The associations between Deltex1 and clinical characteristics of breast cancer

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Contributions: (I) Conception and design: X Liu, H Wang; (II) Administrative support: X Liu; (III) Provision of study materials or patients: X Liu, Y Xian, H Xu; (IV) Collection and assembly of data: Y Xian, M Xiang, K Che; (V) Data analysis and interpretation: X Liu, Y Xian, H Xu, M Hu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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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

Submitted Oct 26, 2021. Accepted for publication Nov 18, 2021.
doi: 10.21037/gs-21-739

View this article at: https://dx.doi.org/10.21037/gs-21-739

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 °C in 5% CO₂.

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 RNaseasy 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⁻ΔCt, where ΔCt = Ct_Target − Ct_18s. The primer sequences were as follows: DTX1, forward primer: 5′-GGGCTGATGCCT GTGAATG-3′, reverse primer: 5′-CCTGGCGAAACTGGTG-3′. β-Actin, forward primer: 5′-CGTGACATTAAGGAGAAGCTG-3′, reverse primer: 5′-CTAGAAGCATTTGCGGTGGAC-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.

Migration and wound healing assay
Migration assay was performed using modified Boyden chamber units with polycarbonate filters of 8 mm porosity (Costar, Vitasir, 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 °C. 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), 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 °C 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/DTX1 cells 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/shDTX1 cells 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.

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 overexpression, 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 4A). The migration of HCC1937 cells with GSI was 48% of HCC1937 cells without GSI (P<0.01, Figure 4B).
analysis revealed that \textit{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, \textit{Table 3}).

\textbf{Discussion}

The role of \textit{DTX1} in oncogenesis has not been fully elucidated. It has been reported that \textit{DTX1} is a potential biomarker for GBM diagnosis (8). Mutations of \textit{DTX1} are associated with poor prognosis in patients with small cell
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. Over-expression 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...
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 adenocarcinoma 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 promotor 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 DTX1 resulted in a strong difference in activation of the Notch signaling pathway in BC samples. Potentially, DTX1 promotes BC

| Characteristics                      | Control | Fibroadenoma | 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/m²), 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 (22.6) |
| 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.
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   |

Table 2 (continued)

| Clinical                          | n  | DTX1       | P value |
|-----------------------------------|----|------------|---------|
| 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.
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-kB, PI3K, AKT, and Wnt (26,27). The PI3K/AKT pathway has been reported to be involved with NF-kB activation in a wide variety of tumors (28-30). Activated Notch-1 might induce activation of the PI3K/AKT pathway, then upregulate NF-kB 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 ERa-dependent transcription, demonstrating that Notch may promote endocrine resistance by affecting ERa 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.

Acknowledgments

We appreciated all pathologists in this study. Funding: This research was supported by project of Youth Research Fund in the Affiliated Hospital of Qingdao University in 2018 (grant number: QDFY20180021) and Clinical + X scientific research project in the Affiliated Hospital of Qingdao University.  

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.
Hospital of Qingdao University in 2019 (grant number: QDFY+X20190059).

**Footnote**

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at [https://dx.doi.org/10.21037/gs-21-739](https://dx.doi.org/10.21037/gs-21-739)

*Data Sharing Statement:* Available at [https://dx.doi.org/10.21037/gs-21-739](https://dx.doi.org/10.21037/gs-21-739)

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at [https://dx.doi.org/10.21037/gs-21-739](https://dx.doi.org/10.21037/gs-21-739)). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. 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.

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(English Language Editor: J. Jones)

Cite this article as: Liu X, Xian Y, Xu H, Hu M, Che K, Liu X, Wang H. The associations between Deltex1 and clinical characteristics of breast cancer. Gland Surg 2021;10(11):3116-3127. doi: 10.21037/gs-21-739