UBE2T knockdown inhibits cell growth and metastasis of breast cancer through Wnt/β-catenin pathway

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Research

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Abstract

**Background:** Emerging evidences have demonstrated that Ubiquitin-conjugating enzyme E2T (UBE2T) is dysregulated and play critical roles in various cancers. With the development of sequencing technology, studies have discovered that UBE2T is overexpressed in breast cancer tissues. However, the biological roles of UBE2T in breast cancer are still far to clear. In the present study,

**Methods:** We analyzed the UBE2T expression in the Cancer Genome Atlas (TCGA) database. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to assess the expression of UBE2T in breast cancer cell lines. Cell proliferation, invasion and epithelial-mesenchymal transition (EMT) were determined by using CCK-8, EdU, Transwell and western blot assays.

**Results:** UBE2T was highly expressed in breast cancer cell lines. Functional analysis revealed that silence or elevation of UBE2T inhibited or promoted the proliferation, migration, invasion and EMT and Wnt/β-catenin signaling pathway related markers of MCF-7 cells. Mechanically, blocking of Wnt/β-catenin pathway by XAV939 reversed the promotion effect of UBE2T overexpression on breast cancer cells’ proliferation, migration and invasion.

**Conclusion:** Our findings emphasized that UBE2T may act as an oncogene via activating the Wnt/β-catenin pathway, which may provide a potential therapeutic target for the treatment of breast cancer.

Introduction

Breast cancer is one type of the most common malignant tumors in women worldwide (1, 2). The incidence of breast cancer has increased annually in recent years. With the continuous improvement of diagnosis and treatment, including chemotherapy, radiotherapy and molecular targeted biomarkers in the last few decades, the mortality of breast cancer patients remains relatively high (3-5). Hence, it is urgent to elucidate the molecular mechanism underlying the initiation and development of breast cancer.

Ubiquitin-conjugating enzyme E2T (UBE2T) belongs to the member of the ubiquitin-proteasome family, which located on chromosome 1q32.1 (6, 7). Many studies have proved that UBE2T serves important roles in in various cancers progression. Yan et al. discovered that silenced the UBE2T inhibits proliferation and induces cell cycle arrest and apoptosis in bladder cancer cells (8). UBE2T has been reported to be inhibits gastric cancer progression (9). In addition, knockdown of UBE2T could inhibit cell proliferation and invasion via regulating the PI3K/Akt signaling pathway in osteosarcoma (10). UBE2T have reported to accelerate glioblastoma invasion via regulating GRP78 (11). Yin et al. found that UBE2T promotes epithelial-mesenchymal transition through mediating FOXO1 degradation in non-small cell lung cancer (12). More importantly, the study demonstrated that UBE2T facilitated the cell proliferation and metastasis by activating the AKT/GSK3β/β-catenin pathway in nasopharyngeal carcinoma (13). However, the potential mechanism of UBE2T in breast cancer remains unclear.
In the present study, we found that UBE2T is simultaneously over-expressed in breast cancer tissues and cells. We then focused on exploring the functions of UBE2T in the progression of breast cancer and demonstrated that inhibition of its expression could markedly attenuate the proliferation and epithelial-mesenchymal transition (EMT) of breast cancer cells. Mechanistically, we elucidated the mechanism of UBE2T in the breast cancer progression and indicated that it could regulate breast cancer cell invasion and EMT through Wnt/β-catenin signaling pathway. Our findings illustrate a new target and underlying mechanism of breast cancer progression, which provide an effective target for the treatment and diagnosis of breast cancer, and extend the understanding mechanism-related functions of UBE2T in breast cancer.

**Materials And Methods**

**Cell culture and treatment**

MDA-MB-468, SUM149PT, MCF-7, SKBR3, MCF-10A cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and routinely cultured in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (FBS, LifeTechnologies, Inc., USA) in a humidified cell incubator at 37°C with 5% CO₂.

**Cell transfection**

Cells were transduced with small interference RNA (siRNA) specifically targeting UBE2T (si-UBE2T) as well as their corresponding negative control, UBE2T overexpression vector (UBE2T) and empty vector (pcDNA3.1) were purchased from GenePharma (Shanghai, China). For transfection, cells were plated into six-well plates and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequences were used as follows: UBE2T siRNA-1: 5′-TTGTCTGGATGTTCTCAAATT-3′, siRNA-2: 5′-CTCCTCAGATCCGATTTCT-3′, siRNA-3: 5′-GCTGCTCATGTCAGAACCCAA-3′.

**Cell Counting Kit 8 (CCK8) assay**

Cell viability was detected by MTT assay. MCF-7 cells (1 × 10⁵ cells/well) were seeded in 96-wells plate and cultured for 24 h, 48 h and 72 h. Then, cell viability was examined by CCK-8 kit (Beyotime Biotechnology, China). In brief, 50 µL of CCK-8 solution was added into each well and incubated at 37°C for 4 h. The absorbance value was measured by a microplate reader at 490nm wavelength. The experiment was repeated for three times.

**Transwell assay**

Cells were collected and plated the upper chamber (Corning) coated with (invasion) or without (migration) Matrigel (0.1%, Millipore, MO, USA). 1 × 10⁵ cells in serum-free medium in the upper chamber of transwell. Culture medium containing 20% FBS was supplemented into the lower chamber. Following 24 h
incubation, the non-migrated and non-invading cells were removed. At last, cells were fixed with 4% formaldehyde and stained using crystal violet. Cells were counted under a microscope (Olympus, Tokyo, Japan) at 200× magnification. The number of cells was the average value from six representative fields. The migratory or invaded cells were counted and photographed under a light microscope (Nikon, Tokyo).

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay**

Total RNA extraction was harvested utilizing TRIzol (Invitrogen). RNA quantification were amplified and detected on ABI PRISM 7900 Real-time PCR system (Applied Biosystems). The reaction conditions were as follows: 94°C 3 min, 94°C 30s, 56°C 40 s, 72°C 30 s, a total of 32 cycles, and finally extended at 72°C for 10min. GAPDH used for normalization and the relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers were as followed: UBE2T: 5′-GGCAAGATAAAGACCAAATGGA-3′ (Forward), 5′-CCTACTAGCTGACTGGCCTT-3′ (Reverse); E-cadherin: 5′-CGATTCAAAGTGGCACAGATG-3′ (Forward), 5′-GTAGGGAGTCCCAGGCGTAG-3′ (Reverse); Vimentin: 5′-TCTGGATCCCTCTGTGGTT-3′ (Forward), 5′-ATCGTGATGCTGAGAAGTTTCGT-3′ (Reverse). GAPDH: 5′-GAAGGTGAAGGTCGGAGTC-3′ (Forward) and 5′-GAAGATGATGGGATGTTCC-3′ (Reverse).

**Western blot analysis**

Total protein was extracted from cells using ice-cold RIPA lysis buffer (Beyotime Bitech, China). The concentration was estimated through a BCA protein assay kit (Beyotime Bitech, China). Equivalent samples (20 mg) were separated by 10% SDS-PAGE, and then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Subsequently, the membrane was blocked utilizing non-fat milk for 2 h and incubated with the following primary antibodies overnight at 4°C, including E-cadherin (ab231303, 1: 1, 000, abcam), N-cadherin (ab76057, 1: 1, 000, abcam), β-catenin (ab68183, 1: 1, 000, abcam), p-GSK-3β (ab93926, 1: 1, 000, abcam), GSK-3β (ab32391, 1: 1, 000, abcam), GAPDH (ab128915, 1: 1, 000, abcam). Then the membrane was washed three times with PBST. Then the membrane was cultured with HRP adjusted second antibody for about 2 h at room temperature. Protein bands were visualized with Enhanced Chemiluminescence Detection Kit (Thermo Fisher Scientific, USA) and the density of the bands was quantified by ImageJ software. GAPDH was used as the loading control.

**Statistical analysis**

Graphpad prism 8.0 (GraphPad, San Diego, CA, USA) was applied for statistical analysis. Data are presented as the mean ± standard deviation (SD). Comparisons between two groups were determined using Student’s t-test. One-way ANOVA analysis followed by Turkey’s post hoc test was used for multiple comparisons. P < .05 meant statistically significant.

**Results**

UBE2T expression was up-regulated in breast cancer
After browsing the Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/), we found that the UBE2T expression level was significantly higher in BRCA tissues than normal tissues (Figure 1A). Subsequently, the association of UBE2T expression levels with overall survival was analyzed. It was observed that the overall survival rate was significantly higher in low UBE2T level group than in high UBE2T level group (Figure 1B). Furthermore, the expression level of UBE2T in BC cell lines and human normal epithelial cell line MCF-10A was examined through qRT-PCR analysis. Consistently, the results indicated that the expression level of UBE2T was dramatically elevated in BC cell lines compared to MCF-10A cells, especially in MCF-7 cells that were used in the subsequent experiments (Figure 1C).

**UBE2T knockdown inhibits cell proliferation of breast cancer**

To probe the role of UBE2T in breast cancer, the UBE2T overexpression plasmid and UBE2T knockdown plasmid were transfected into MCF-7 cells, in order to up-regulated or down-regulated the UBE2T expression. The transfection efficacy was determined via qRT-PCR. Likewise, the data showed that UBE2T expression was conspicuously increased in MCF-7 cells transfected with pc-UBE2T, while decreased in MCF-7 cells transfected with si-UBE2T relative to NC group (Figure 2A). The efficacy of siRNA-3 and UBE2T-3 was better, so we chose si-UBE2T and UBE2T-3 for the next experiment.

Next, the effect of UBE2T on breast cancer cell proliferation was evaluated by CCK-8 assay. As shown in Figure 2B, the result indicated that compared with control groups, silenced UBE2T expression led to decreased cell proliferation rate. However, the viabilities of MCF-7 cells when transfected with pc-UBE2T was relatively promoted compared with that of the control group. Similarly, the results of EdU experiment unveiled that UBE2T silencing or promotion markedly suppressed or facilitated the proliferation of MCF-7 cells in comparison with the control group (Figure 2C). Additionally, UBE2T overexpression led to the opposite trend in the ability of MCF-7 cells.

**UBE2T knockdown suppresses the invasion and EMT of breast cancer cells**

Transwell migration and invasion assays were performed to assess the effects of UBE2T on the migration and invasion properties in MCF-7 cells. Compared with the control groups, UBE2T down-regulation led to inhibition, while UBE2T overexpression led to promotion of both migration and invasion of MCF-7 cells (Figure 3A, B). Epithelial-to-mesenchymal transition (EMT) is a process by which epithelial cells acquire the characteristics of mesenchymal cells in tumor progression [12, 13]. EMT related markers containing E-cadherin and N-cadherin were assessed. As shown in Figure 3C and 3D, the E-cadherin mRNA and protein expression level was increased while N-cadherin expression level was decreased when UBE2T was knocking down. Whereas the E-cadherin mRNA and protein expression levels in pc-UBE2T group was lower, while N-cadherin expression level was higher than those in the control group.

**UBE2T facilitated breast cancer cell invasion and EMT through activation of the Wnt/β-catenin signaling pathway**
Wnt/β-catenin signaling pathway plays an essential role in cancer progression, and we speculated that UBE2T facilitated breast cancer progression through Wnt/β-catenin signaling pathway. To test our hypothesis and investigate the possible mechanism of UBE2T in regulating the invasion and EMT of breast cancer, we investigated the expression of the related mRNA and proteins in Wnt/β-catenin pathway. We constructed UBE2T plasmid to force expression of UBE2T in MCF-7 cells (Figure 4A), and treated with XAV-939 (Wnt/β-catenin signaling inhibitor). Compared with the control group, ectopic of UBE2T significantly increases β-catenin and GSK-3β mRNA expression levels and can be reversed by XAV-939 treatment (Figure 4A). As shown in Figure 4B, the overexpression of UBE2T group showed an up-regulation in the protein expression levels of β-catenin and phosphorylation levels of GSK-3β. Meanwhile, these effects were reversed by XAV-939 treatment.

To investigate whether UBE2T plays its role in promoting tumor progression through Wnt/β-catenin signaling pathway, rescue experiments were performed. Followed functional analysis indicated that overexpression of UBE2T facilitated the proliferative capacity of MCF-7 cells, while were partly suppressed by XAV-939 treatment compared with control group (Figure 5A). In addition, the migration and invasion abilities of MCF-7 cells transfected with pc-UBE2T was higher than those in the control group. These effects were reversed in MCF-7 cells treated with XAV-939 (Figure 5B, C).

Discussion

In recent years, with the development of molecular biology, individualized cancer management has developed rapidly. Breast cancer has become one of the most challenging malignant tumors in woman. Interest in finding useful biomarkers for diagnosis and treatment of breast cancer has been accumulating. Increasing data indicated that aberrant UBE2T expression has serve important roles in almost all cell biological behaviors, including growth, cell cycle, apoptosis, differentiation, apoptosis and metastasis (14-17). However, little is known about the function of UBE2T in breast cancer. The purpose of our study was to explore the role of UBE2T in breast cancer.

By performing a series of bioinformatics analyses, we found that UBE2T was notably up-regulated in breast cancer tissues. According to the clinical evidence, we deduced that UBE2T may affect breast cancer cell functions. Based on the results of qRT-PCR, UBE2T expression was higher in breast cancer cells than that in normal epithelial cells. In vitro experiments were performed to evaluate the role of UBE2T in cell proliferation and invasion of breast cancer by using the CCK-8, EdU and colony formation and transwell assays with MCF-7 cells exhibiting overexpression or knockdown of UBE2T. As expected, we found that knocking down of UBE2T could inhibit cell proliferation, migration and invasion, indicating that UBE2T exerted a significant inhibitory role in these biological processes.

Tumor metastasis involves many tumor processes and EMT is a key initial step for tumor cells to acquire the potential of metastasis and invasion (18, 19). EMT is an evolutionary process in which cells lose epithelial properties and acquire mesenchymal properties. E-cadherin and Vimentin as the epithelial biomarkers, have been proved to play an important role in tumor metastasis (20-23). For example, Zhang
et al. found that GRIM-19 could inhibit colorectal cancer cell invasion and EMT by inactivation of STAT3/HIF-1α signaling axis (24). REC8 have been proved to inhibit gastric cancer cell EMT by down-regulating EGR1 expression (25). In the present study, the results of qRT-PCR and western blot assays demonstrated that knockdown of UBE2T markedly repressed the expression level of mesenchymal marker N-cadherin, while the expression level of epithelial marker E-cadherin was increased.

The role of Wnt/β-catenin signaling played in the growth and metastasis of tumor has been investigated in many researches (26-28). Above these findings led us to consider the potential association of UBE2T with the Wnt/β-catenin pathway. The results indicated that ectopic of UBE2T expression activated Wnt/β-catenin pathway with the change of β-catenin and GSK-3β. In addition, disruption of Wnt/β-catenin signaling by XAV-939 treatment reversed the stimulative effect of UBE2T overexpression on breast cancer cell proliferation, migration and invasion.

In summary, our results demonstrated that UBE2T was significantly stimulated the proliferation, migration, invasion and EMT of breast cancer by activating Wnt/β-catenin pathway. These findings indicated that UBE2T may be a potential candidate predictive factor in breast cancer, which may contribute to the diagnosis and treatment of breast cancer.

Declarations

Contributions of all authors

MJ conceived and designed the study. TX drafted the manuscript and analyzed the data. HY performed the experiments.

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Conflict of interest

The authors state that there is no conflict of interest.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.
Consent for publication

Not applicable.

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Figures
Figure 1

The expression levels of UBE2T were up-regulated in breast cancer. A. The expression levels of UBE2T in 1085 breast cancer tissues and 291 normal tissues in GEPIA database. B. The overall survival rates of patients with breast cancer were analyzed in TCGA database using the GEPIA online tool.
Figure 2

UBE2T knockdown inhibits cell proliferation of breast cancer. A. The transfection efficacy was determined via qRT-PCR. B, C. The effect of UBE2T on breast cancer cell proliferation was evaluated by CCK-8 and EdU assays.
Figure 3

UBE2T knockdown suppresses the invasion and EMT of breast cancer cells. A, B. Cell migration and invasion were analyzed by transwell assay. C, D. qRT-PCR and western blot analyses were used to determine the mRNA and protein level of EMT related markers.
Figure 4

UBE2T facilitated breast cancer cell invasion and EMT through activation of the Wnt/β-catenin signaling pathway. A. Wnt/β-catenin signaling pathway associated mRNA and protein expressions were assessed using qRT-PCR and western blot assays.
Figure 5

UBE2T facilitated breast cancer cell invasion and EMT through activation of the Wnt/β-catenin signaling pathway. A. The proliferative capacity of MCF-7 cell was evaluated by CCK-8 analysis. B. The migration of MCF-7 cells was estimated by transwell assay. C. Transwell experiment was conducted to assess the invasion ability of two cells.