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

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Research article

Keywords: BTBD7, SLUG, Epithelial-mesenchymal transformation, Triple-negative breast cancer, HER-2 overexpression breast cancer

Posted Date: May 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-30238/v1

Abstract

There is a poor prognosis of metastasis in hormone receptor negative breast cancer (HRNBC), including triple-negative breast cancer (TNBC) and HER-2 overexpression breast cancer. Recent studies have indicated that BTB/POZ domain-containing protein 7 (BTBD7) regulates SLUG which is a key EMT (epithelial-mesenchymal transformation)-associated protein. The role of BTBD7 in HRNBC, however, has not been identified. In this study, The Cancer Genome Atlas (TCGA) was used to identify BTBD7 mRNA expression in breast cancer. In addition, GO and Metascape were used to identify differentially expressed genes. Immunohistochemical staining were applied to determine the protein expression of Btbd7 and Slug in paraffin-embedded samples from 144 HRNBC patients and 30 benign lesion patients. In cancer tissue, 64.9% in TNBC and 70.0% in HER-2+ overexpression breast cancer of Btbd7 protein was expressed when compared with a 20% expression in benign lesion tissues. Increased Btbd7 expression was further associated with larger tumor volume and poor TNM stages in HRNBC patients. Higher BTBD7 mRNA expression level was associated with shorter disease free survival (DFS) time from TCGA data. Higher Btbd7 protein expression in HRNBC tissue was associated with shorter DFS and OS time. Btbd7 protein expression significantly correlated with Slug expression in HRNBC tissue. Combining Btbd7 and Slug expression had a high predictive value for 3-year and 5-year DFS. As such, the positive expression of Btbd7 may contribute towards the development of breast cancer, specifically HRNBC via the up-regulation of Slug expression. Btbd7 was concluded to be an important predictor for the diagnosis of HRNBC patients.

Introduction

According to the International Agency for Research on Cancer, breast cancer is the most common malignant tumor and the leading cause of cancer-related death among women worldwide.[1] Although the incidence of breast cancer in China is significantly lower when compared with North America, Australia and Western Europe, its prevalence has rapidly increased with higher diagnoses in the younger population in recent decades.[2-4] Breast cancer is clinically categorized into four therapeutic groups (luminal A and luminal B, triple negative, and HER-2 overexpression) according to the hormonal receptor and human epidermal growth factor receptor 2 (HER-2) status, and both the HER-2 overexpression and TNBC are the hormone receptor negative breast cancer.

HER-2 overexpression, which is defined as the lack of the expression of hormonal receptor (HR) and overexpression of human epidermal growth factor receptor 2 (HER-2) in breast cancer cells, accounts in 18-30% of the breast cancer.[5] The over-expression of the HER-2 gene used to be poor prognostic factor, till the development of HER-2 targeted therapy has revolutionized the treatment of these cancers. However, the drug resistance of the trastuzumab may lead to the transient benefits of the targeted therapy in these breast cancer patients. Triple-negative breast cancer (TNBC), which is defined as the lack of the expression of HR and (HER-2) in breast cancer cells[6-8], accounts for approximately 10 – 20% of all breast cancer cases worldwide.[9] TNBC is characterised by a younger age of onset (age < 50), larger

tumors, increased lymphatic and distant metastases, especially located in the lungs, liver and bone when compared with other molecular subtypes.[10-11] Existing therapeutic options for TNBC, however, are surgery, chemotherapy and radiotherapy which often have limited effect.

Recent studied have confirmed the importance of epithelial-mesenchymal transition (EMT) towards tumor invasion and metastases in breast cancer; as such, the polarity, adhesion ability and differentiation characteristics of mesenchymal tissues is 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 from the repression of 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 protein family and is identified as a critical regulatory factor in epithelial cell dynamics and branching morphology.[17] Research of embryonic development found that highly focal expressions of Btbd7 leads to the local regulation of Slug, E-cadherin and epithelial cell motility.[18] Btbd7 has been proved to promote metastasis and be an adverse prognostic factors in Non-Small-Cell Lung Cancer (NSCLC)[19], hepatocellular carcinoma[20] and as human salivary adenoid cystic carcinoma (SACC)[21]. Moreover, in SACC, BTBD7 expression significantly correlated with SLUG expression whilst BTBD7 silencing down-regulated the expression of SLUG in SACC-LM cells[21-22]. However, whether BTBD7 participates in the development of metastasis and affects the prognosis of HRNBC requires clarification. In this study, we analysed the genetic expression of BTBD7 in breast cancer from The Cancer Genome Atlas (TCGA) as well as immunohistochemistry to investigate the expression of BTBD7 and SLUG in HRNBC.

Materials And Methods

Tissue samples

Paraffin-embedded tissue specimens of 144 hormone receptor negative breast cancer tissues and 30 benign breast lesions were obtained from The Third Affiliated Hospital of Sun Yat-sen University and The First Affiliated Hospital, Shantou University Medical College, archives between 2007 and 2016. The pathological pattern was the invasive ductal carcinoma (IDC). The clinicopathological data were collected retrospectively from the inpatient record and pathology department. Of the 144 HRNBC cases, variable factors including age, sex, type of surgery, HER-2 receptor, tumour size, location, TNM staging, histology and lymphatic invasion were analysed. All biopsies were collected from women at the time of surgery. All patients completed a telephone follow-up interview after initial surgery. The TNBC and HER-2 overexpression breast cancer patients were categorised according to the tumour-node-metastasis staging system classification (American Joint Committee on Cancer, AJCC). Tumors exhibiting greater than or equal to 10% positivity for ER or PR at any staining intensity among the total tumor cells were considered positive. The HER-2 staining score was evaluated from 0 to 3+ . A HER-2 score 0–1+ was considered negative; and when HER-2 score was 2+ and 3+, further examination of fluorescence in situ hybridization (FISH) was performed. Of the 144 hormone receptor negative breast cancer tissues, 94 (62.3%) were

TNBC tissues, and 50 (34.7%) were HER-2 overexpression breast cancer tissues. Of the 30 benign breast lesions, 15 of them were identified to be fibrocystic mastopathy whilst the remaining 15 were breast fibroadenoma.

This study was approved by 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 the local ethical guidelines (as stipulated by the Declaration of Helsinki)

Immunohistochemical staining and scoring for BTBD7 and SLUG expression

Each tissue section (5 μm x 5 μm) were dewaxed, rehydrated and treated with 0.3% hydrogen peroxide to block endogenous peroxidase followed by an antigen retrieval. The sections were incubated with rabbit anti-Btbd7 (1:100 dilution, ab204362; Abcam, Cambridge, MA) and mouse anti-Slug (1:20 dilution, sc-166476, Santa Cruz biotechnology) overnight at 4°C. After being washed, the bound antibodies were detected with horseradish peroxidase-conjugated secondary antibody (Maxin, Fujian, China) and diaminobenzidine (Xilong Scientific, China), followed by counterstaining with Mayer's hematoxylin (Keygen Biotech, Nanjing, China). The primary antibody was omitted in 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 immunohistochemistry results of Btbd7 and Slug 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 positive staining.

Bioinformatics Analysis

Gene expression data of breast cancer in the TCGA database were downloaded from UCSC Xena (https://xenabrowser.net/datapages/). 1082 samples were obtained and divided into BTBD7 low-expression group and high- expression group according to the median expression value of BTBD7. R package 'edgeR' (R version 3.5.2) was used to identify differentially expressed genes (DEGs). Genes with |log2(fold-change) |> 2 and false discovery rate (FDR) < 0.05 were considered as DEGs. To explore the function roles of BTBD7, GO oncology (GO) was performed on the DEGs using DAVID (https://david.ncifcrf.gov/).[23] Terms with FDR< 0.05 were selected and visualized using R language. KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/kobas3/?t=1)[24] 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 were listed.

Statistical analysis

SPSS for Windows version 21.0 was used for data analyses. All experiments were performed in triplicate and the data were expressed as means ± SD and analysed by Student's t-test. For the overall survival (OS) and relapse free survival (DFS), the Kaplan–Meier method was used. The Log-rank test was used for group comparison. The 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 to be significant.

Results

The demographic and clinicopathological characteristics of HRNBC patients

144 HRNBC patients were studied, and the demographic and clinicopathological characteristics was showed in the table 1. All biopsies were collected from women from 24 to 86 years of age at the time of surgery, garnering an average age of 50.4 years old. The time of follow-up ranged from 27 to 130 months, with the average of 75 months. 113 (78.5%) and 31 (21.5%) patients had tumors of TNM stages I-II and III, respectively. 30 (20.8%) and 114 (79.2%) patients had T classification of T1 and T2-T4, respectively. In total, 57 (39.6%) patients were devoid of lymph node invasion. 48(33.3%) and 96 (66.7%) patients had well + moderate cell differentiation and poor cell differentiation of IDC, respectively. 50(34.7%) patients had HER-2 positive. 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 relationships with clinicopathological parameters

Immunohistochemistry (IHC) analysis was performed to investigate Btbd7 expression in HRNBC and benign breast lesions tissues. Positive immunohistochemical staining of Btbd7 was mainly observed in the cytoplasm of the cells. Benign breast lesions cells exhibited negative or weak staining when compared with the staining of HRNBC cells (Figure 1 A). The total positive rate of Btbd7 expression in TNBC and Her-2+ patients was 64.9% (61/94) and 70% (35/50), respectively, which was higher than that in benign breast lesions tissues (20%) (6/30) (Figure 1 B, $p < 0.001$). Increased Btbd7 expression in HRNBC was significantly associated with larger tumour volume and poorer TNM stage (Figure 1 B $p < 0.05$). No significant association, however, could be described for the expression of Btbd7 and other clinicopathological factors in HRNBC.

Survival analysis

The data from TCGA showed that in HRNBC patients and HER-2 overexpression subgroup breast cancer, high BTBD7 mRNA expression tended to have shorter DFS ($P < 0.001$) than low expression (Figure 1C-D). However, the DFS for patients with TNBC were insufficient to adequately analyse prognosis.

As shown in the Kaplan-Meier survival curves in Figure 1E-F, the median estimated DFS time was 93.5 ± 5.0 months for HRNBC patients with low Btbd7 expression. For HRNBC patients with high Btbd7

expression, however, the DFS time was only 72.7 ± 5.8 months (P = 0.003). What's more, the median estimated OS time was 98.4±3.8 months for HRNBC patients with low Btbd7 expression, which is significant longer than those with high Btbd7 expression (92.2 ± 5.2 months, P=0.028).

In order to analyse the function of Btbd7 in TNBC and HER-2+ patients, respectively, we had the subgroup analysis of these two molecular subtyping.

In the TNBC subgroup analysis, the patients with high Btbd7 expression had significant shorter DFS (P=0.049) and OS (P=0.048) than those with low Btbd7 expression (Figure 2 G-H). As shown in the figure 2 A-J, among the patients aged less than 50 years and TNM stages 1–2 and well + moderately differentiated IDC, a higher Btbd7 expression level was associated with shorter DFS time. As shown in the figure 2 K-T, among the patients age >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 significant shorter DFS (P=0.026) than those with low Btbd7 expression (Figure 2 I). No significant differences, however, were identified between the median estimated survival time between HER-2+ patients with low and high Btbd7 expression (P=0.338) (Figure 1 J). As shown in the figure 3 A-J, among the patients aged more than 50 years, tumour diameter more than 2cm, with lymph node metastasis and poor differentiated invasive ductal carcinoma, a higher Btbd7 expression level was associated with shorter DFS time. However, as shown in the figure 3 K-T, a higher Btbd7 expression level was not associated with shorter OS time among any subgroup.

Tumour recurrence and metastasis were the main cause of death in breast cancer patients. In our study, we found that high Btbd7 expression was significantly associated with high lung metastases (P = 0.001，r = 0.282) while no association could be linked between Btbd7 expression and bone, liver, brain metastatic and chest wall recurrence (P > 0.05) (Table 2).

Correlation between Btbd7 expression and Slug expression in HRNBC

As Slug is recognised to be an important regulatory factor in EMT, in order to verify the level of influence of Btbd7 on EMT, a correlation analyses on Btbd7 and Slug was performed. The staining of Slug was observed mainly in the cytoplasm and in the cell nucleus (Figure 4 A).

The data from TCGA showed that in HRNBC patients, high SLUG mRNA expression tended to have shorter DFS (P =0.014) than low expression (Figure 4B). Our IHC experiment also showed that high Slug protein expression in the HRNBC patients' tissue was associated with shorter DFS (P=0.001) and OS (P=0.003) time (Figure 4C-D), which indicated that SLUG was also the prognostic indicator in HRNBC patients. As shown in Figure 4E, patients with high expression of Btbd7 subsequently exhibited high Slug expression (P = 0.001); 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 patients with low Btbd7. 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 positively correlated with Slug expression. (Figure 4F)

Considering the prognostic significance of Btbd7 and Slug, we generated ROC curves to assess the predictive value on 3-year, and 5-year recurrence-free survival rate. AUC combining Btbd7 and Slug was 74.5% for 3 years, and73.5% for 5 years, which was more accurate than prediction using pT, pN, and Btbd7 or Slug alone (Figure 4G-H).

Univariate and multivariate analysis

Furthermore, the factors potentially affecting HRNBC prognosis including DFS (Table 3) and OS (Table 4) of the patients were analysed by 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, 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 overall survival, only a 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 the age (P = 0.001) the Btbd7 (P=0.036) and Slug (P=0.021) were the independent prognostic factors.

BTBD7 was involved in regulating extracellular components.

To explore the function roles of BTBD7 in breast cancer, we screened out 171 DEGs according to the expression level of BTBD7. DAVID was used to analyse the DEGs and enriched several GO terms, such as extracellular space and extracellular region (Fig 5A). To enlarge this observation, KOBAS 3.0 was performed. Similar GO term ‘extracellular region’ (Figure 5B) and several KEGG pathways such as Ras signalling pathway, MAPK signalling pathway, Estrogen signalling pathway and Breast cancer (Figure 5C) were obtained. These results revealed BTBD7 was associated with extracellular components and indicated it is closely related to tumor invasion and metastasis.

Correlation Between Btbd7 Expression And Slug Expression In Hrnbc

As Slug is recognised to be an important regulatory factor in EMT, in order to verify the level of influence of Btbd7 on EMT, a correlation analyses on Btbd7 and Slug was performed. The staining of Slug was observed mainly in the cytoplasm and in the cell nucleus (Fig. 4A).

The data from TCGA showed that in HRNBC patients, high SLUG mRNA expression tended to have shorter DFS (P = 0.014) than low expression (Fig. 4B). Our IHC experiment also showed that high Slug protein expression in the HRNBC patients’ tissue was associated with shorter DFS (P = 0.001) and OS (P = 0.003) time (Fig. 4C-D), which indicated that SLUG was also the prognostic indicator in HRNBC patients. As shown in Fig. 4E, patients with high expression of Btbd7 subsequently exhibited high Slug expression

(P = 0.001); 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 patients with low Btbd7. 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 positively correlated with Slug expression. (Fig. 4F)

Considering the prognostic significance of Btbd7 and Slug, we generated ROC curves to assess the predictive value on 3-year, and 5-year recurrence-free survival rate. AUC combining Btbd7 and Slug was 74.5% for 3 years, and73.5% for 5 years, which was more accurate than prediction using pT, pN, and Btbd7 or Slug alone (Fig. 4G-H).

Discussion

Cancer relapse and metastases are the leading cause of death in malignant tumours[25-28]. Previous research[29] has shown that differences in BRCA molecular subtypes have significantly different prognoses. Our study meant to found out the predictive factor for the HER-2 overexpression and TNBC patients.

In the immunohistochemical studies, Btbd7 protein was mainly expressed in the cytoplasm of tumour cell with the average expression levels in TNBC and HER-2 overexpression breast cancer tissue being significantly higher when compared to that observed in benign lesions. Our results were similar to that derived from Chuifeng Fan et al.[30] whereby Btbd7 expression was elevated in non-small cell lung cancer tissues when compared with normal lung tissues. As such, this suggests that Btbd7 is a probable biomarker for malignant cancer. Chuifeng Fan et al. [30] further observed that an increased Btbd7 expression in NSCLC was significantly associated with lymph node metastasis and advanced TNM stages. Yunxia Liu et al.[31] found that in salivary adenoid cystic carcinoma, positive rates of Btbd7 expression were significantly associated with the lymph node metastasis. In our study, the pathological analysis showed that the overexpression of Btbd7 in HRNBC was associated with larger tumour volume, as well as poorer TNM stages. As such, the results herein suggested that Btbd7 may be an important molecule to promote the malignant behaviour of tumours.

In our study, we found that in all breast cancer from the TCGA database, the prediction value of BTBD7 mRNA expression level in DFS was good. HER-2 overexpression patients with high BTBD7 mRNA expression level had a shorter DFS time. A study conducted by Yi-Ming Tao et al.[20] showed that BTBD7 mRNA expression in hepatocarcinoma was capable of promoting cancer cell proliferation. Herein, we analysed the clinical outcome of HRNBC patients and found that patients with Btbd7 protein expression in HRNBC tissues have a shorter DFS and OS time than those without Btbd7 expression. In addition, Fan-Yan Luo et al.[19] observed that NSCLC patients with negative Btbd7 expression had a longer OS time than patients with positive Btbd7 expression.

TNBC subgroup analyses indicated that in patients aged less than 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 among the patients aged more than 50 years, tumour diameter more than 2cm, with lymph node metastasis and poor differentiated invasive ductal carcinoma, a higher Btbd7 expression level was associated with shorter DFS time. However, we did not find any significant association in the OS time among the HER-2 + patients, thus, a larger sample is required. This may consequently suggest that clinical doctors should pay more attention to this patient population who exhibit BTBD7 expression.

A study by Tomohiro Onodera et al.[18] 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[19, 30], Btbd7 may contribute to lung cancer development and a poor clinical patient outcome in non-small-cell lung cancer. 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 CT examination should be performed in the HRNBC patients with high Btbd7 expression.

Slug is an important transcriptional factor regulating expression of genes responsible for the EMT.[32-33] Slug had been demonstrated to down-regulate epithelial markers such as E-cadherin as well as the up-regulation of mesenchymal markers such as vimentin and fibronectin. Moreover, Liu Yang et al.[21-22] identified a positive correlation between Btbd7 and Slug expression in SACC tissues; silencing BTBD7 down-regulated the expression of SLUG in SACC-LM cells which subsequently supressed the migration and invasion abilities of SACC cells. TCGA data and IHC experiments in our article illustrates a common result that both in mRNA and protein level, the high Slug expression was associated with poor prognosis in HRNBC. Our results herein further showed that a Btbd7 expression was significant higher in HRNBC patients with positive Slug expression than those when compared with patients without Slug expression (77.8% versus 48.1% (P < 0.05)). Bbtbd7 expression was further found to be significantly associated with increased Slug expression in HRNBC (r = 0.304). The ROC curves of the three and five-year DFS showed that combining the Btbd7 and Slug had the best predictive value on the HRNBC recurrence. As aforementioned, Btbd7 may play a significant role in EMT in HRNBC, but the mechanism remains unclear. More researches are required to elucidate the relationship between Btbd7 expression and EMT in HRNBC.

The multivariate cox regression analyses revealed that AGE, Btbd7, and Slug were the independent prognostic factors for DFS and OS. In AGE, Btbd7, and Slug, a low value was associated with better outcome. Although in the univariate cox regression analyses, the lymphatic metastasis, TNM, IDC grading were the prognostic factors for DFS, which has been widely accepted clinically, we firstly found that Btbd7 and Slug was the better prognostic factors, which may indicate that Btbd7 and Slug was more sensitive in forecasting HRNBC prognosis.

Following GO oncology and the Metascape analyses, it was identified that BTBD7 was associated with extracellular matrix (ECM) which further proved that BTBD7 play an important role in the tumour microenvironment. ECM proteins including fibrillar collagens, fibronectin, specific laminins and proteoglycans may play an important role in the progression and metastasis of breast cancer.[34-35] Qian et al.[36] found that the up-regulation of the fibronectin may resulting in the acquisition of metastatic behaviour in breast cancer. Barkan et al.[37] showed that type I collagen can induce breast cancer cell proliferation and increase the breast cancer metastasis in the mouse model. However, some studies have found that some ECM can, however, prevent the metastasis of the breast cancer. For example, Oded Komemi et al.[38] demonstrated that placental ECM can prevent the attachment of breast cancer and induce their motility and aggregation. The mechanism that the BTBD7 perform in the ECM is still unclear therefore further work is required.

In conclusion, the study herein reports that high Btbd7 expression is significantly related to HRNBC tumour proliferation and lung metastasis. We further identified that there was a positive correlation between Btbd7 and Slug expression in HRNBC tissues which may indicate that Btbd7 is an important contributor towards EMT regulation. Moreover, Btbd7 is associated with extracellular matrix which may influence the metastasis of the breast cancer. However, further *in vitro* investigations are still required to fully elucidate the mechanism of Btbd7 in HRNBC.

Declarations

Acknowledgments

This work was supported by National Natural Science Foundation of China (No. 81402509 to Tian-tian Wang, No. 81672640 to Lin Ling), the Grant for Key Disciplinary Project of Clinical Medicine under the Guangdong High-level University Development Program, the Project of Innovating and Strengthening Universities in Guangdong Province (No. 2018KTSCX066 to Lin Ling) and the Special Funds for Innovation Strategy of Science and Education in Guangdong Province (No. 2018-157 to Lin Ling).

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1 Demographic and clinicopathological characteristics of 144 hormone receptor negative breast cancer.

Variables	No.	%
Age(years)		
50	76	52.8
≥50	68	47.2
TNM stage		
I-II	113	78.5
III	31	21.5
T classification		
T1	30	20.8
T2-T4	114	79.2
pN		
N0	87	60.4
N1-3	57	39.6
IDC Grading		
Well + Moderate	48	33.3
poor	96	66.7
HER-2		
negative	94	65.3
positive	50	34.7
Metastatic sites		
Lung	29	20.1
Bone	19	13.2
Liver	10	6.9
Chest wall	16	11.1
Brain	3	2.1

Table 2 Btbd7 expression level and metastatic and recurrence in HRNBC patients

Metastatic and recurrence sites		Btbd7		Correlation coefficient	P value
		LOW	HIGH		
Lung	_	46	69	0.282	**0.001**
	+	2	27		
Bone	_	44	81	0.102	0.223
	+	4	15		
Liver	_	45	89	0.019	0.817
	+	3	7		
Chest wall	_	42	86	-0.031	0.708
	+	6	10		
Brain	_	47	94	0.000	1.000
	+	1	2		

Table 3. RFS of the HRNBC patients based on univariate and multivariate Cox proportional regression analyses.

Factor	RFS			
	univariate		multivariate	
	HR (95%CI)	P value	HR (95%CI)	P value
Age(≤50vs>=50)	1.71(0.99-2.97)	0.056	2.05(1.16-3.64)	**0.014**
Tumor diameter (<2cmvs>=2cm)	1.47(0.72-3.02)	0.291	/	/
Lymphatic metastasis(Yes vs no)	1.78(1.03-3.08)	**0.038**	/	/
TNM(I-II vs III-IV)	2.20(1.24-3.98)	**0.007**	/	/
Her-2(- vs +)	1.20(0.67-2.10)	0.549	/	**/**
IDC grading（I-II vs III）	2.00(1.09-3.68)	**0.026**	/	**/**
Btbd7(Low vs High)	3.13(1.47-6.64)	**0.003**	2.70(1.23-5.91)	**0.013**
Slug(Low vs High)	3.66(1.72-7.78)	**0.001**	2.75(1.27-6.00)	**0.011**

Table 4. OS of the HRNBC patients based on univariate and multivariate Cox proportional regression analyses.

Factor	OS			
	univariate		multivariate	
	HR (95%CI)	P value	HR (95%CI)	P value
Age(<50vs>=50)	2.83(1.35-5.95)	**0.006**	3.66(1.70-7.89)	**0.001**
Tumor diameter (<2cmvs>=2cm)	1.24(0.51-3.01)	0.634	/	/
Lymphatic metastasis(Yes vs no)	1.37(0.69-2.76)	0.372	/	/
TNM(I-II vs III-IV)	1.79(0.85-3.78)	0.125	/	/
Her-2(- vs +)	1.09(0.52-2.29)	0.811	/	**/**
IDC grading(I-II vs III)	1.94(0.92-4.08)	0.194	/	/
Btbd7(Low vs High)	2.59(1.07-6.29)	**0.035**	2.73(1.07-7.01)	**0.036**
Slug(Low vs High)	4.35(1.53-12.4)	**0.006**	3.55(1.21-10.4)	**0.021**

Figures

Figure 1

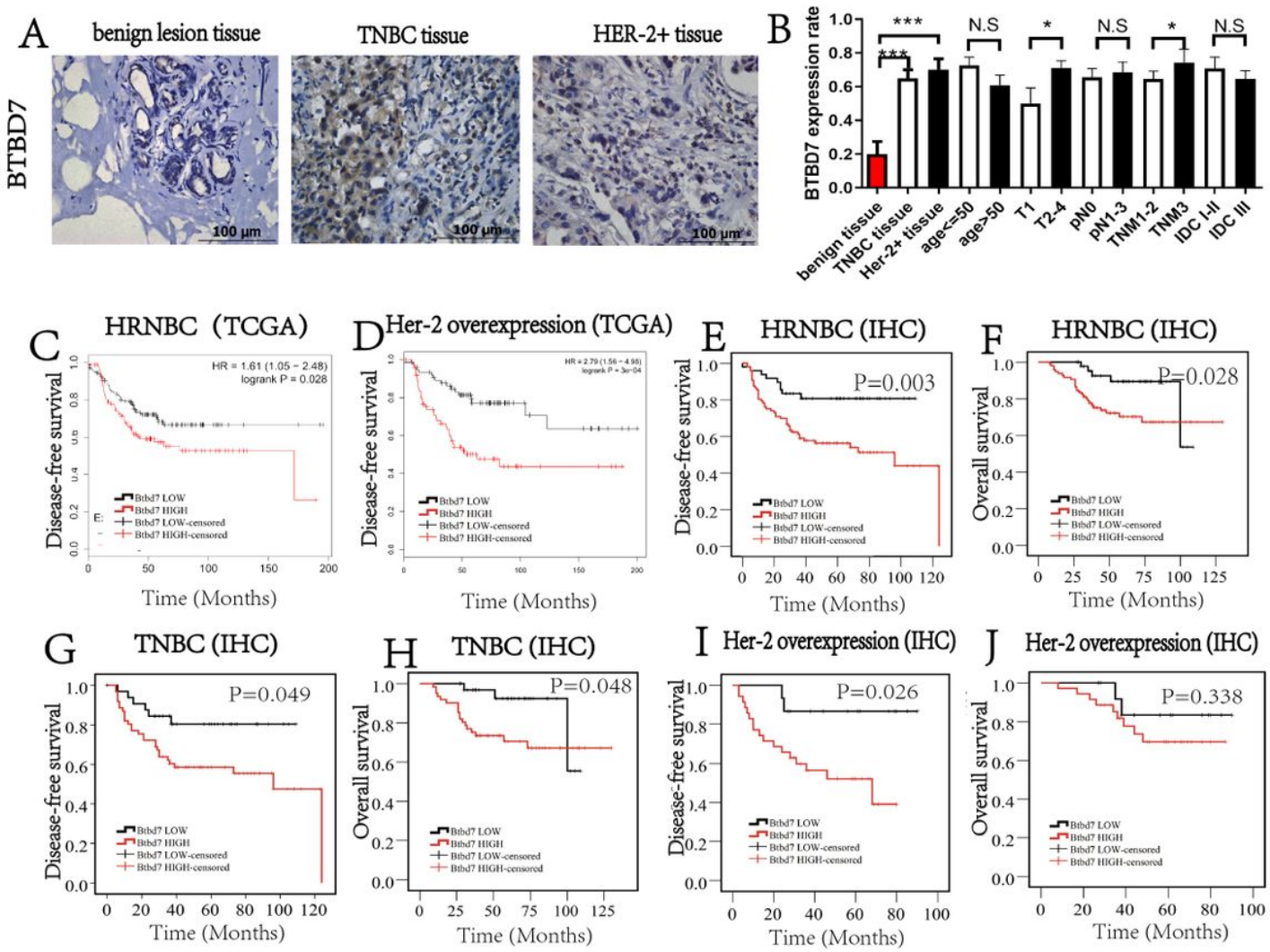


Figure 1

Expression level and prognostic value of Btbd7 in HRNBC. (A) Immunohistochemical staining of Btbd7 in benign breast lesions tissues, TNBC and HER-2 overexpression breast cancer tissue. (B) Expression of Btbd7 differed by cancer or benign tissue, age, tumour volume, lymph node metastasis, TNM stage and IDC differentiated degree (I-II for well to Moderate, III for poor). (C-D) High BTBD7 mRNA level influenced breast cancer prognosis in TCGA cohorts. (E- J) Kaplan-Meier analysis shows the DFS and OS of HRNBC, TNBC and HER-2 overexpression patients with high and low Btbd7 protein expression by Immunohistochemical staining. (N.S. no significant difference, * $p < 0.05$, *** $p < 0.001$).

Figure 2

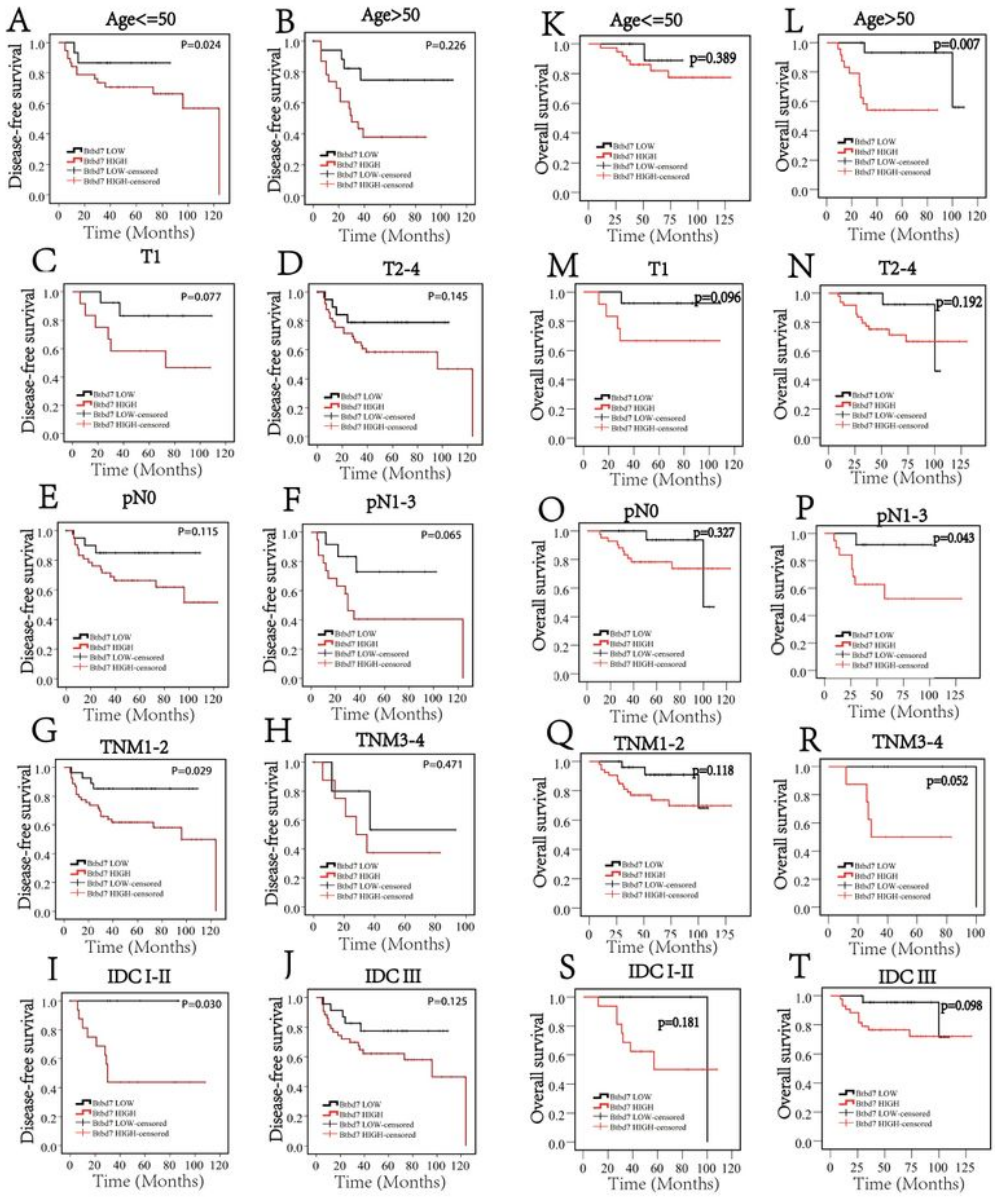


Figure 2

Subgroup analysis of DFS and OS of TNBC patients according to Btbd7 by Kaplan-Meier curves. (A-I) Kaplan–Meier curves of DFS according to Btbd7 differed by age, tumour volume, lymph node metastasis, TNM stage and IDC differentiated degree. (K-T) Kaplan–Meier curves of OS according to Btbd7 differed by age, tumour volume, lymph node metastasis, TNM stage and IDC differentiated degree.

Figure 3

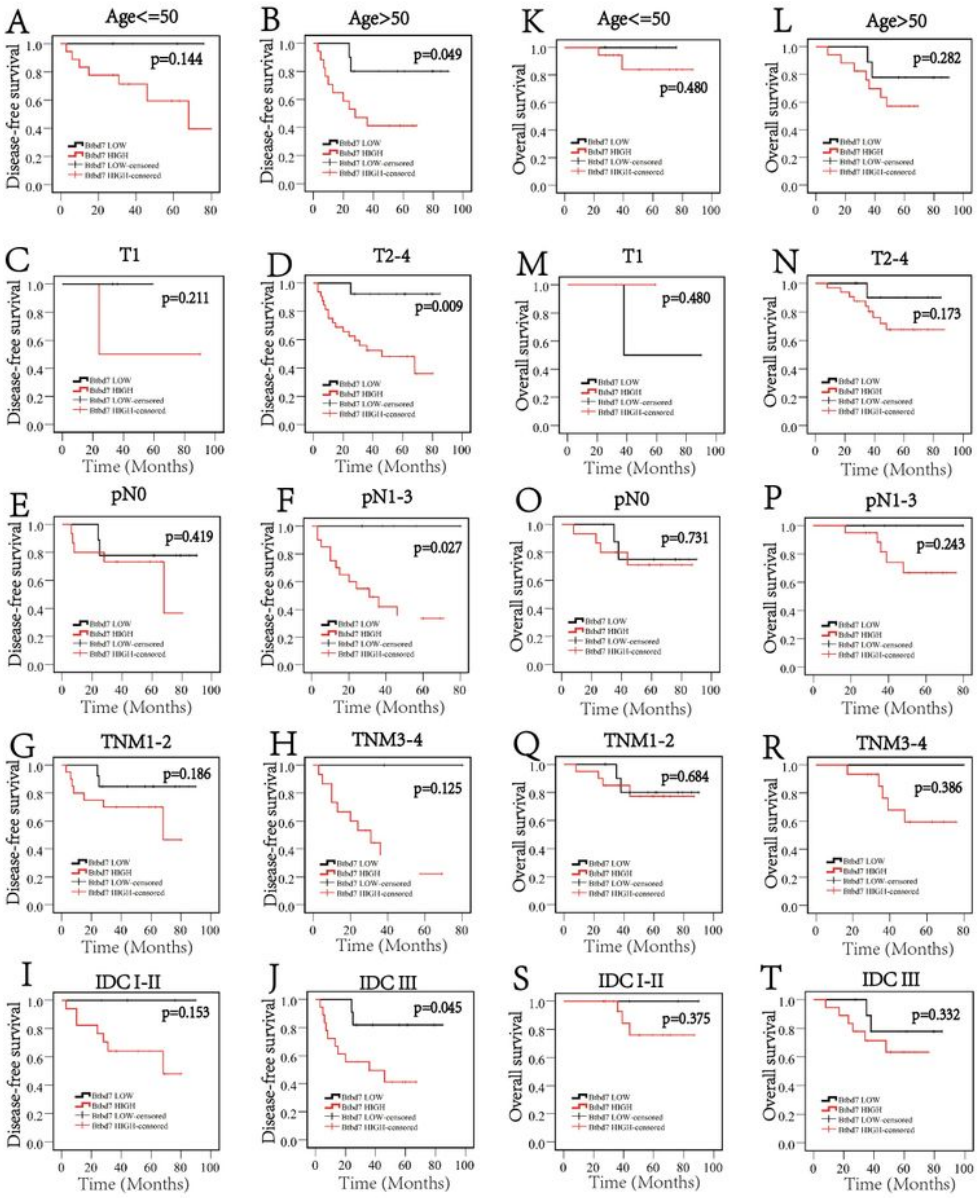
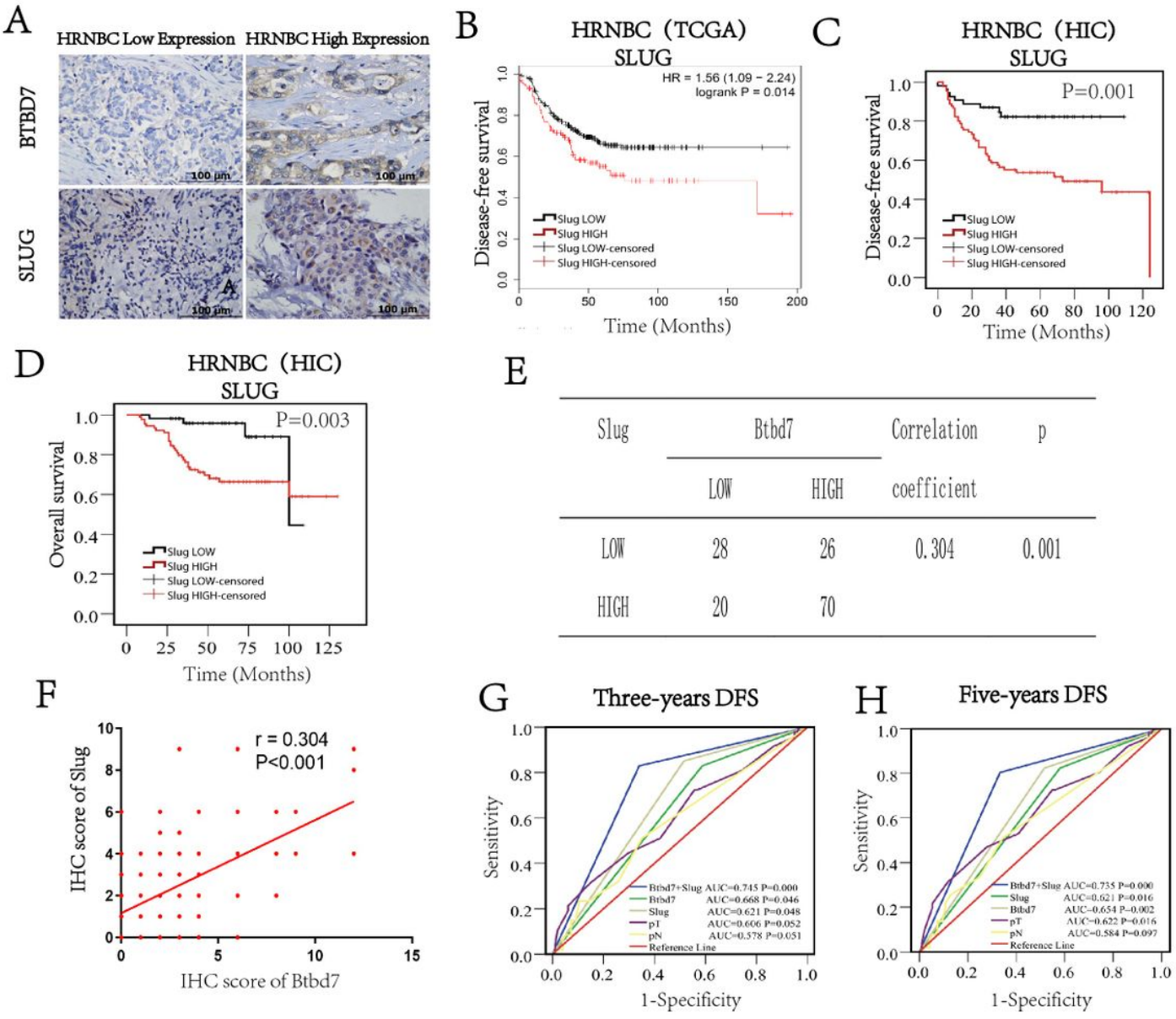


Figure 3

Subgroup analysis of DFS and OS of HER-2 overexpression patients according to Btbd7 by Kaplan-Meier curves. (A-I) Kaplan–Meier curves of DFS according to Btbd7 differed by age, tumour volume, lymph node metastasis, TNM stage and IDC differentiated degree. (K-T) Kaplan–Meier curves of OS according to Btbd7 differed by age, tumour volume, lymph node metastasis, TNM stage and IDC differentiated degree.

Figure 4



Slug	Btbd7		Correlation coefficient	p
	LOW	HIGH		
LOW	28	26	0.304	0.001
HIGH	20	70		

Figure 4

Immunohistochemical staining and Correlation analysis of Btbd7 and Slug. (A) Low and High Btbd7 and Slug expression in HRNBC tissues. (B) high SLUG mRNA level influenced HRNBC patients' prognosis in TCGA cohorts. (C-D) Kaplan-Meier analysis shows the DFS and OS of HRNBC patients with high and low Slug protein expression by Immunohistochemical staining. (E-F) Linear regression and correlation analysis of the expression level of Btbd7 and Slug in HRNBC tissue by fourfold table and scatter diagram. (G-H) Slug and Btbd7 predicted 3-year, and 5-year of DFS in HRNBC patients by ROC curves.

Figure 5

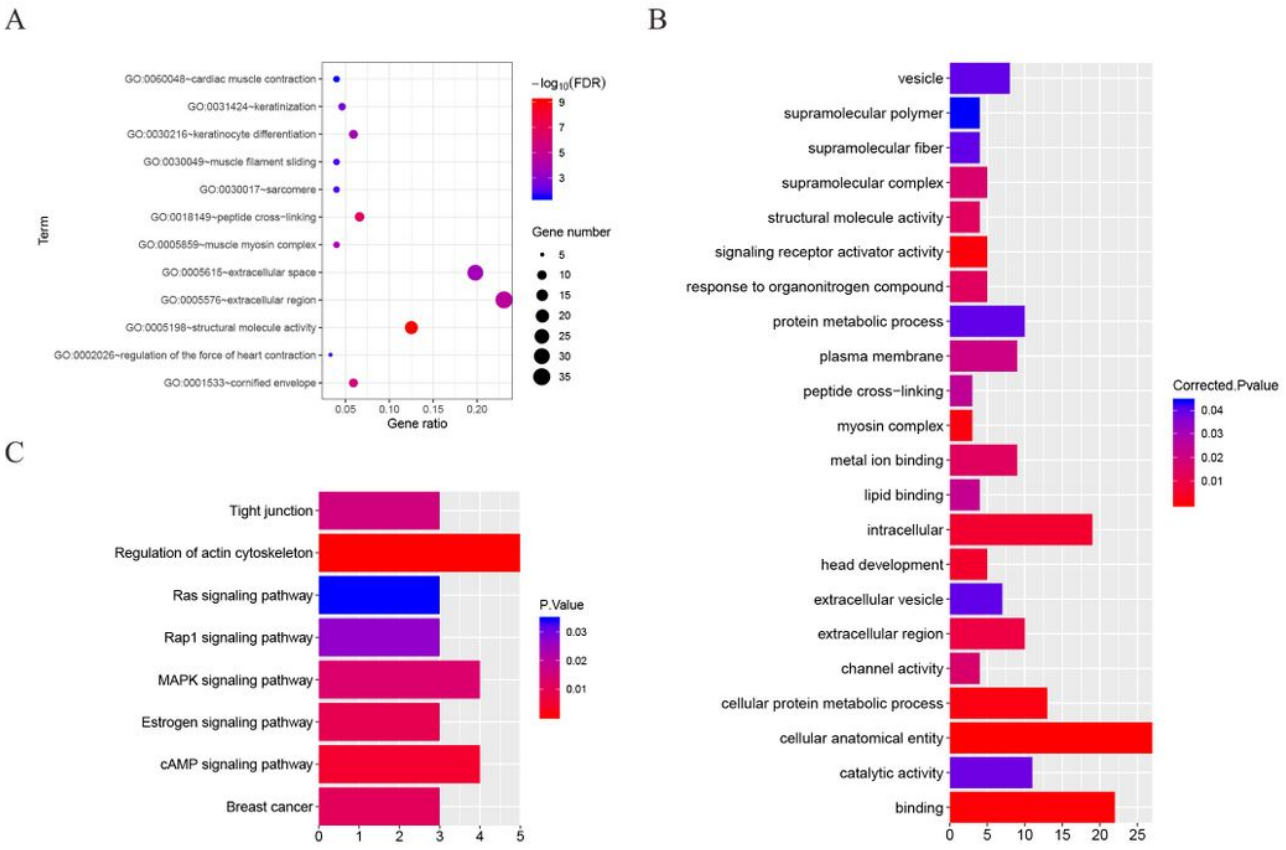


Figure 5

DAVID was used to explore the function roles of BTBD7 based on its DEGs (A). Gene Oncology (B) and KEGG pathway analysis (C) were performed using KOBAS 3.0.