Original Article

The associations between Deltex1 and clinical characteristics of breast cancer

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Background: Deltex 1 (*DTX1*) is a single transmembrane protein with ubiquitin E3 ligase activity which has been found to play a role in the development of several cancers. We aimed to investigate the associations between *DTX1* and breast cancer (BC).

Methods: We explored the roles and mechanisms of *DTX1* in BC by using BC cell lines *in vitro*. Levels of *DTX1* in serum and tissues were determined in 316 patients with BC, 102 patients with fibroadenoma, and 113 healthy controls by immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR). The associations between *DTX1* and clinical characteristics of BC were analyzed using multivariate analysis and Cox regression survival analysis.

Results: Lower levels of *DTX1* promoted BC cell proliferation, migration, and invasion. The cell growth and survival of BC might be regulated by DTX1 via the Notch signaling pathway. Levels of *DTX1* in BC tissues were lower compared to fibroadenoma tissues and peri-neoplastic breast tissues (P<0.01). A lower level of *DTX1* was shown to be associated with advanced tumor grade (P=0.017), advanced clinical stage (P=0.031), positive lymph node metastasis (LNM) (P=0.009), and high Ki-67 index (P=0.023). Lower *DTX1* expression was recognized as an impact factor for metastasis-free survival (MFS) in BC.

Conclusions: Lower levels of *DTX1* could promote BC cell proliferation and migration, and are associated with advanced BC. There is potential for *DTX1* as a marker to assist the selection of new BC treatment.

Keywords: Breast cancer (BC); Deltex 1 (*DTX1*); Notch signaling pathway; clinical characteristics; prognosis

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Introduction

Breast cancer (BC) is the most common malignancy in women. Due to the development and improvement of novel therapeutic schemes, BC is one of the most researched cancers of the past 20 years (1). Currently, therapy for BC is individualized and multimodal, and surgical treatment is still an essential part of the therapeutic scheme. However, several issues such as disease recurrence and distant metastases remain (2). It is vitally necessary to investigate and find new biomarkers to explore better drug or therapy for BC.

Deltex 1 (*DTX1*) is a single transmembrane protein with

ubiquitin E3 ligase activity (3,4), which has roles in the development and differentiation of lymphocytes. During the course of enhanced B lymphocyte development and suppressed T cell development, over expression of *DTX1* has been shown to inhibit Notch signaling (5,6). The expression of *DTX1* induced in T-cell anergy and was found to inhibit T-cell activation in E3-dependent and E3-independent mechanisms (5).

Recently, *DTX1* was found to play a role in the development of several cancers. The downregulation of *DTX1* in gastric cancer tissues has been linked to better prognosis in gastric cancer (3). Mutations of *DTX1* have been identified in patients with primary and relapsed diffuse large B-cell lymphoma (7). Huber *et al.* found that glioblastoma (GBM) patients with low *DTX1* levels had a more favorable prognosis (8). However, little has been reported about associations between *DTX1* and BC.

In mammals, *DTX1* is a downstream molecule and negative regulator of Notch signaling pathway (3,4). It regulates osteosarcoma invasiveness through Notch/HES1 signaling, and low *DTX1* expression might be useful as a marker to select osteosarcoma patients who could benefit from Notch inhibitor treatment (9). Reintroduction of *DTX1* into gastric cancer cells increased TRAIL-induced apoptosis (3). This might be a new approach for therapy in breast cancer. However, there is little report about associations between *DTX1* and BC.

In the present study, we investigated the associations between *DTX1* and BC, the role and possible mechanisms of *DTX1* in the development of BC, and the relationships between *DTX1* and clinical characteristics of BC.

We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/gs-21-739).

Methods

Cell culture and transfection

The human BC cell lines, HCC1937 (CPB60418), T47D (CPB60397), MDA-MB-468 (CPB60387), BT474 (CPB30103L), and normal breast cell line MCF-10A (CPB60419) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). These cell lines were derived from women and cultured at Passage 3. Each cell line was authenticated using a short tandem repeat (STR) Identifiler kit (Applied Biosystems, Waltham, MA, USA), and cultured in Dulbecco's modified Eagle medium (DMEM; 11885, Thermo Fisher Scientific, Waltham, MA, USA) with 5% fetal bovine serum (FBS; SH30910.03, HyClone, Logan, UT, USA) at 37 ℃ in 5% CO_2.

For knockdown assays, expression of the *DTX1* gene was downregulated by ON-TARGET plus small interfering (si) RNA SMART pool RNA (Thermo Scientific) using RNAi MAX transfection reagent (13778150, Life Technologies, Carlsbad, CA, USA). Non-targeting SMART pool RNA (Thermo Scientific, USA) was used as the control. The ectopic overexpression of *DTX1* was achieved with pCMV6-Entry-DTX1 plasmid (RC208338, Origene, Rockville, MD, USA) using FuGENE Extreme 9 transfection reagent (Roche, Nutley, NJ, USA). Empty pCMV6-Entry (PS100001, Origene) was used as control. Transfection efficiency was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot.

RNA extraction and RT-PCR

Total RNA from cell lines, breast tissues, and peripheral blood was extracted using RNeasy Mini Kit (74104, Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA purity was assessed by the integrity of 18S and 28S rRNA using an Agilent microfluidic chip (Agilent, Santa Clara, CA, USA). The concentrations and quantity of total RNA were determined based on the absorbance at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, USA).

From each sample, 1 μg RNA was reverse transcribed using the Omniscript RT kit (205111, Qiagen), and PCR was performed in triplicate using an ABI-Prism 7900 (Applied Biosystems) with SYBR Green I detection (218073, Qiagen) according to the manufacturer's protocol. The expression of each target gene was presented as the ratio of the target gene to β-Actin expression calculated using the formula, $2^{-\Delta Ct}$, where $\Delta Ct = Ct^{Target} - Ct^{18s}$. The primer sequences were as follows: *DTX1*, forward primer: 5'-GGGCTGATGCCT GTGAATG-3', reverse primer: 5'-CCTGGCGAAAC TGGTGC-3'. β-Actin, forward primer: 5'-CGTGACATTAAGGAGAAGCTG-3', reverse primer: 5'-CTAGAAGCATTTG CGGTGG AC-3'.

Cell proliferation analysis

Cell proliferation was analyzed using the 'Amersham Cell Proliferation Biotrak enzyme-linked immunosorbent assay (ELISA), version 2' system (GE Healthcare, Amersham,

UK) according to manufacturer's instructions. In short, 5,000 cells were seeded in the well of a 96-well plate and grown for 2 days, labeled with bromodeoxyuridine (BrdU) for 3–4 h, fixed and labeled with a peroxidase labeled anti-BrdU antibody. After coloring reaction, the optical density was measured with a 'Spectra MAX 250' plate reader and analyzed with accompanying 'Soft Max Pro' software (Molecular Devices, MDS Analytical Technologies, Toronto, Canada). For cell counting, equal amounts of cells were seeded in triplicates and cultured under standard conditions for 3 days. Cells were then harvested, and each biological replicate was counted 3 times using a 'Neubauer'-chamber (hemocytometer).

Migration and wound healing assay

Migration assay was performed using modified Boyden chamber units with polycarbonate filters of 8 mm porosity (Costar, Vitaris, Switzerland). The lower side of the filter was coated with 25 mg/mL collagen I (C9879, Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 ℃. The bottom chamber was filled with DMEM containing 10% FBS. Cells (4×10^4 per well in serum-free DMEM) were plated in the upper chamber in 100 µL medium and incubated for 24 h in standard conditions. After removal of the remaining cells from the upper surface of the filter insert, migrated cells at the bottom of the filter were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and stained with 0.1% crystal violet. For every individual filter, the cells in 9 fields of view were counted. Every experiment was conducted in triplicate.

For wound healing analysis, cells were grown to above 90% confluency under standard conditions. A wound was inflicted by scratching over the surface of the culture flask with a 200-µL pipette tip. The wounds were documented immediately after scratching, 24 and 48 h. Quantification of wound closing was performed with Image J software (https://imagej.net/) according to the manufacturers' instructions.

Western blot analysis

Protein lysates from tissues or cells were resolved on denaturing 8–12% sodium dodecyl-sulfate (SDS) polyacrylamide gels and transferred to nitrocellulose membranes (LC2000, iBlot Gel transfer stacks, Invitrogen). The following primary antibodies were used: anti-Actin (ab8227, Sigma Aldrich), anti-Notch1 (07-1231, AB Biotech, Bloomington, IN, USA), anti-Jagged1 (07-220, AB Biotech), anti-HES1 (AB15470, Millipore, Burlington, MA, USA), and anti-DTX1 (ab198877, AB Biotech). Decorated proteins were revealed using horseradish peroxidase-conjugated anti-mouse, anti-rabbit, anti-rat (New England Biolabs, Ipswich, MA, USA) secondary antibodies and visualized by the chemiluminescence detection system Super-Signal West Pico (Thermo Scientific). Densitometry of western blots was performed using Image J software according to manufacturers' instructions.

Patients and BC specimens

This study was approved by the Institutional Review Board of the Affiliated Hospital of Qingdao University (approval No. QYFYWZLL26477), and conformed with the Code of Ethics of the World Medical Association (Declaration of Helsinki, 2013 version). The written informed consent of each participant was provided before enrollment.

All patients and healthy controls enrolled in the study were from the same geographic area (Qingdao, Shandong). The enrolment time was from March 2015 to February 2017. All patients were diagnosed according to their clinical and pathologic manifestation, as defined by the WHO classification criteria (10). The main exclusion criteria were inflammatory BC; other concurrent or previous malignant disease; life-threatening disease, such as uncontrolled cardiac diseases; a pregnant or lactating status; and radiotherapy or chemotherapy administration before surgery. The healthy controls were women who had undergone physical examination in our hospital.

Histopathological examination and immunohistochemistry (IHC)

Surgical specimens or tumor tissues acquired by biopsy were microscopically examined by at least two experienced pathologists. The following histopathologic factors were assessed: cell type of the main lesion, primary tumor size, location, multiplicity, margin involvement, lymph node metastasis (LNM), and status of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and Ki-67.

Breast tissues were excised and formalin-fixed, then, paraffin-embedded sections were prepared for hematoxylin and eosin (H&E) staining and IHC by the Department of Histopathology, the Affiliated Hospital of Qingdao

University. Antigen retrieval was carried out by incubation of tissue sections in 10 mM sodium citrate buffer (pH 6.0) for 20 min at a sub-boiling temperature. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min, followed by incubation for 30 min with blocking solution [10% goat serum in Tris-buffered saline (TBS)], and then incubation overnight at 4 ℃ with the appropriate primary antibody diluted in blocking solution. Biotin-conjugated secondary antibodies were diluted in TBS containing 0.1% Tween-20 and incubated for 30 min at room temperature using the ABC Vectastain detection system (Vector Labs, Burlingame, CA, USA) and diaminobenzidine, and slides were counterstained with Harris-modified hematoxylin, dehydrated, and mounted in Permount™ (Thermo-Fisher).

Statistical analysis

The software SPSS 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Wilcoxon test was used to analyze significance between data presented as median (range). Multivariate analysis was used to analyze significance between different groups. To adjust the survival for other known confounding variables, Cox regression survival analysis was used. The initial date was the date of diagnosis, the date of event was the date of death for 5-year overall survival (OS). The median follow-up time was 30 months. Results where P<0.05 were considered statistically significant.

Results

Lower DTX1 promoted BC cell proliferation

Levels of *DTX1* in HCC1937, T47D, MDA-MB-468, and BT474 were lower than those detected in MCF-10A cells. As shown in *Figure 1A*, HCC1937 cells with lower *DTX1* expression and BT474 cells with higher *DTX1* expression were used for *DTX1* overexpression and knockdown experiments. The transfection efficiency was 75% for BT474/shDTX1 cells and 60% for HCC1937/DTX1 cells. After selection with puromycin, a high purity of HCC1937/DTX1 cells, HCC1937/scr cells, BT474/scr cells, and BT474/shDTX1 cells were acquired (*Figure 1B*). The growth of BT474/shDTX1 cells was 141% of the rate of BT474/scr cells while HCC1937/DTX1 cells grew in monolayer culture at 62% of HCC1937/scr cells (P<0.01, as shown in *Figure 1A*).

Lower DTX1 promoted BC cell migration and invasion

To determine the role of *DTX1* in invasion of BC cells, we performed transmembrane invasion assays with HCC1937/DTX1 cells, HCC1937/scr cells, BT474/scr cells, BT474/shDTX1 cells, and MCF-10A control cells. Low expression of *DTX1* significantly promoted the invasive behavior of BT474/shDTX1 cells by126% of BT474/scr cells, while HCC1937/DTX1cells showed an average reduction of invasion by 71% of HCC1937/scr cells (P<0.01, *Figure 2A*).

Wound closure was massively accelerated in BT474/shDTX1 cells compared to BT474/scr cells and MCF-10A controls cells (P<0.01, *Figure 2B*). The healing area of BT474/shDTX1 cells was 59% at 24 h after wound scratching 24 h and 80% after 48 h. The healing area of BT474/scr cells was 40% at 24 h after wound scratching and 53% after 48 h. Wound closure was massively reduced in HCC1937/DTX1 cells compared to HCC1937/scr cells and MCF-10A controls cells (P<0.01, *Figure 2B*). The healing area of HCC1937/DTX1 cells was 26% at 24 h after wound scratching and 49% after 48 h, while the healing area of HCC1937/scr cells was 49% at 24 h after wound scratching and 77% after 48 h. These results confirmed the findings of the transmembrane invasion assay.

DTX1 regulated BC cell growth and survival via Notch signaling pathways

Compared with MCF-10A control cells and HCC1937/scr cells, as shown in *Figure 3*, expressions of Notch1, Jagged1, and HES1 of the Notch signaling pathway were significantly down-regulated in HCC1937/DTX1 cells (P<0.01), while expressions of these molecules increased in BT474/shDTX1 cells compared to BT474/scr cells (P<0.01).

To explore whether *DTX1* regulated cell growth and migration via the Notch/DTX1 signaling pathways, we treated HCC1937 cells with gamma-secretase inhibitor (GSI), a Notch blocker. Cells were cultured for 3 days with GSI (compound E, 10 μM) to establish a Notch-off state. After GSI treatment, proliferation of HCC1937 cells was 62% of HCC1937 cells without GSI (P<0.01, *Figure 4A*). The wound closing was 40% *vs.* 25% (HCC1937 cells without GSI *vs.* HCC1937 cells with GSI) at 24 h after wound scratching, and 70% *vs.* 40% (HCC1937 cells without GSI *vs.* HCC1937 cells with GSI) at 48 h after wound scratching. The wound closing of HCC1937 cells with GSI reduced by 15% and 30% compared to HCC1937 cells without GSI after 24 and 48 h, respectively (P<0.05,

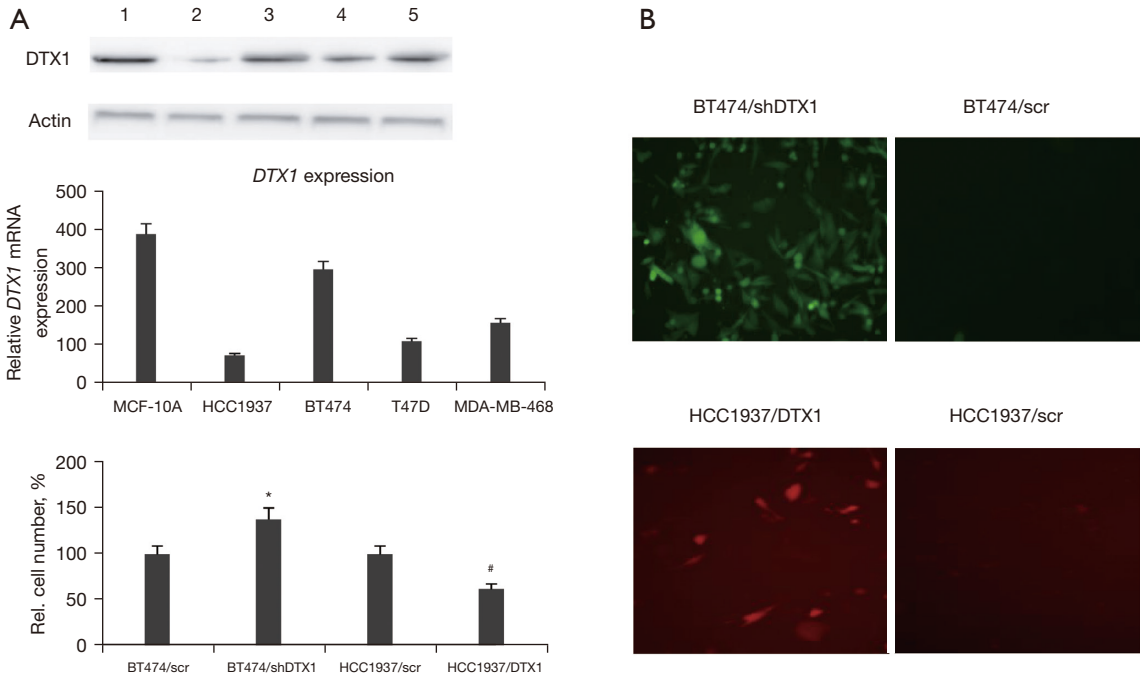

Figure 1 Lower *DTX1* promoted breast cancer cell proliferation. (A) Levels of *DTX1* in different breast cell lines by Western blot and RT-PCR (1, MCF-10A control cells; 2, HCC1937 cells; 3, BT474 cells; 4, T47D cells; 5, MDA-MA-468 cells). The proliferation of BT474/shDTX1cells increased while HCC1937/DTX1 cells decreased compared to control cells by cell proliferation analysis (*, P<0.01; #, P<0.01). (B) HCC1937 and BT474 cell lines for *DTX1* overexpression and knockdown studies (fluorescent color, green: GFP, red: mCherry. amplification 40×). RT-PCR, reverse transcription-polymerase chain reaction.

Figure 4A). The migration of HCC1937 cells with GSI was 48% of HCC1937 cells without GSI (P<0.01, *Figure 4B*).

Patients and clinical characteristics

We enrolled 316 patients with BC, 102 patients with fibroadenoma, and 113 healthy controls in the present study. The mean ages of healthy controls and patients with BC were 50.9±11.5 years (range, 23–65 years) and 50.8± 9.7 years (range, 20–66 years), while the mean age in patients with fibroadenoma was 42.4±14.3 years (range, 21–50 years) due to the younger peak age of this disease. The BC patients were staged according to their tumor-node-metastasis (TNM) classification. There were 88 patients with stage I (27.8%), 144 patients with stage II (45.6%), and 84 patients with stage III (26.6%). All the clinical characteristics are shown in *Table 1*.

DTX1 was expressed in breast tissues

There were no significant differences in serum levels of *DTX1* expression between controls, patients with fibroadenoma, and patients with BC. In breast tissues, levels of *DTX1* in BC were significantly lower compared to fibroadenoma tissues and peri-neoplastic breast tissues by RT-PCR and IHC (P<0.01, *Figure 5*). We also analyzed the levels of *DTX1* in breast tissues between the four subtypes of BC, including Luminal A, Luminal B, HER2 over-expression, and triple negative subtype, among which no significant differences were found (P>0.05).

Associations between DTX1 and clinical characteristics of BC

There were no positive associations between levels of *DTX1* and age, body mass index (BMI), tumor size, status of hormone, and HER2 (P>0.05, as shown in *Table 2*). Lower levels of *DTX1* were associated with advanced tumor grade (grade III, P=0.017), advanced clinical stage (stage III, P=0.031), positive lymph node status (positive, P=0.009), and high Ki-67 index (≥20%, P=0.023). These results indicated that lower *DTX1* was associated with advanced disease condition. In addition, multivariate Cox regression

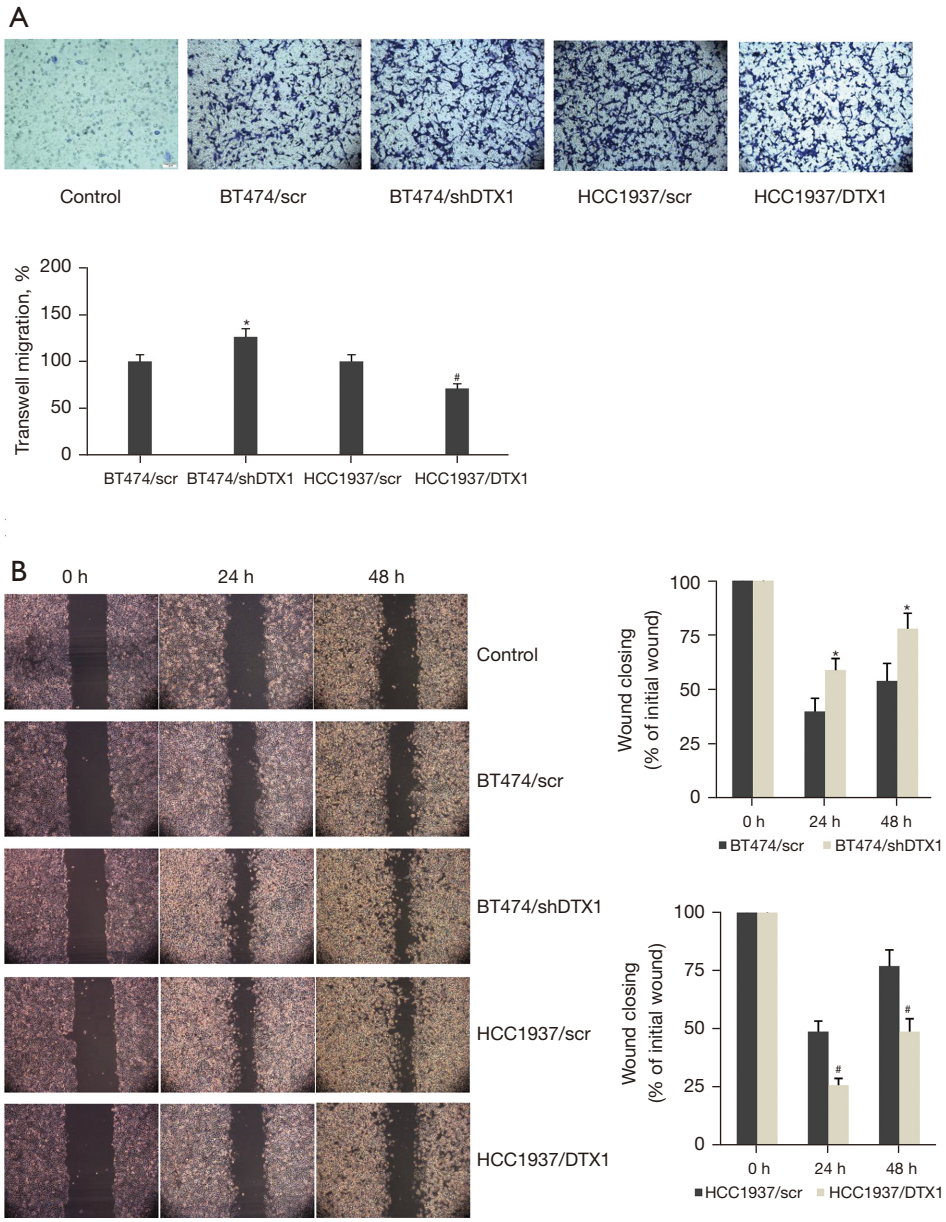

Figure 2 Lower *DTX1* promoted breast cancer cell migration and invasion. (A) Compared with control cells, low expression of *DTX1* significantly promoted the invasive behavior of BT474/shDTX1 cells, while higher *DTX1* expression decreased the invasion behavior of HCC1937/DTX1 cells (crystal violet staining, amplification 10×). *, P<0.01; #, P<0.01. (B) Wound closure massively accelerated in BT474/shDTX1 cells while reduced in HCC1937/DTX1 cells compared to controls by wound healing assay (amplification 10×). *, P<0.01; #, P<0.01.

analysis revealed that *DTX1* expression, LNM, clinical stage, and Ki-67 index were associated with 5-year survival of BC, and each might be prognostic factor for metastasis-free survival (MFS) in BC (P=0.002, P=0.003, P=0.002, P<0.001, *Table 3*).

Discussion

The role of *DTX1* in oncogenesis has not been fully elucidated. It has been reported that *DTX1* is a potential biomarker for GBM diagnosis (8). Mutations of *DTX1* are associated with poor prognosis in patients with small cell

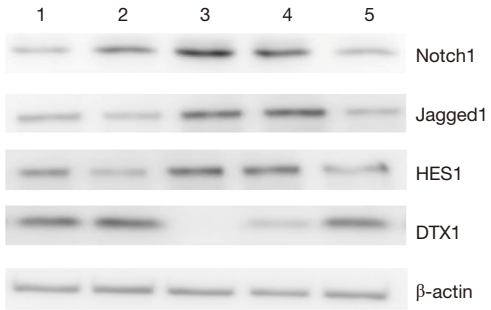

Figure 3 The associations of Notch pathways and DTX1 in breast cancer cells. Expressions of Notch1, Jagged1, and HES1 of the Notch signaling pathway were significantly down-regulated in HCC1937/DTX1 cells, while expressions of these molecules increased in BT474/shDTX1 cells compared to BT474/scr cells (1, MCF-10A control cells; 2, BT474/scr cells; 3, BT474/shDTX1 cells; 4, HCC1937/scr cells; 5, HCC1937/DTX1 cells).

lung cancer (SCLC) (11). In the present study, we explored the associations between *DTX1* and BC, and found lower *DTX1* could promote BC cell proliferation, migration, and invasion.

Expression of the *DTX1* gene has been detected not only in a variety of normal tissues such as lymphoid tissues, heart, liver, and kidney (12), but also in tumors with a centroblast expression profile (13). It has been shown to play different roles in different types of cancers. Overexpression of *DTX1* increases cell migration and invasion in GBM (8), while downregulated *DTX1* might be associated with Notch pathway activation and increased migration potential in head and neck squamous cell carcinoma (14). In our experiment, lower expression of *DTX1* promoted BC cell proliferation, migration, and invasion.

The Notch signaling pathway participates in neoplastic

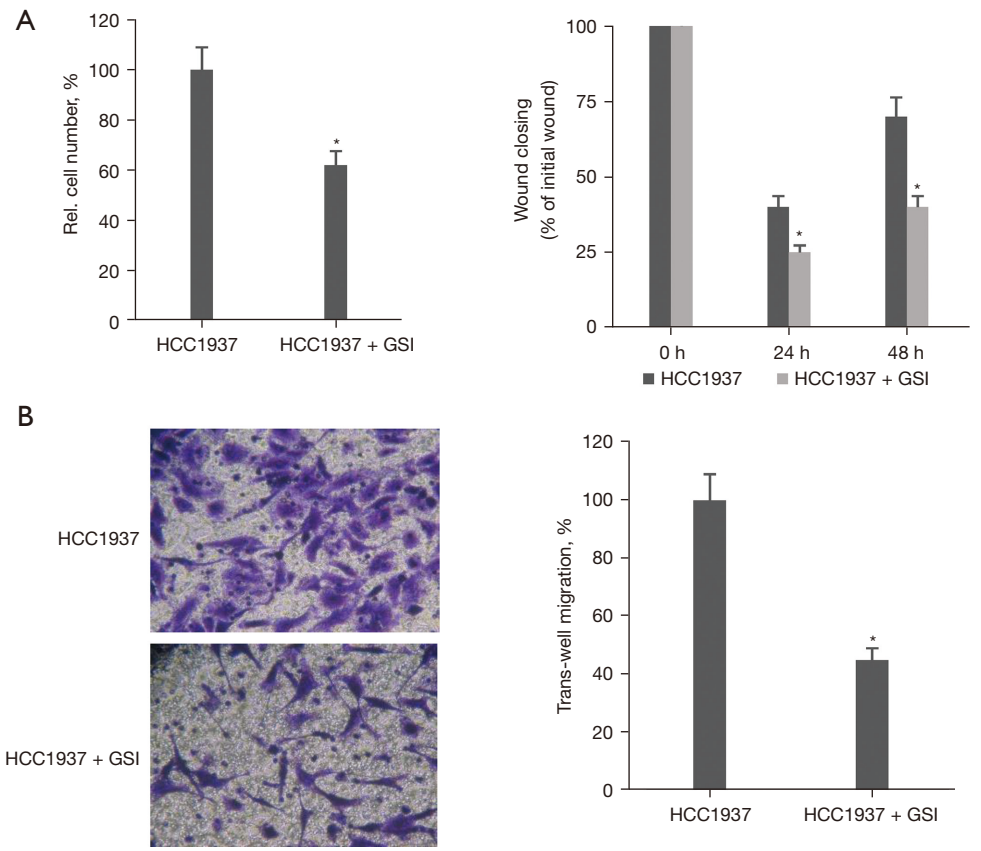

Figure 4 GSI inhibited proliferation and migration of HCC1937 cells. (A) After GSI treatment, the proliferation of HCC1937 cells with GSI were 62% of HCC1937 cells without GSI. The wound closing of HCC1937 cells with GSI reduced 15% and 30% compared to HCC1937 cells without GSI after 24 h and 48 h, respectively (*, P<0.01). (B) The migration of HCC1937 cells with GSI was 48% of HCC1937 cells without GSI (*, P<0.01). Crystal violet staining, amplification 40×. GSI, gamma-secretase inhibitor.

Table 1 Clinical characteristics of controls, patients with fibroadenoma, and patients with breast cancer

Characteristics	Control	Fibroadenoma	Breast cancer (subtypes)			
			Luminal A	Luminal B	HER2$^+$	Basal-like
Number	113	102	108	67	84	57
Age, years (range)	50.9 [23–65]	42.4 [21–50]	50.3 [20–64]	50.7 [21–66]	51.1 [20–65]	50.9 [20–62]
BMI (kg/m^2), mean ± SD	25.7±6.3	24.2±6.9	26.9±7.0	28.2±6.1	27.6±5.9	27.1±6.3
Clinical stage, n (%)						
I			26 (24.1)	20 (29.9)	25 (29.8)	17 (29.8)
II			46 (42.6)	31 (46.3)	40 (47.6)	27 (47.4)
III			36 (33.3)	16 (23.8)	19 (22.6)	13 (22.8)
Tumor size, n (%)						
PT1 >1 mm, <20 mm			31 (28.7)	22 (32.8)	30 (35.7)	21 (36.8)
PT2 ≥20 mm, <50 mm			54 (50.0)	32 (47.8)	45 (53.6)	30 (52.7)
PT3 ≥50 mm			23 (21.3)	13 (19.4)	9 (10.7)	6 (10.5)
Lymph node involved, n (%)						
0			24 (22.2)	15 (22.4)	20 (23.8)	12 (21.1)
1–3			31 (28.7)	21 (31.3)	29 (34.5)	15 (26.3)
4–9			31 (28.7)	19 (28.4)	25 (29.8)	17 (29.8)
≥10			22 (20.4)	12 (17.9)	10 (11.9)	13 (22.8)
Distant metastasis, n (%)						
M0			108 (100.0)	67 (100.0)	84 (100.0)	57 (100.0)
M1			0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

BMI, body mass index.

transformation in various cell types, such as cervical cancer, glioma, osteosarcoma, and BC (15-18). High expressions of Notch-1 and its ligand Jagged-1 are associated with poor prognosis in BC (19). Levels of Notch-1, DLL1, and Jagged-1 were upregulated in renal cell carcinoma and lung adeno carcinoma *in vitro* and *in vivo* models (20-22). Higher Notch 4 in triple-negative BC (TNBC) cells caused increased proliferation and invasiveness. It was reported that Notch 4 was a novel ubiquitin substrate of DTX3, DTX3 promoted the ubiquitination and degradation of Notch 4, thus decreasing TNBC cells proliferation and invasion (23). Also, Notch inhibition results in decreased proliferation and self-renewal of GBM cells (24). However, the mechanisms of Notch signaling in the promotion of cancer cell invasion and metastasis are not fully understood.

The *DTX1* gene is a downstream molecule and critical regulator of the Notch signaling pathway (25). Gene expression analysis of Notch pathway molecules suggested that *DTX1* downregulation is correlated with downregulation of some Notch pathway genes including *DTX3*, *DLL3*, etc. Furthermore, *HES1* can inversely regulate *DTX1* expression through binding to transcription promoter region of *DTX1*. *DTX1* carries a putative SH3-binding domain, it binds to the intracellular domain of Notch (ICN) and negatively regulates Notch through ubiquitination (14). In the present experiment, Notch1, Jagged1, and HES1 in the Notch pathway were significantly upregulated in cells with lower *DTX1* expression compared to those with *DTX1* high expression cells. It is possible that GSI (an inhibitor of Notch signaling pathway) could inhibit the proliferation and migration of HCC1937 cells. These findings inferred that downregulation of D*TX1* resulted in a strong difference in activation of the Notch signaling pathway in BC samples. Potentially, *DTX1* promotes BC

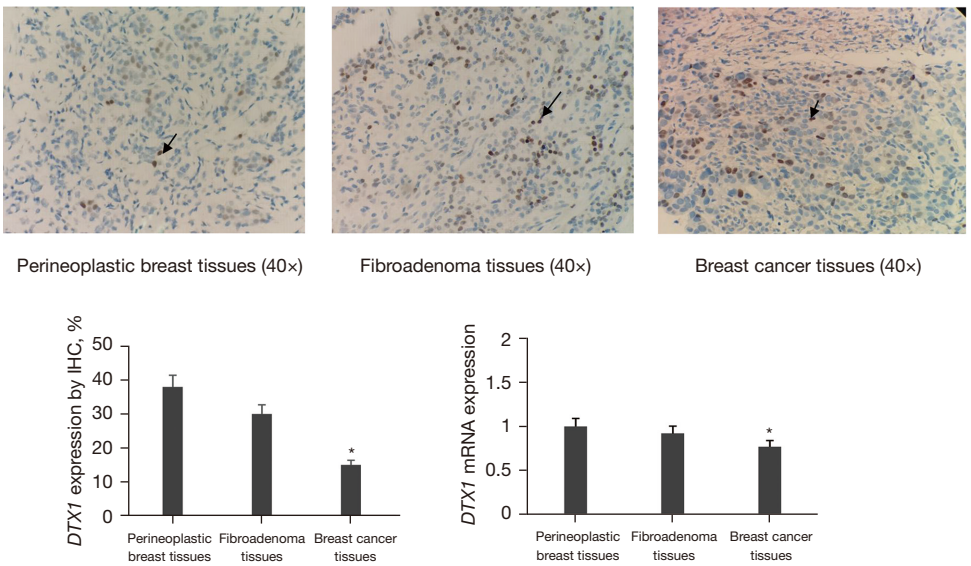

Figure 5 *DTX1* expression in breast tissues. Levels of *DTX1* in breast cancer tissue, fibroadenoma tissues, and perineoplastic breast tissues were determined by IHC (arrows showed DTX1 positive cells, 40×). Level of *DTX1* in breast cancer tissues was lower compared to fibroadenoma tissues, and perineoplastic breast tissues by IHC and RT-PCR (*, P<0.01). IHC, immunohistochemistry; RT-PCR, reverse transcription-polymerase chain reaction.

Table 2 Association of *DTX1* expression with clinical characteristics of breast cancer

Clinical	n	*DTX1*	P value
Age, years			
<50	131	1.89 (0.33–4.09)	0.183
≥50	185	1.61 (0.21–3.93)	
BMI			
<28	213	1.83 (0.21–4.95)	0.099
≥28	103	1.59 (0.16–4.62)	
Tumor size (mm)			
≤20	110	1.69 (0.22–4.97)	0.078
>20	206	1.53 (0.16–4.32)	
Tumor grade			
I–II	249	1.64 (1.04–3.87)	0.017
III	67	0.92 (0.22–3.84)	
Clinical stage			
I–II	232	1.78 (0.25–5.39)	0.031
III	84	0.85 (0.12–4.12)	
Lymph node status			
Negative	71	1.61 (0.27–4.84)	0.009
Positive	245	0.93 (0.11–4.09)	
HR (ER or PR)			
Negative	137	1.82 (0.35–4.99)	0.412
Positive	179	1.69 (0.23–4.19)	
Her-2			
Negative	187	1.53 (0.16–4.97)	0.127
Positive	129	1.69 (0.38–5.19)	
Ki-67			
<20%	173	2.01 (0.37–5.72)	0.023
≥20%	143	0.95 (0.22–4.51)	

Data were presented by median (range). Wilcoxon test or Kruskal-Wallis test (nonparametric tests) was used. BMI, body mass index; HR, hormone receptor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.

Table 3 Multivariate analysis between *DTX1* and MFS in breast cancer

Characteristic	5-year survival		MFS	
	P value	HR (95% CI)	P value	HR (95% CI)
Tumor grade	0.923	1.003 (0.489–1.928)	0.893	1.082 (0.613–2.079)
Clinical stage	0.002	1.928 (1.173–3.026)	0.001	1.794 (1.292–3.189)
LNM	0.003	1.827 (1.214–2.981)	<0.001	2.213 (1.545–3.582)
Ki-67	<0.001	1.935 (1.413–3.127)	0.001	2.015 (1.351–3.493)
DTX1	0.002	2.162 (1.382–3.693)	<0.001	1.973 (1.376–3.125)

BMI, body mass index; MFS, metastasis-free survival; CI, confidence interval; HR, hazard ratio; HR, hormone receptor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.

cell proliferation and migration via the Notch signaling pathway.

The Notch pathway is usually influenced by other signaling pathways which it cross-talks with, including NF-κB, PI3K, AKT, and Wnt (26,27). The PI3K/AKT pathway has been reported to be involved with NF-κB activation in a wide variety of tumors (28-30). Activated Notch-1 might induce activation of the PI3K/AKT pathway, then upregulate NF-κB activity to facilitate cell invasion (31). Endocrine therapy is the most efficacious drug for the treatment of ER-positive breast cancer patients, but there are still some patients with endocrine resistance. Notch1/4 play an essential role in endocrine resistance, E2 can inhibit Notch activation and SERMs reactivates Notch in breast cancer cells. Meanwhile Notch activates ERα-dependent transcription, demonstrating that Notch may promote endocrine resistance by affecting ERα activity (32). More research is needed to investigate further mechanisms involved.

In the present study, levels of *DTX1* in BC tissues were lower compared to fibroadenoma tissues and perineoplastic breast tissues. As it was well known, the progression and prognosis of BC is different for different subtypes, so we also explored the associations between *DTX1* and four BC subtypes, but no significant differences were found. This might indicate that lower levels of *DTX1* promoted BC proliferation and invasion regardless of subtypes.

We also analyzed the associations between *DTX1* and clinical characteristics of BC. Lower level of *DTX1* was associated with advanced tumor grade, advanced clinical stage, positive lymph node status, and high Ki-67 index. These findings indicated that lower *DTX1* was associated with advanced disease condition. In addition, multivariate Cox regression analysis revealed that *DTX1* expression was associated with 5-year survival of BC, and might be considered as a prognostic factor for MFS in BC.

The mechanisms of *DTX1* to promote breast cancer cell proliferation and migration are still unclear. *DTX1* reduces GBM tumor growth through ubiquitination of Notch1 receptors (8). *DTX1* promotes degradation of c-FLIP through the endosome-lysosomal pathway in gastric cancer. Reintroduction of *DTX1* into gastric cancer cells reduced c-FLIP and increased TRAIL-induced apoptosis (3). Induction of *DTX1* could be a new approach to enhancing the benefits of TRAIL-mediated cancer therapy, it might also play a role in breast cancer.

There were some limitations in the present research. First, the specific mechanisms by which *DTX1* promotes BC cell proliferation and invasion were not fully elucidated. Second, the associations between *DTX1* and prognosis of BC still need further investigation. There is a long way to go to clarify the role and mechanisms of *DTX1* in BC and further to find new target drug to evaluate in clinical trials.

In conclusion, lower level of *DTX1* might influence the proliferation, migration, and invasion of BC cells through the Notch signaling pathway. Lower level of *DTX1* was negatively correlated with tumor grade, clinical stage, LNM status, and high Ki-67 index, and might be an impact factor of MFS in BC. There is potential for *DTX1* to be used as a marker for the development of new BC therapy.

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Footnote

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