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Knockdown of XB130 Restrains Cancer Stem Cell-Like Phenotype Through Inhibition of Wnt/β-Catenin Signaling in Breast Cancer

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ABBREVIATIONS

CSC, cancer stem cell; **EMT**, epithelial-mesenchymal transition; **SP$^+$ cells**, side population cells; **Src**, Proto-oncogene tyrosine-protein kinase; **PI3K**, Phosphoinositide 3-kinase; **shRNA**, a short hairpin RNA; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide; **NANOG**, Nanog homeobox; **OCT4**, organic cation/carnitine transporter 4; **SOX2**, sex determining region Y box 2; **ABCG2**, ATP binding cassette subfamily G member 2; **c-Myc**, MYC proto-oncogene; **MMP-9**, matrix metallopeptidase 9.

RUNNING TITLE

Silencing XB130 Decreases Cancer Stem Cells in Breast Cancer

KEYWORDS

Cancer Stem Cells, EMT, Mammosphere, SP$^+$ cells

Abstract

The cancer stem cells (CSCs) is a subset of cancer cells that possess stem cell properties, which plays a crucial role in the occurrence, metastasis, and recurrence of the tumor. XB130 is a novel adapter protein potentially serves as a functional factor in CSCs. To determine the role of CSCs in breast cancer, we focused on the study of XB130. In our study, we found that XB130 expression was significantly upregulated in breast cancer and was closely related to the clinicopathologic characteristics, overall survival and poor prognosis of breast cancer patients. Functionally, we found that knockdown of XB130 was not only played an important role in proliferation, epithelial-mesenchymal transition (EMT), and metastasis in breast cancer cells but also exhibited potent antitumor activity in animal tumor models. Moreover, we demonstrated that silencing endogenous XB130 regulated the cancer stem cell-like properties of breast cancer, including the formation of self-renewing spheres and the proportion of breast cancer SP^+ cells. Mechanistically, our studies indicated that downregulation of XB130 restrained the EMT and Wnt/β-catenin signaling, so as to weaken the tumor-initiating cell-like phenotype of breast cancer cells. This study indicates that XB130 plays an important role in maintaining the EMT and stem cell-like characteristics of breast cancer cells, supporting the significance of XB130 as a new potential therapeutic target for early diagnosis and prognosis of breast cancer.

1 INTRODUCTION

Breast cancer is the most prevalent malignancies and the leading cause of cancer death among women[1]. According to the statistics of the World Health

Organization, about 2.1 million women suffer from breast cancer and an estimated 627,000 women died of breast cancer in 2018[2]. Although the progress of early diagnosis and treatment of breast cancer has greatly improved the survival rate of patients with breast cancer, there are still about one-third patients died of tumor metastasis and recurrence within 5 to 10 years[3]. At present, the most significant problem in the treatment of breast cancer is the recurrence and metastasis caused by self-renewal of breast cancer cells. There is a small subpopulation of cancer cells with strong self-renewal ability, multidirectional differentiation potential and high tumorigenicity in cancer, which is known as cancer stem cells (CSCs)[4]. CSCs are closely related to tumor invasion, metastasis, recurrence, anti-radiotherapy, and anti-chemotherapy, so it has become a hot and difficult study point in current research[5]. The discovery of CSCs provides a new direction for the study of the pathogenesis of breast cancer. Therefore, it is of great significance to explore the biological characteristics and gene regulatory signaling pathway of CSCs, which may help greatly to solve the problem of metastasis and recurrence and develop new therapeutic treatments for breast cancer.

CSCs, which can lead to tumor development, recurrence, metastasis, and drug resistance[6], can be isolated and identified by cell surface markers and can be found in breast cancer, gastric cancer, glioma, and many other tumors[7]. The treatment failure of a large number of tumors indicated that CSCs showed stronger resistance to radiotherapy and chemotherapy, which suggested that the CSCs subpopulation could escape from traditional treatment, and the abnormal differentiation enhanced the tumorigenicity and metastasis of cancer cells[8]. It is reported that CSCs could be isolated from both the primary and metastatic tumor

tissues of colorectal cancer patients with liver metastasis, and the existence of CSCs in primary colorectal cancer tissues could predict the occurrence of distant metastasis[9]. Another study showed that a subpopulation of CSCs isolated from a metastatic pancreatic cancer cell line may be associated with tumor initiation and play a crucial role in tumor metastasis[10]. Recent research has found that the epithelial-mesenchymal transition (EMT) is related to the acquisition of stem cell properties[11]. Many other studies have shown that EMT cells have the characteristics of mesenchymal stem cells and can differentiate into a variety of cell lineages and form new tissues, indicating these EMT cells got increased stemness[12].

The human XB130 gene, which participates in a variety of cellular functions, is located on the 10q25.3 chromosome and encodes an adaptor protein of 818 amino acids[13]. As the N-terminal region of XB130 protein contains multiple tyrosine kinase phosphorylation sites and a proline-rich region, XB130 protein probably may bind to the SH2 and SH3 domains of Src protein[14]. Shiozaki et al. found that silencing the expression of XB130 in esophageal squamous cell carcinoma inhibited the operation of the cell cycle[15]. It was also found that XB130 promoted the tumorigenesis of thyroid carcinoma by binding to P85 α of PI3K[16]. Moreover, studies have shown that overexpression of XB130 may promote lung metastasis of breast cancer patients, and the expression of XB130 in sphere culture cells is higher than that in adherent cells[17]. These data suggest that XB130 may play an important role in the regulation of breast cancer metastasis and stem cells. However, the specific biological characteristics and molecular mechanism of XB130 on the regulation of breast cancer metastasis and self-renewal

characteristics remain to be elucidated. In the current study, we found that XB130 is significantly overexpressed in breast cancer cell lines and tissues and the high expression of XB130 is closely associated with shorter overall survival of breast cancer patients. Furthermore, we found that silencing XB130 decreases the metastasis and self-renewal ability of breast cancer cells through inhibiting EMT and the Wnt/β-catenin pathway. Taken together, our results indicate that XB130 plays an important role in maintaining stem cell-like properties of breast cancer cells, and XB130 may become a new therapeutic target for the diagnosis and prognosis of breast cancer.

2 MATERIALS AND METHODS

2.1 Cell lines

A non-tumorigenic epithelial cell line MCF-10a, and breast cancer cell lines BT549, MCF-7, MDA-MB-468, MDA-MB-231, T47D, ZR-75-30 and HCC1954 were purchased from ATCC. The MCF-10a cells were maintained in MEGM^{TM} mammary epithelial cell growth medium bulletKit^{TM} (Lonza, Basel, CHE), and 7 breast cancer cell lines were cultured in DMEM medium (Gibco, Grand Island, NY, USA) according to the ATCC protocol.

2.2 Patient information and tissue specimens

This study was conducted on a total of 205 cases of paraffin-embedded, archived breast cancer samples, which had been histopathologically and clinically diagnosed at the Sun Yat-sen University Cancer Center from 2002 to 2007. Clinical and pathological classification and staging were determined according to

the American Joint Committee on Cancer (AJCC) criteria. Ethics approval and prior patient written consent had been obtained from the Institutional Research Ethics Committee of Sun Yat-sen University Cancer Center for the use of the clinical specimens for research purposes. This research was carried out in accordance with the Declaration of Helsinki.

2.3 Immunohistochemistry analysis

Immunohistochemical staining (IHC) was used on paraffin-embedded tissue sections made from 205 cases of breast cancer samples, using anti-XB130 (1:300, Epitomics, Burlingame, CA), anti-CD44 (1:50), anti-β-catenin (1:100) and anti-Ki67 (1:600, Cell Signaling, Danvers, MA) antibodies according to the IHC kit's instruction (ZSGB-BIO, China). The stained sections were examined and scored by two independent pathologists, based on both the proportion of positively stained tumor cells and the intensity of the staining. The proportion of positively stained tumor cells were scored as follows: 0, no positive tumor cells; 1, <10%; 2, 10–50%; 3, 51–75%; 4, > 75%. The intensity of staining was graded according to the following criteria: 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow brown); and 3, strong staining (brown). The staining index was calculated as the staining intensity score multiplied by the proportion of positive tumor cells.

2.4 Vectors and retroviral infection

A short hairpin RNA (shRNA) oligonucleotides targeting human XB130 were cloned into pSuper-retro-puro to generate pSuper-retro-XB130-RNAi(s). The targeting sequence is 5′-GCTGAAGATCACACCGATG-3′, and the sequence of

scramble shRNA is 5′-GCCAGCTTAGCACTGACTC-3′ (Invitrogen, Carlsbad, CA)[18]. Retroviral production and infection were performed as previously described. Stable cell lines expressing XB130 shRNA were selected for 10 days with 0.5 μg/ml puromycin.

2.5 RNA extraction, reverse transcription (RT) PCR, and Real-Time PCR

Total RNA from cultured cells using the Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instruction. cDNAs were amplified and quantified in ABI Prism 7500 Sequence Detection System (Thermo Fisher Scientific) using dye SYBR Green I (Thermo Fisher Scientific). The primers were selected as the following: XB130 (forward: 5'-AAGCAGCAGCTCTGATGAGG-3' and reverse: 5'-GGTCTGGAAGGCTCTTCTGA-3')[20]; the housekeeping gene GAPDH (forward: 5'-ACCACAGTCCATGCCATCAC-3' and reverse: 5'-TCCACCACCCTGTTGCTGTA-3') was used as the internal control and all RT-PCR reactions were done in triplicate. Relative mRNA expression level was calculated by the $2^{-[(\text{Ct of } gene)-(\text{Ct of } GAPDH)]}$ method, where Ct represents the threshold cycle for each transcript.

2.6 Western blotting

Western blotting was performed according to standard methods as described previously[19], using anti-XB130 antibody (1:1000, Epitomics), anti-E-cadherin, anti-Vimentin, anti-β-catenin (1:1000, CST). Anti-GAPDH antibody (Sigma-Aldrich, Saint Louis, MO, USA) was used as a loading control.

2.7 MTT cell viability assay

Cells were seeded in 96-well plates at a density of 2×10^3 cells/well. At each time point, cells were stained with 100μl sterile MTT dye (0.5 mg/ml, Sigma-Aldrich) for 4 hours at 37°C, followed by removal of the culture medium and addition of 100μl of dimethyl sulphoxide (Sigma-Aldrich). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. Each experiment was performed in triplicates.

2.8 Colony formation assay

Cells were plated in 6-well plates (5×10^2 cells) and cultured for 10 days. The colonies were stained with 1% crystal violet for 30 seconds after fixation with 4% formaldehyde for 5 minutes. Colonies were counted and the results were shown as the fold change compared to vector control cells.

2.9 Immunofluorescence and Confocal Imaging

Cells were seeded on the coverslips in 24-well plate, and fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.1% Triton X-100 for 10 min, and then blocked with 2% BSA for 30 min. Cells were incubated with anti-E-cadherin (1:200, CST), anti-vimentin (1:1000, CST) and anti-β-catenin (1:200, CST) antibodies at 4 ℃ overnight. The washed cells were incubated with FITC labeled goat anti-rabbit secondary antibody (1: 5000, Abcam, Cambridge, MA) for 1 h. After washing, cells were treated with 6-diamino-2-phenylindole (DAPI, 1:5000, Sigma-Aldrich) for 10min. Cells were observed by a fluorescence

microscope (Olympus). Image-Pro Plus 6.0 (Olympus) was used for image analysis.

2.10 Wound healing assay

Cells were seeded into 6-well plates at 90% confluence and incubated overnight for adherence. Then a wound was made along the center of each well by scratching the cell layer with the tip of a 10 μl pipette. Next, the wells were washed twice with PBS to remove loose cells and fresh medium was added. Photographs were taken at 0 h, 12 h, and 24 h to assess cell migration into the wound.

2.11 Transwell migration assay

The migration potential of breast cancer cells was assessed by migration assay using 24-well chambers (BD Biosciences, San Jose, CA, USA). Briefly, breast cancer cells (5×10^4/ml in 0.5 ml of serum-free medium) were added to the upper chambers, and 0.75 ml of medium supplemented with 20% fetal bovine serum was added to each of the lower chambers as a chemoattractant. After incubation for 20 h, the cells remaining in the upper chambers were removed by scraping, and the invading cells in the lower chambers were fixed with 4% paraformaldehyde. Then the cells were washed twice with PBS, stained with hematoxylin for 1 h at room temperature, and photographed under a microscope.

2.12 Tumor xenografts

All experimental procedures were approved by the IACUC of Sun Yat-sen University cancer center. The NOD/SCID mice were randomly divided into 3

groups (n = 5 per group). Indicated cells of 3 doses (1×10^6, 1×10^5, 1×10^4) were inoculated with Matrigel (final concentration of 25%) into the inguinal folds of NOD/SCID mice. Tumor volume was determined using an external caliper and calculated using the equation $(L \times W^2)/2$. The mice were sacrificed 42 days after inoculation and the tumors were excised and subjected to pathologic examination.

2.13 Mammosphere formation assays

Five hundred cells were seeded in 6-well ultra-low cluster plates and 100 cells were seeded in 24-well ultra-low cluster plates for 10 days. Spheres were cultured in DMEM/F12 serum-free medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 20 ng/ml EGF, 20 ng/ ml bFGF (PeproTech), 0.4% BSA (Sigma-Aldrich), and 5 μg/ml insulin.

2.14 Flow cytometric analysis

Cells were dissociated with trypsin and resuspended at 1×10^6 cells per milliliter in DMEM containing 2% FBS and then preincubated at 37°C for 30 minutes with or without 100 μmol/l verapamil (Sigma-Aldrich) to inhibit ABC transporters. The cells were subsequently incubated for 90 minutes at 37°C with 5 μg/ml Hoechst 33342 (Sigma-Aldrich). Finally, the cells were incubated on ice for 10 minutes and washed with ice-cold PBS before flow cytometric analysis. The data were analyzed by Summit 5.2 software (Beckman Coulter, Fullerton, CA).

2.15 Statistical analysis

SPSS16.0 statistical software package was used for statistical analysis. The relationship between XB130 expression and clinicopathological features was

analyzed by chi-square test. The survival curve was drawn by the Kaplan-Meier method and compared by log-rank test. Univariate and multivariate analysis of potential prognostic factors and OS using Cox proportional hazard regression analysis.

3 RESULTS

3.1 Expression of XB130 is upregulated in breast cancer and is associated with poor prognosis in patients

We first measured XB130 expression level in breast cancer cells and tissues to determine the potential role of XB130. Both mRNA and protein levels of XB130 in breast cancer cells were higher than that in a non-tumorigenic epithelial cell line MCF-10a (Fig. 1A-1B). Consistently, IHC analysis showed that the expression of XB130 protein in the breast cancer tissues was also significantly higher than in the paired adjacent non-tumor tissues (Fig. 1C).

To investigate the relationship between XB130 expression and the clinicopathological features of breast cancer, the tissue samples of 205 cases of human breast cancer were analyzed by IHC analysis. With the increase of clinical stage, the expression level of XB130 was also upregulated in the tissues (Fig. 1D). Statistical analysis further revealed that XB130 expression was strongly associated with the clinical stage ($P < 0.01$), T classification ($P < 0.01$), N classification ($P < 0.01$) and M classification ($P < 0.01$) (Table 1). Kaplan-Meier survival curves and the log-rank test showed that XB130 expression was significantly negatively correlated with the overall survival (OS) of breast cancer patients ($P < 0.01$; Fig. 1E). Moreover, univariate and multivariate analyses

indicated that XB130 expression were independent prognostic factors for the patients (Table 2), which suggests that XB130 might be a useful predictive biomarker for prognosis in patients with breast cancer.

3.2 Downregulation of XB130 inhibits the proliferation of breast cancer cells

Because the results from the clinical specimens indicated that there is a correlation between XB130 expression and proliferation, so IHC assay was used to detect the expression of proliferation marker Ki67. The result showed that in the breast cancer specimens, the areas that displayed high levels of XB130 staining also showed strong Ki67 staining, while areas with low XB130 expression also displayed weakly detectable Ki67 expression (Fig. 2A). Statistical analysis showed that the expression level of XB130 was closely related to the expression level of Ki67 ($P < 0.01$) (Table 1), suggesting that XB130 may be a pro-proliferative factor in breast cancer.

We used stable XB130-knockdown cells to further investigate the effect of XB130 on the proliferation of breast cancer cells. The western blotting result showed the expression level XB130 was effectively downregulated (Fig. 2B). As shown in Fig. 2C and 2D, MTT and colony formation assays indicated that the proliferation rate of shXB130 cells was significantly decreased compared with the shNT control cells. Collectively, our results provided strong evidence that XB130 plays a vital role in promoting cell growth and proliferation of breast cancer cells.

3.3 Silencing XB130 reduces epithelial-mesenchymal transition (EMT) and tumor metastasis

The changes of cell migration ability commonly involve the epithelial-mesenchymal transition (EMT) process, and the main characteristics of EMT are the loss of epithelial marker (E-cadherin) and the increase of mesenchymal marker (Vimentin)[20]. Accordingly, the effect of XB130 on EMT was investigated by detecting the expression levels of EMT markers. As shown in Fig. 3A-3B, immunofluorescence staining and western blotting analysis showed that shXB130 could upregulate epithelial marker (E-cadherin) and downregulate mesenchymal marker (vimentin). Moreover, our study of clinical samples indicates that XB130 is associated with tumor metastasis. To verify this, we used wound healing assay and transwell assay to assess the role of XB130 in breast cancer cell migration and invasion. Our results showed that shXB130 cells inhibited the migration and invasion ability of breast cancer cells (Fig. 3C and 3D). Collectively, these results suggest that XB130 plays an important role in the motility and invasiveness of breast cancer cells.

3.4 Knockdown of XB130 restrains the tumorigenicity of breast cancer cells in vivo

To investigate the effect of XB130 on the tumorigenicity of breast cancer cells, three doses (1×10^6, 1×10^5 and 1×10^4) of XB130-silenced ZR-75-30 cells and corresponding shNT control cells were subcutaneously inoculated in NOD/SCID mice. As shown in Fig. 4A, compared with the shNT control cells, the tumorigenicity of XB130-silenced cells was weakened and the tumor growth rate

was decreased. In the group of 1×10^4 doses, none of the five mice formed visible tumors in the NOD/SCID mice implanted with XB130-silenced cells, by contrast, one of the five mice formed visible tumors in the NOD/SCID mice implanted with shNT control cells. In the group of 1×10^5 doses, one of the five mice implanted with XB130-silenced cells formed visible tumors, however, three of the mice implanted with shNT control cells formed visible tumors. In the group of 1×10^6 doses, all the five mice implanted with XB130-silenced cells and shNT control cells formed visible tumors (Fig. 4B), however, the transplanted tumor of XB130-silenced cells was smaller than that of the corresponding shNT control cells. In addition, immunohistochemical analysis results revealed that XB130 silencing decreased the level of tumor stemness-related marker (CD44) and inhibited the ability of β-catenin to transfer into the nucleus (Fig. 4C). These results suggested that silencing XB130 may reduce the number of CSCs in breast cancer cells, so as to strongly weaken the tumorigenicity of breast cancer cells and inhibit the recurrence of breast cancer in vivo.

3.5 Downregulation of XB130 attenuates a stem cell-like phenotype in breast cancer cells

Studies have shown that cells with stem cell characteristics in breast cancer have higher expression of pluripotent markers[21]. Consistent with these studies, we found that the mRNA expression levels of pluripotency-associated markers related to cancer stemness, including NANOG, OCT4, SOX2, and ABCG2, were significantly decreased in shXB130 stably transduced cells (Fig. 5A). To study whether XB130 is involved in promoting the formation of the CSC population in breast cancer, we used a mammosphere formation assay to detect the effect of

XB130 on the self-renewal ability of breast cancer cells. Notably, the results showed that XB130-silenced breast cancer cells formed smaller and fewer spheres compared with shNT control cells (Fig. 5B).

Previous studies have shown that the side population (SP) assay is a widely used method for isolating stem cell-like cells from cancer cell lines[22]. Then, we examined the effect of XB130 on the regulation of the proportion of SP^+ cells. As shown in Fig. 5C, silencing XB130 decreased the proportion of SP^+ cells from 1.13% to 0.12% in ZR-75-30 cells, and from 1.07% to 0.16% in MDA-MB-231 cells. Taken together, our data suggested that XB130 promotes the stem-like characteristics of breast cancer cells.

3.6 Mechanism of XB130-Mediated Expansion of Cancer Stem-Like Cells

The Wnt signaling pathway is considered crucial for the maintenance of the self-renewal ability of tumor stem cells and involved in the carcinogenesis of breast cancer cells[23]. Therefore, we next examined whether the effect of XB130 on the self-renewal ability of breast cancer stem cells is through the Wnt signaling pathway. We found that in the breast cancer specimens, the areas that displayed high levels of XB130 staining also showed strong β-catenin staining, while areas with low XB130 expression also displayed weakly detectable β-catenin expression (Fig. 6A). Statistical analysis also showed that the expression level of XB130 was closely related to the expression level of β-catenin ($P < 0.01$) (Table 1). Moreover, silencing XB130 significantly decreased the expression of four downstream target genes of the Wnt/β-catenin pathway and the activity of the luciferase reporter (TOP/FOP) driven by Wnt/β-catenin signals in breast cancer cells (Fig. 6B and

6C). Meanwhile, subcellular fractionation and immunofluorescence staining assays showed that β-catenin could not aggregate in the nucleus of breast cancer cells after knockdown of XB130 gene (Fig. 6D and 6E), similar as the result of Fig. 4C. Next, we further examined the role of Wnt activation in XB130-induced stemness. As shown in Fig. 6F, overexpressed β-catenin in XB130-silenced cells strikingly reversed the spheroidizing ability of breast cancer cells. Taken together, our results suggested that downregulation of XB130 may inhibit the stemness and tumorigenesis of breast cancer cells by inhibiting Wnt/β-catenin signaling.

4 DISCUSSION

XB130, an adaptor protein, is discovered in the molecular cloning of actin filament associated protein (AFAP)[24]. Adapter proteins are a unique group of protein binding partners. They do not contain enzyme activity, but they contain at least two modular domains which can connect signal elements to form macromolecular complexes which in turn transmit cell signals. XB130, as a novel adapter protein, plays an important role in signal transduction and cell functions. Many studies have shown that the expression of XB130 is higher in tumor tissues than in adjacent nontumor tissues and it promotes cell proliferation and tumor formation[15,25-27]. Chen et al. found that XB130 was remarkably overexpressed in prostate cancer tissues and significantly correlated with the increased prostate specific antigen, and knockdown of XB130 may attenuate invasion and metastasis of prostate cancer[27]. Shi et al. reported that XB130 is an oncogene in gastric cancer cells and might be an important regulator of the metastasis through its role in an EMT-like process, and perhaps is the potential target for treatment of this cancer[28]. Oskarsson et al. found that knockdown of XB130 significantly reduces

the lung metastatic efficiency of breast cancer cells[29]. We found that XB130 was overexpressed in breast cancer cells and tissues, and the overexpression of XB130 was closely correlated with poor prognosis of clinical breast cancer patients. In addition, through our functional studies, we found that downregulation of XB130 inhibited the proliferation of breast cancer cells. Taken together, these data suggest that XB130 may function as an oncogene, and promote the development and progression of breast cancer, similarly to its function in other types of cancers.

The statistical analysis results of clinical specimens showed that the expression of XB130 was closely related to the metastasis of breast cancer. The EMT process, which plays a key role in tumor metastasis, includes the loss of cell-cell adhesion, the activation of mesenchymal markers, and the increased motility of tumor cells[30,31]. To better elucidate the molecular mechanism of XB130 in enhancing the metastatic abilities of breast cancer, we investigated the effect of XB130 silencing on the process of EMT. As we expected, in XB130-silenced cells, the expression of epithelial marker E-cadherin was upregulated, whereas the mesenchymal marker vimentin was decreased. Furthermore, silencing XB130 expression in breast cancer cells inhibited the migration ability of EMT phenotype, indicating that XB130 plays an important role in the migration and metastasis of breast cancer cells.

It is reported that the induction of EMT in cancer cells could result in the acquisition of cancer stem cell properties, including the expression of the stem cell-associated $CD44^{+}/CD24^{-}$/low antigenic profile, the ability to self-renew, the initiation of tumors and the resistance to conventional therapies[32]. Some studies have also suggested that breast cancer cells could acquire the mesenchymal-like

phenotype via EMT, which leads to an increase in the number of stem cells with self-renewal and spheroidizing ability [32-35]. Additionally, a part of cancer cells develops the ability of invasion and metastasis through EMT when leaving primary tumor, while establishes the critical self-renewal ability as cancer stem cell [36-39]. As shown by our results, compared with the shNT control cells, knockdown of XB130 inhibited the spheroidizing ability of stem cells and decreased the proportion of SP^+ cells in vitro. Simultaneously knockdown of XB130 lowered the tumorigenicity and decreased tumor growth rates in vivo. The role of Wnt/β-catenin signaling in regulating EMT during tumor metastasis and the development of stem cell-like properties was well defined in a wide variety of cancer types, suggested that targeting aberrant Wnt signaling activity in CSCs may represent an important approach to cancer therapy [40-42]. In the presence of Wnt ligands, the co-activation of Frizzled receptor leads to the stability of β-catenin. The accumulation of intracellular β-catenin eventually leads to its nuclear translocation. In the nucleus, β-catenin binds to members of the TCF/LEF transcription factor family to regulate the expression of target genes [40]. In our current study, the results of IHC showed that the expression of XB130 was significantly correlated with the expression of β-catenin, and there was statistical significance. Furthermore, silencing XB130 inhibited the transcription level and nuclear translocation of β-catenin, and meanwhile, the expression of down signaling target genes of β-catenin was also decreased.

Taken together, these results suggest that knockdown of XB130 restrains EMT and the ability of stem cell self-renewal via inhibiting Wnt/β-catenin signaling pathway, thus inhibited the metastasis and stem cell-like properties of breast

cancer. In particular, this study suggests that XB130 may be a novel prototype therapeutic agent that can target Wnt/β-catenin signaling in breast CSCs to suppress tumorigenesis and relapse. In conclusion, XB130 exhibits correlations with both poor prognosis and overall survival time of patients with breast cancer. XB130 is an independent risk prognostic factor in breast cancer patients, which indicates that it has great potential for application as a useful prognostic indicator. Knockdown of XB130 attenuates the invasion, metastasis and stem cell self-renewal of breast cancer cells by modulating the EMT process and inhibiting Wnt/β-catenin signaling. It is of great significance to explore the role and molecular mechanism of XB130 to improve the diagnosis and treatment of breast cancer.

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CONFLICT OF INTERESTS

No potential conflicts of interest were disclosed.

AUTHOR CONTRIBUTIONS

XHZ, XBS, TX, JC, and TD contributed to the project design and execution of experiments, analysis of results, and writing of manuscript. TX, JC, and TD contributed to the execution of experiments and data analysis. TD, RX and XZ contributed to overlooking and following up with experiments and results analysis. XHZ, XBS, TX, JC, and TD contributed to project design, result analysis, manuscript writing, and proofreading. All authors have read and approved the final draft.

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DATA AVAILABILITY OF STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURES

Figure 1 Expression of XB130 is upregulated in breast cancer and is associated with poor prognosis in patients. (A and B) Real-Time PCR and western blotting analysis of XB130 expression in a non-tumorigenic epithelial cell line (MCF-10a) and 7 breast cancer cell lines. (C) Immunohistochemical analysis of XB130 protein expression in 6 pairs matched breast cancer tissues (T) and adjacent noncancerous tissues (ANT) from the same patients. Scale bars, 100μm. (D) The expression of XB130 in normal breast tissues and breast cancer tissues at different clinical stages. (E) Kaplan-Meier Analysis of the correlation between XB130 level and 5-year overall survival in 205 patients with Breast Cancer. *P < 0.01.

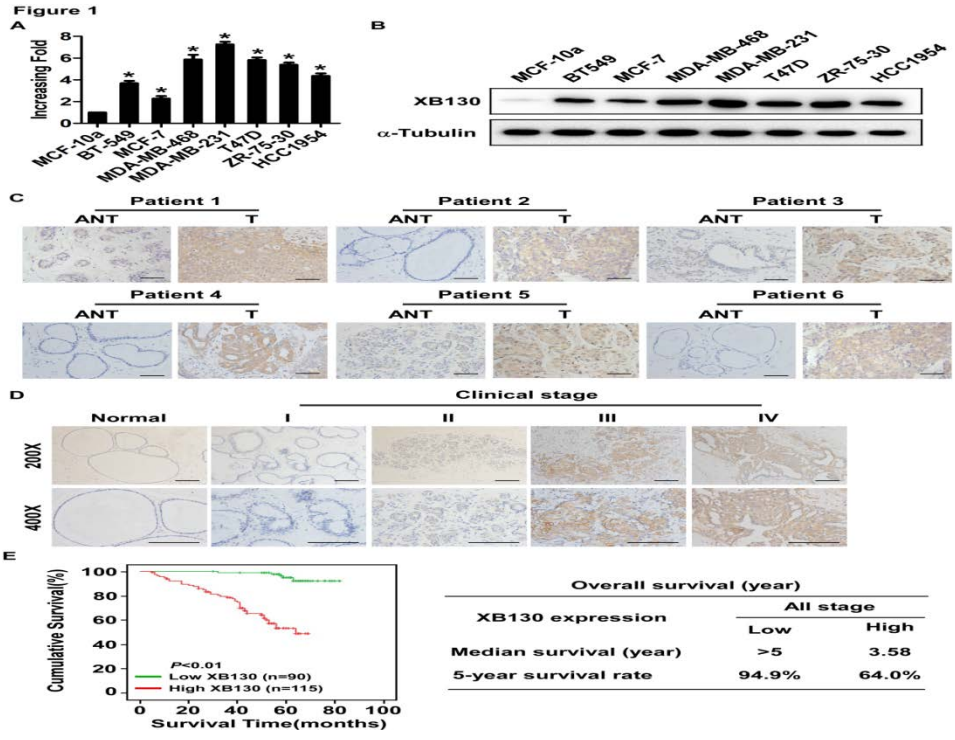


Figure 2 Downregulation of XB130 inhibits the proliferation of breast cancer cells. (A) XB130 expression levels significantly correlated with Ki67 expression in breast cancer tissues (n=205; $P < 0.01$). Two representative cases are shown (left) and percentage of specimens with low or high XB130 expression, relative to the levels of Ki67 staining (right). (B) Confirmation of the downregulation of XB130 in ZR-75-30 and MDA-MB-231 cells by western blotting; α-Tubulin was used as a loading control. (C and D) MTT and colony formation assay indicate that the growth rates decreased in XB130-silenced breast cancer cells. The number of colonies was quantified in the colony formation assay. Error bars represent the means ± SD of three independent experiments. *$P < 0.05$.

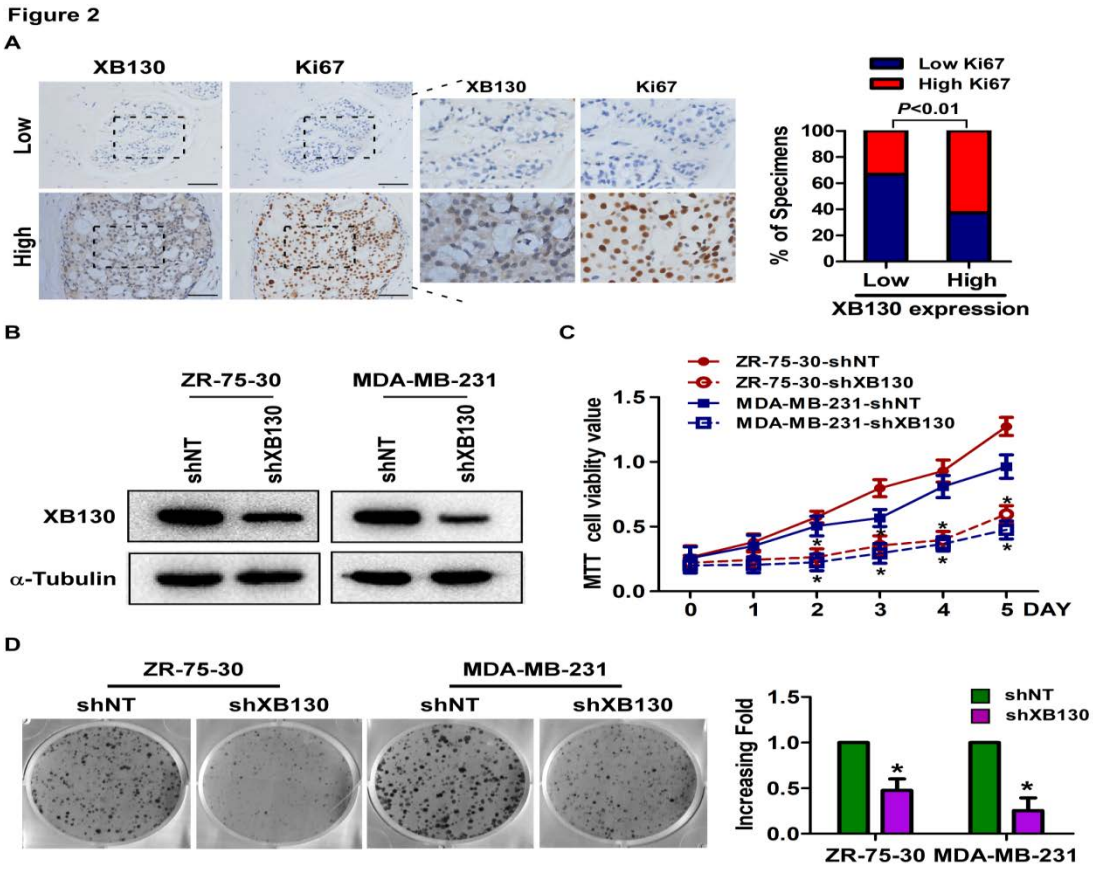


Figure 3 silencing XB130 reduces epithelial-mesenchymal transition (EMT) and tumor metastasis. (A and B) Representative immunofluorescence staining and western blotting assay show the expression of E-cadherin and vimentin in breast cancer cells. Nuclei were counterstained with DAPI. Scale bars, 20μm (C) The migration of breast cancer cells at the indicated time points were evaluated by wound healing assay. Scale bars, 200μm. (D) Transwell assay without Matrigel was performed to detect the migration of XB130-silenced breast cancer cells. Scale bars, 100μm. Error bars represent the means ± SD of three independent experiments. *$P < 0.05$.

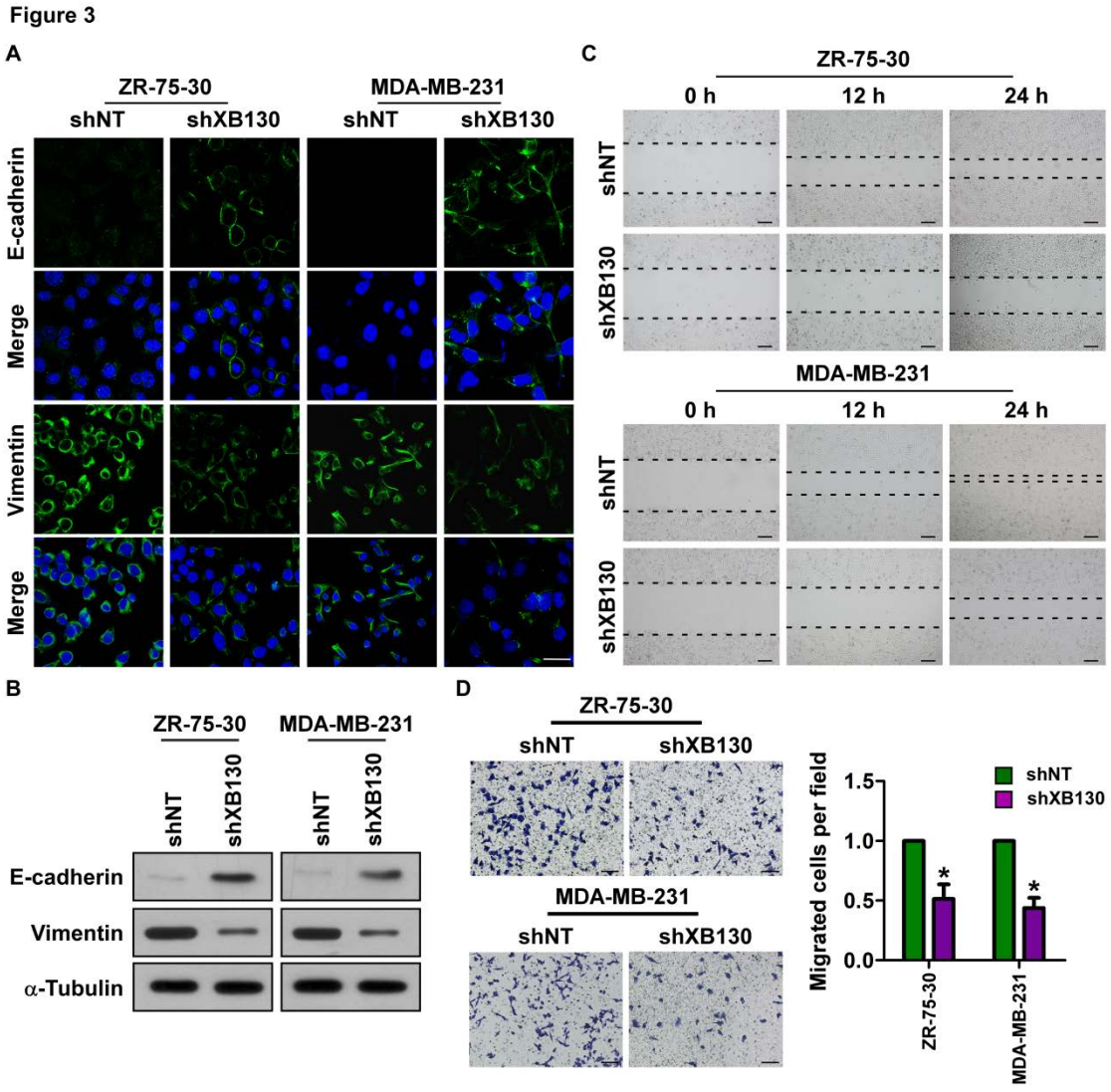
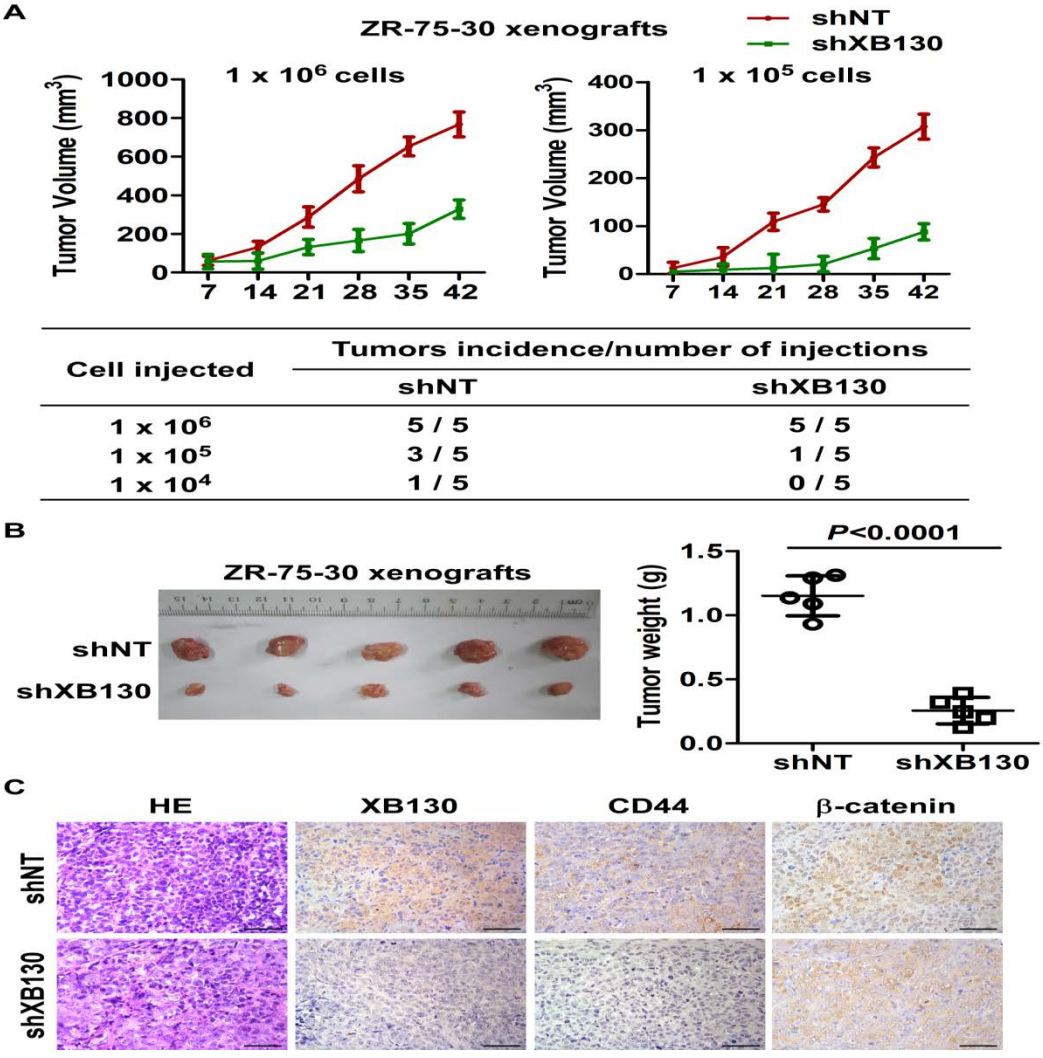


Figure 4 Knockdown of XB130 restrains the tumorigenicity of breast cancer cells in vivo. (A) Tumor growth curve after injection of the different number of the indicated cells. Draw the tumor volume (upper panel). The frequency of tumor formation for different numbers of indicator cells (lower panel). (B) The indicated cells (1×10^6) were injected subcutaneously into NOD/SCID mice. Tumor weight was measured at 42 days of tumor growth. Scale bar, 1 cm. (C) Representative images of tumor tissue formed by the indicated cells stained with H&E or antibodies against XB130, CD44 and β-catenin. Scale bars, 100 μm.

Figure 4



Cell injected	Tumors incidence/number of injections	
	shNT	shXB130
1×10^6	5 / 5	5 / 5
1×10^5	3 / 5	1 / 5
1×10^4	1 / 5	0 / 5

Figure 5 Downregulation of XB130 attenuates a stem cell-like phenotype in breast cancer cells. (A) Real-Time PCR analysis of the expression levels of cancer stemness associated markers, including NANOG, OCT4, SOX2 and ABCG2 in XB130-silenced cells compared with the vector control cells. (B) Representative images and quantification of spheres formed by the indicated cells. Scale bars, 100μm. (C) Hoechst 33342 dye exclusion assay showing SP cell proportions in the indicated cells. Error bars represent the means ± SD of three independent experiments. *$P < 0.01$.

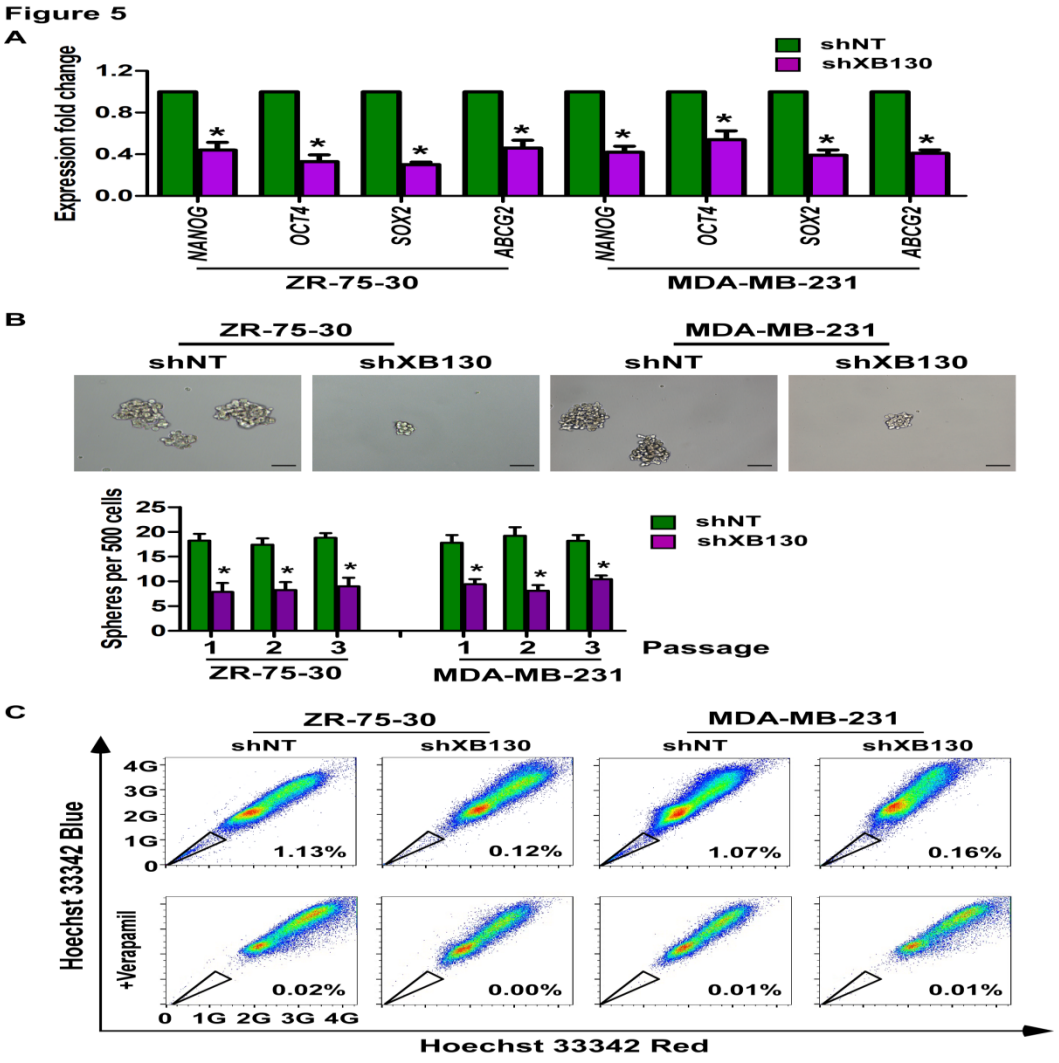


Figure 6 Mechanism of XB130-Mediated Expansion of Cancer Stem-Like Cells. (A) XB130 expression levels significantly correlated with β-catenin expression in breast cancer tissues (n=205; $P < 0.01$). Two representative cases are shown (left) and percentage of specimens with low or high XB130 expression, relative to the levels of β-catenin staining (right). (B) Real Time PCR analysis of the expression of the established downstream targets of the Wnt/β-catenin pathway, including c-MYC, MMP-9, CD44 and CD133 in the indicated cells. (C) The indicated cells were transfected with TOP or FOP reporter and Renilla pRL-TK plasmids and subjected to dual-luciferase assays 48 h after transfection. The detected reporter activity was normalized to the Renilla activity. (D) Silencing XB130 changed the nuclear translocation of β-catenin. Western blotting analysis showed the nuclear components of the cells. P84 was used as a loading control. (E) Immunofluorescence staining showed the localization of β-catenin in cells. Scale bars, 50μm (F) Representative images and quantification of cellular spheres formed by the indicated cells. Error bars represent the means ± SD of three independent experiments. *$P < 0.05$.

Figure 6

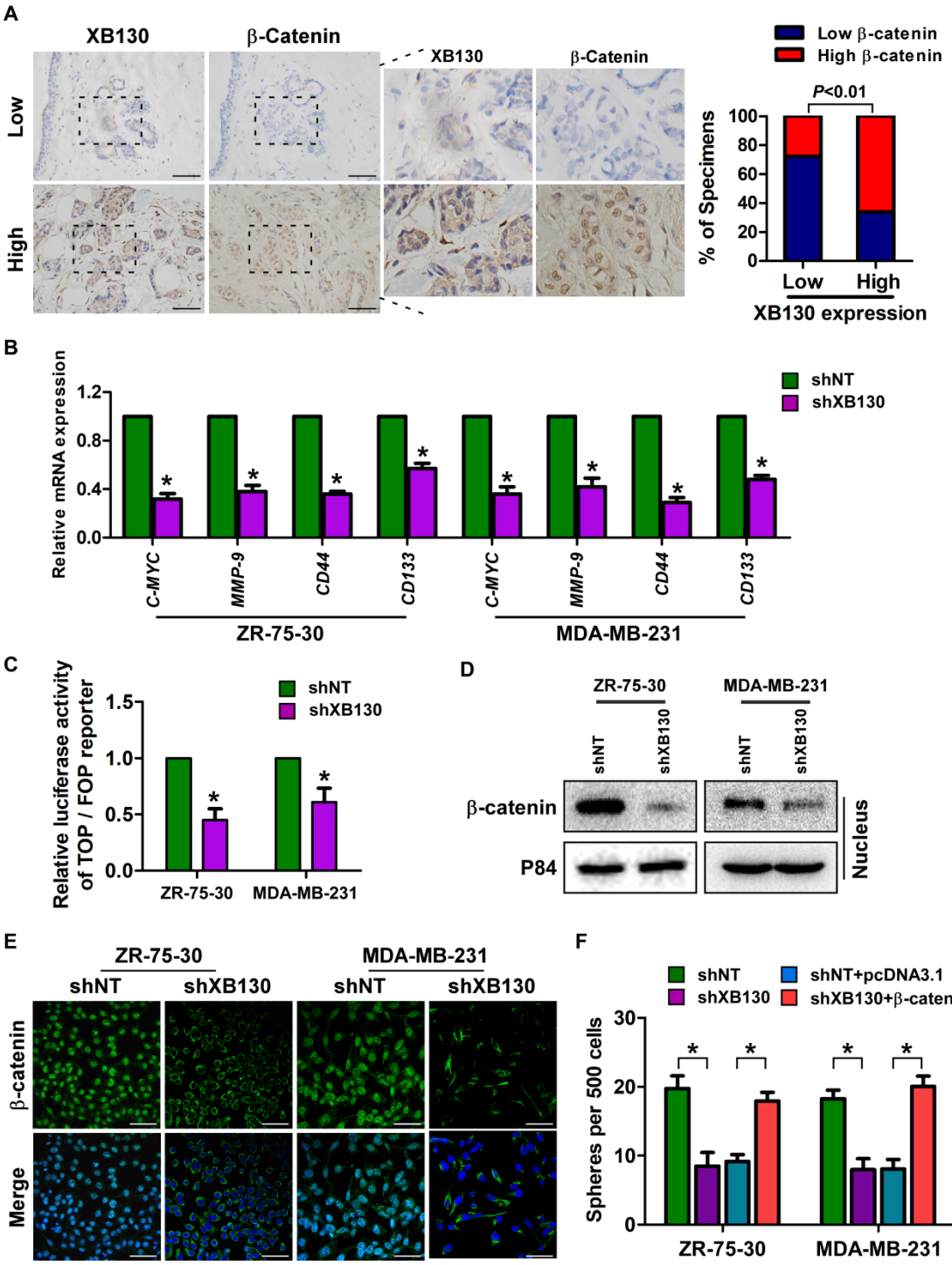


Table 1. Correlation between XB130 expression and clinicopathologic characteristics of Breast Cancer

Characteristics		XB130		Chi-square test (*P* value)
		Low (%)	High (%)	
Age (years)	>=47	52(57.8)	45 (39.1)	0.127
	<47	38(42.2)	70(60.9)	
Clinical Stage	I	34(37.8)	5(4.3)	0.000
	II	47 (52.2)	44(38.3)	
	III	8(8.9)	59 (51.3)	
	IV	1 (1.1)	7 (6.1)	
T classification	T1	38(42.2)	30 (26.1)	0.000
	T2	45(50.0)	50 (43.5)	
	T3	5 (5.6)	27 (23.5)	
	T4	2(2.2)	8 (6.9)	
N classification	N0	79 (87.8)	3 (2.7)	0.000
	N1-4	11(12.2)	112 (97.3)	

M classification	No	88(97.8)	108(93.9)	0.000
	Yes	2(2.2)	7(6.1)	
Ki67	Low	60(66.7)	43(37.4)	0.000
	High	30(33.3)	72(62.6)	
β-catenin	Low	65(72.2)	39(33.9)	0.000
	High	25(27.8)	76(66.1)	
ER	0	47(52.3)	52(45.2)	0.577
	1	20 (22.2)	19 (16.6)	
	2	11(12.2)	8 (6.9)	
	3	12(13.3)	36 (31.3)	
PR	0	44(48.9)	46 (40.0)	0.570
	1	13(14.4)	24 (20.9)	
	2	14 (15.6)	18 (15.6)	
	3	19(21.1)	27(23.5)	
HER2	0	48(53.3)	61(53.0)	0.097

1	9 (10.0)	17 (14.8)
2	8(8.9)	10 (8.7)
3	25 (27.8)	27 (23.5)

Table 2. Univariate and multivariate analyses of various prognostic parameters in patients with Breast cancer Cox-regression analysis

		Univariate analysis		Multivariate analysis		
	No. patients	*P*	Regression coefficient (SE)	*P*	Relative risk	95% confidence interval
Clinical stage						
I	39					
II	81	0.000	1.625(0.296)	0.000	3.263	1.826-5.829
III	67					
IV	8					
T classification						

T1	68					
T2	95					
		0.000	1.624(0.292)	0.000	3..569	1.979-6.438
T3	32					
T4	10					
N classification						
N0	82					
		0.000	2.043(0.438)	0.004	5.137	1.349-5.251
N1-4	123					
M classification						
M0	196					
		0.000	1.893(0.476)	0.003	4.286	1.646-11.158
M1	9					
Expression of XB130						
Low expression	90					
		0.000	2.512(0.476)	0.000	9.032	3.480-23.444
High expression	115					