multivariate Cox regression analyses revealed that AGE, Btbd7, and Slug were independent prognostic factors for DFS and OS. In AGE, Btbd7, and Slug, a low value was associated with better outcomes. Although in the univariate Cox regression analyses, the lymphatic metastasis, TNM, and IDC grading were the prognostic factors for DFS, which has been widely accepted clinically, we first found that Btbd7 and Slug were better prognostic factors, which may indicate that Btbd7 and Slug were more sensitive in forecasting HRNBC prognosis.

As mentioned above, Btbd7 may play a significant role in EMT in HRNBC; in order to confirm this conjecture, we knocked down *BTBD7* in MDA-MB-231 cells.

After the *BTBD7* expression was silenced, the Slug protein was significantly down-regulated, and as a result, the EMT process was inhibited. These findings suggest that *BTBD7* positively regulates the EMT process. Our results are consistent with those of other reports.[21, 22, 32] The CCK-8 and invasion assays identified that *BTBD7* can promote the proliferation and invasion ability of the cells, which was in accordance with the IHC results.

To further explore the mechanism by which the Btbd7 gene plays a role in breast cancer, we screened out 332 DEGs according to the expression level of *BTBD7* by bioinformatics analysis. The top three up-regulated genes were *TRIP11*, *DICER1*, and *ATG2B*, and the top 3 down-regulated genes were *LSM4*, *PAM16*, and *SNRPA*. More research is required to elucidate the relationship between the altered expression of these genes and Btbd7. Following GO and KEGG analyses, it was identified that *BTBD7* was associated with the generic transcription, integral component of the membrane, signal transduction, and so on. Tian et al. [17] showed that the promoter region of the BTB/POZ domain contains binding sites for notable transcription factors such as alpha-fetoprotein (AFP)-1 and GATA in HepG2 cells, which was consistent with our results. However, further in vitro investigations are still required to fully elucidate the mechanism of Btbd7 action in cancer cells.

**Conclusions**

In summary, this study reports that high levels of Btbd7 and Slug expression were significantly related to metastasis OS in HRNBC patients and were poor prognostic factors. We further identified the co-expression of Btbd7 and Slug in HRNBC tissues and cells, and Btbd7 was an important contributor to EMT regulation. Moreover, Btbd7 was associated with generic transcription, which may influence the proliferation and metastasis in breast cancer. *BTBD7* might act as a potential molecular target for gene therapy in HRNBC patients.

**Abbreviations**

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<th>Abbreviation</th>
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<tr>
<td>HRNBC</td>
<td>hormone receptor-negative breast cancer</td>
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<td>TNBC</td>
<td>triple-negative breast cancer</td>
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HER-2  human epidermal growth factor receptor 2
HR    hormonal receptor
Btbd7  BTB/POZ domain-containing protein 7
EMT   Epithelial-mesenchymal transition
GO    Gene ontology
KEGG  Kyoto Encyclopedia of Genes and Genomes
DEGs  Differentially expressed genes
TCGA  The Cancer Genome Atlas
NAT   Normal adjacent tissue
BTB   Bric-a-brac tramtrack broad complex
NSCLC Non-small cell lung cancer
SACC  Human salivary adenoid cystic carcinoma
IHC   Immunohistochemistry
IDC   Invasive ductal carcinoma
ER    Estrogen receptor
PR    Progesterone receptor
TNM   Tumor node metastasis
AJCC  American Joint Committee on Cancer
FISH  Fluorescence in situ hybridization
ATCC  American Type Culture Collection
CCK-8 Cell counting kit-8
FDR   False discovery rate
OS    Overall survival
DFS   Disease-free survival
Declarations

Ethics approval and consent to participate

Ethics approval was obtained from the ethics committee of The Third Affiliated Hospital of Sun Yat-sen University and the First Affiliated Hospital, Shantou University Medical College. Samples were anonymously coded in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki). Written informed consent was obtained from study participants.

Consent for publication

Not applicable.

Availability of data and material

The data used or analyzed during this study are included in this article and available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors’ contributions


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Not applicable.
References

References:


**Tables**

Due to technical limitations, the tables are only available as a download in the supplemental files section.

**Figures**
Figure 2

Immunohistochemical and immunofluorescence staining and correlation analysis of Btbd7 and Slug. (A) Representative immunohistochemical staining showing co-expression of Btbd7 and Slug in HRNBC tissues. (400x) (B-C) high SLUG mRNA level influences prognosis in HRNBC patients in TCGA cohorts. (D-E) Kaplan-Meier curve analysis shows the DFS and OS in patients with HRNBC with high and low Slug protein expression by immunohistochemical staining. (F) Representative immunofluorescence staining of
co-expression of Btbd7 and Slug in HRNBC tissues. (400×) (G) Linear regression of the expression level of Btbd7 and Slug in HRNBC tissue by scatter diagram. (H-I) Slug and Btbd7 predict 3- and 5-year DFS in HRNBC patients by ROC curves. DFS, disease-free survival; OS, overall survival

**Supplementary Files**

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- table2.pdf
- FigS2.tif
- SupplementaryTable3.xlsx
- SupplementaryTable4.xlsx