

Role of ubiquitin-conjugating enzyme E2T in the carcinogenesis and progression of pancreatic cancer

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Abstract. Ubiquitin-conjugating enzyme E2T (UBE2T) is a recently discovered oncogenic protein. Numerous studies reported that UBE2T is highly expressed in various types of human cancer; however, its role in the carcinogenesis and progression of pancreatic cancer remains unknown. The aim of the present study was to investigate the role of UBE2T in pancreatic cancer progression through *in vitro* experiments in pancreatic cancer tissues and cell lines. The results obtained in the present study demonstrated that UBE2T served an important role in the initiation and progression of pancreatic cancer. Furthermore, increased expression of UBE2T in human pancreatic cancer tissues and pancreatic cancer cells was observed compared with normal tissues and cells. The effect of upregulating and downregulating UBE2T in pancreatic cancer cell lines was investigated using the MTT, wound-healing and migration and invasion assays. The results demonstrated that overexpression of UBE2T significantly promoted pancreatic cancer cell proliferation, migration and invasion compared with controls. However, UBE2T down-regulation resulted in the inhibition of pancreatic cancer cell proliferation, migration and invasion. In addition, the results demonstrated that UBE2T may promote the epithelial mesenchymal transition in pancreatic cancer cells.

Introduction

Pancreatic cancer is the fifth common malignant tumor in the gastrointestinal tract, with the incidence rate rising four times in the last twenty years in China (1).

Furthermore, most patients with pancreatic cancer are diagnosed with ductal adenocarcinoma, which account for 90% of pancreatic cancer (1). Due to its high degree of malignancy, the prognosis of patients with pancreatic cancer is very poor. The global average annual incidence is 230,000, with an overall 5-year survival rate of <5% (1,2). At present, radical resection remains the best therapeutic option for patients with early stage pancreatic cancer; however, due to the special anatomy of the pancreas, early symptoms of pancreatic cancer remain discrete in most patients and the early diagnosis is therefore difficult (2). The majority of patients are diagnosed at an advanced stage and cannot receive the optimal treatment strategy. The early diagnosis of pancreatic cancer is therefore essential (2-4).

UBE2T has been recently discovered as an oncogenic protein that belongs to the family of ubiquitin-conjugating enzymes (E2) (5). Ubiquitin can be transferred to E2 enzymes via the ubiquitin-activating enzyme (E1), and transferred to substrate proteins by ubiquitin ligase (E3) (5). The protein encoded by the UBE2T gene consists of 197 amino acids and catalyzes the covalent attachment of ubiquitin to protein substrates (6). Previous studies reported that detecting UBE2T expression could directly detect Fanconi anemia (FA) and interfere with the DNA damage-repair response (5,6). Two transcript variants encoding different isoforms have been identified for this gene (6). There are two isoforms including NP-054895.1 and NP-001297255.1, which consist of 197 amino acids and 167 amino acids, respectively. Some of the UBE2T gene-associated pathways are the FA and the ubiquitin proteasome pathway (6). Data from Gene Ontology reported that UBE2T gene is associated with 'chromatin binding' and 'ubiquitin-protein transferase activity' (6) biological processes. Furthermore, the UBE2T gene participates in the protein monoubiquitination catalysis and is involved in the mitomycin-C induced DNA repair (5). In addition, UBE2T protein acts as a specific E2 enzyme for the FA core complex by binding to the E3 ligase named FA Complementation Group L (FANCL) and catalyzing the monoubiquitination of FA Complementation Group D2, which are crucial steps in DNA damage repair (6). The UBE2T gene is also involved in FANCL and FA Complementation Group I monoubiquitination (6). It has been reported that UBE2T might contribute

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to the ubiquitination and degradation of breast cancer type 1 susceptibility protein homolog, which serves an important role in regulating breast cancer cell proliferation and metastasis (7). Furthermore, the reactome pathways in which the UBE2T gene is involved include 'DNA repair', 'Fanconi anemia pathway', 'Metabolism of proteins', 'Post-translational protein modification', 'Protein ubiquitination' and 'synthesis of active ubiquitin-roles of E1 and E2 enzymes' (8). Whether UBE2T serves a role in pancreatic cancer remains unclear.

The present study investigated the role of UBE2T in pancreatic cancer initiation and progression. The results demonstrated that UBE2T is significantly upregulated in human pancreatic cancer tissues compared with normal tissues. Furthermore, the results demonstrated that high expression of UBE2T may promote proliferation, invasion and metastasis of pancreatic cancer cells. In addition, UBE2T may promote epithelial-mesenchymal transition (EMT). These data suggested that UBE2T may be considered as an oncogenic protein and a potential therapeutic target in pancreatic cancer.

Materials and methods

Tissues collection. A total of 121 patients, 76 males and 45 females, with pancreatic cancer were included in this study, aging from 36-78 years old (average age, 44.7 years). Pancreatic cancer resection tissues and normal adjacent tissues were collected from the same patients who underwent surgery at the Department of Organ Transplantation and Second Hepatobiliary Surgery of Shandong Provincial Hospital Affiliated to Shandong University between July 2009 and July 2017. The fresh tissues were stored at liquid nitrogen immediately. The present study was approved by the Ethics Committee of Shandong Provincial Hospital and all patients provided signed informed consent.

Immunohistochemistry (IHC). IHC staining of UBE2T was performed on pancreatic cancer and normal tissues. The tissue sections were fixed using 10% formalin and dehydrated with ethanol of gradient concentration (75, 85 and 95% ethanol for 90 min each at room temperature; waterfree alcohol I and waterfree alcohol II for 30 min each; immersion wax temperature 58-65°C). Then the tissues sections were embedded with paraffin at 60°C. The tissues sections were then cut into 5 µm thickness slides and rehydrated using xylene and ethanol of gradient concentration. Besides, antigen retrieval was performed using citrate buffer for 20 min at 90°C. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide for 30 min at room temperature. Tissues were incubated with a primary antibody against UBE2T (1:200 dilution; cat. no. ab179802; Abcam) for 15 h at 4°C. Tissue slides were then incubated with secondary antibody (goat anti-rabbit IgG H&L horseradish peroxidase-conjugated preadsorbed; 1:500; cat. no. ab7090; Abcam) at room temperature for 1 h. Signal was visualized using 3'-diaminobenzidine that reacts with HRP-labeled secondary antibody. The chromogenic region was observed and images were captured using a fluorescence microscope (Leica Instrument Co., Ltd.; magnification, x200).

Cell lines and cell culture. The human pancreatic cancer cell lines MIApaca-2 and Bxpc-3 were purchased from The Cell

Bank of Type Culture Collection of the Chinese Academy of Sciences. The cell line Panc-1 was donated by Joe Xinzhou, Shanghai. All cells were cultured with RPMI-1640 medium (MIApaca-2 and Bxpc-3 cells) or Dulbecco's Modified Eagle Medium (DMEM) (All purchased from Hyclone; GE Healthcare Life Sciences) for panc-1 cells, supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1x penicillin (100 IU/ml)/streptomycin (100 µg/ml) and placed at 37°C in a humidified incubator containing 5% CO₂.

Cells transfection. All the cells (5x10⁴/ml) were cultured in 6-cm culture dishes without penicillin/streptomycin. Plasmid (2 µg; purchased from GeneCopoeia, Inc.) transfection was performed by the lentivirus transfection system, using REV, GAG and VSV package vectors and Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, once cell density reached ~80% for ~48 h. Cells were divided into four groups, according to the plasmid transfected: Psuper, psuper-UBE2TD, HA-psuper and HA-UBE2T-psuper. HA tag is a tag protein that can be fused to target gene and detected using HA tags. HA-UBE2T is the recombinant plasmid, which contains the HA tag and UBE2T ORF clone, in order to better observe the experimental results. The transfection efficiency is presented in Fig. S1. The following experiments were performed 48 h after transfection.

Cells were also transfected with shRNAs, the sequences were as follows: shUBE2T-A, 5'-GATCCCCAGACCAAA TGGATGACCTGTTCAAGAGACAGGTCATCCATTTGG TCTTTTTTGGAAA-3'; shUBE2T-B, 5'-GATCCCCTG CCAGACAGTGGACAGAGTTCAAGAGACTCTGTCCAC TGTCTGGCATTTTTGGAAA-3' (GENEWIZ, Inc.). The following experiments were performed 48 h after transfection.

Western blotting. Once cell density reached ~80%, cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitor cocktail on ice. Cell lysates were centrifuged at 12,000 x g for 30 min at 4°C following shaking and sonication every 1 min for three times on ice. Protein concentration detection was carried out using the BCA assay (Beijing Solarbio Science & Technology Co., Ltd.) Supernatant was collected and mixed with 5X loading buffer (contain SDS and Bromphenol blue) and diluted to a final concentration of 1X sample buffer (from 5X buffer dilution). The samples were boiled at 95°C for ~5 min, in order to denature the lysate protein. Proteins (40 µg) were separated by 10% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes. Membranes were blocked in 5% bovine serum albumin (BSA; 5 g/l, purchased from Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, and incubated with primary antibody diluted in 5% BSA and against β-actin (1:1,000; cat. no. ab69512; Abcam), UBE2T (1:1,000; cat. no. ab179802; Abcam), vimentin (1:1,000; cat. no. 39325; Cell Signaling Technology, Inc.), E-cadherin (1:1,000; cat. no. 14472; Cell Signaling Technology, Inc.), N-cadherin (1:1,000; cat. no. 13116; Cell Signaling Technology, Inc.) and α-catenin (1:50,000; cat. no. ab64479; Abcam) overnight at 4°C. Membranes were washed three times with TBST (0.2% Tween) and incubated with secondary antibodies: (goat anti-rabbit IgG H&L DyLight488 (cat. no. ab96899; Abcam) and goat

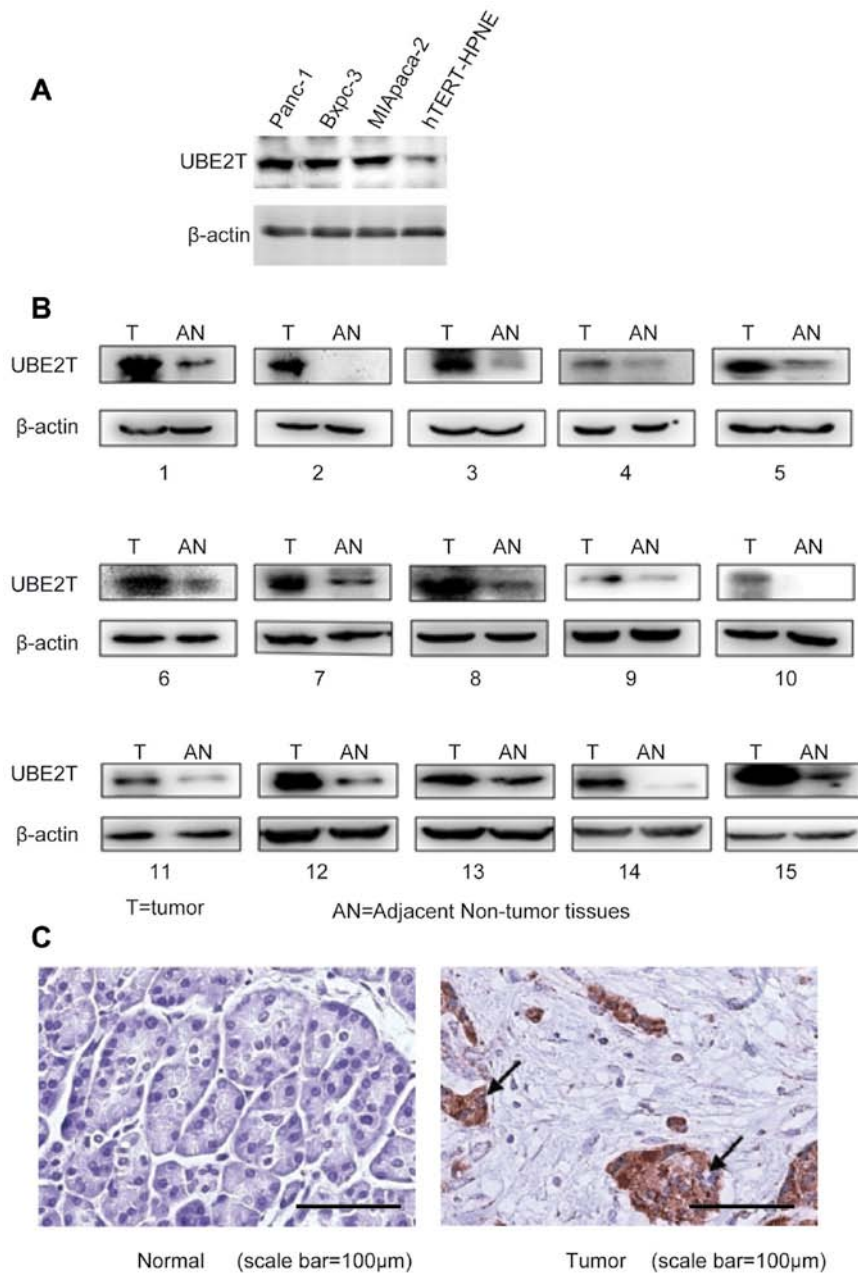


Figure 1. UBE2T was upregulated in pancreatic cancer cells and tissues. (A) Western blotting demonstrated that UBE2T was upregulated in three pancreatic cancer cell lines. (B) UBE2T protein expression in pancreatic cancer tissues (15 cases) was increased compared with adjacent normal tissues. (C) Immunohistochemistry demonstrated that UBE2T expression was higher in pancreatic cancer tissues than in adjacent normal tissues. Arrows point to tumor cells. Magnification, x200. AN, adjacent normal tissue; UBE2T, ubiquitin-conjugating enzyme E2T; T, tumor tissue.

anti-mouse IgG H&L DyLight594 (cat. no. ab96881; Abcam) (all 1:10,000) for 1 h at room temperature. Membranes were washed three times with TBST and bands were detected using Odyssey Imaging system (supplied by LI-COR Biosciences).

MTT assay. In order to detect the effects of UBE2T on cell proliferation, transfected pancreatic cancer cells were seeded into 96-well plate at a density of 3,000 cells per well and placed at 37°C in a humidified incubator containing 5% CO₂ for 48 h. Six biological replicates were analyzed per group and a blank well was also set up with only medium. Cells were cultured for five days until a confluence of 80-90% was reached. Cells were incubated with MTT reagent (10 μl/well) and placed for 4 h at 37°C in the dark. Supernatant was removed and purple

formazan was dissolved with 150 μl DMSO. The plate was oscillated for 10 min and the optical density was measured at 570 nm with a microplate reader.

Wound-healing assay. All transfected pancreatic cancer cell lines were cultured in 6-cm culture dishes at a density of 5x10⁴/ml. Cells were scratched using a 200-μl tip, floating cells were washed with Hank's Balanced Salt Solution (Thermo Fisher Scientific, Inc.) and new medium (DMEM contain 10% bovine serum) was added. Cells were incubated for 48 h at 37°C. The wound width was observed and recorded every 24 h under a microscope (magnification, x100). The results were observed using the Zeiss inverted fluorescence microscope (Zeiss AG).

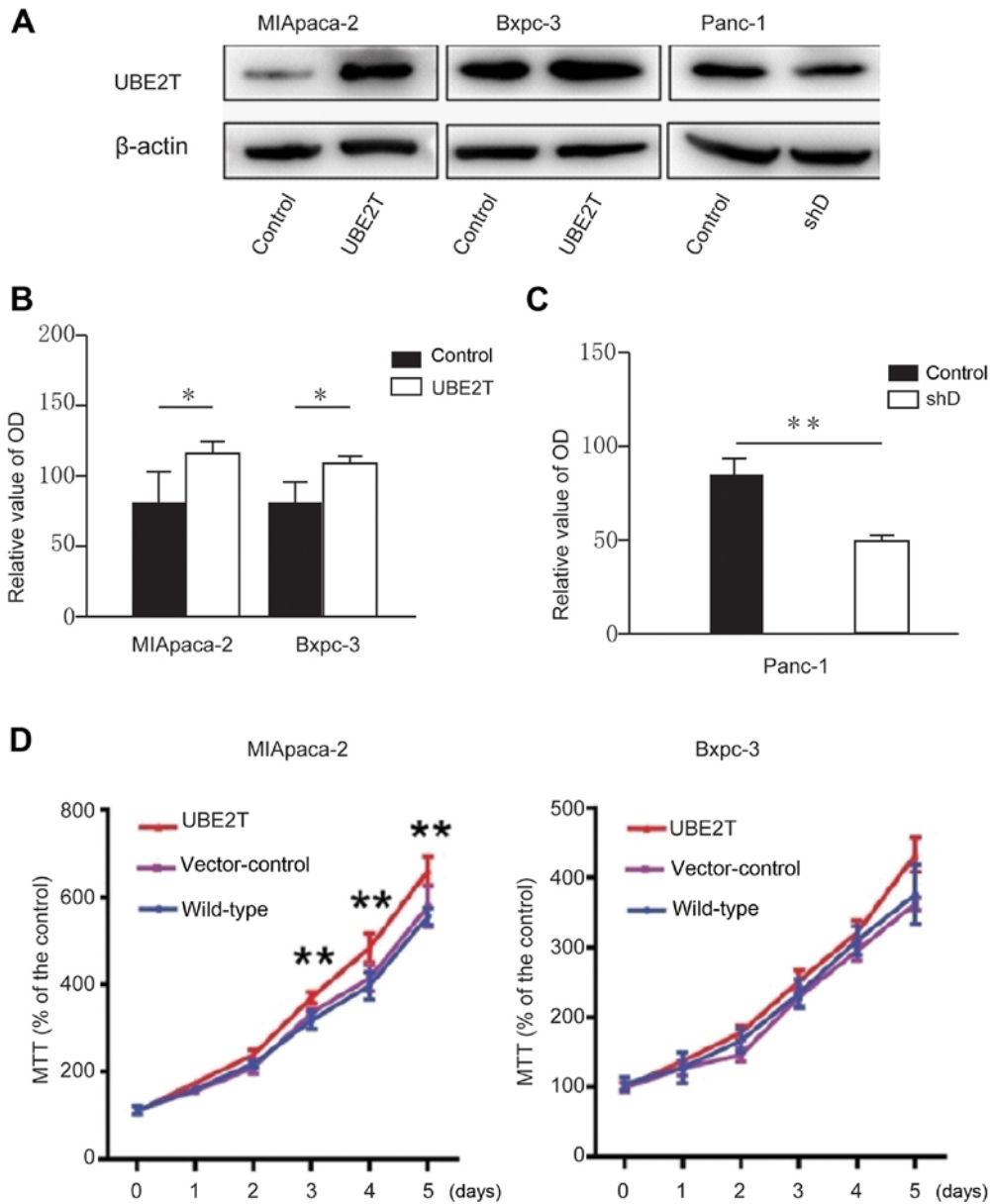


Figure 2. UBE2T promoted pancreatic cancer cell proliferation. (A) Western blotting demonstrated that UBE2T protein expression was higher in cells lines overexpressing UBE2T compared with the control group. (B) The MTT assay demonstrated that the proliferation of MIApaca-2 and Bxpc-3 cells overexpressing UBE2T was higher compared with the control cells. * $P < 0.05$. (C) Panc-1 cell proliferation was decreased following transfection with shRNA compared with the control cells. ** $P < 0.01$. (D) The MTT assay demonstrated that MIApaca-2 and Bxpc-3 cell proliferation was higher following UBE2T overexpression compared with control cells for up to five days. shD, pSuper-shUBE2T.D (shRNA silence UBE2T pSuper); OD, optical density; UBE2T, ubiquitin-conjugating enzyme E2T.

Cell invasion and migration assay. Transwell (pore size, 8 μ m) chambers (BD Biosciences) were coated with Matrigel and incubated for 30 min at 37°C and were inserted into a 24-well plate. Transfected pancreatic cancer cells were cultured with RPMI1640 medium or DMEM supplemented with 1% FBS. Cell suspension (~ 8×10^4 cells/200 μ l) was added in the upper chamber and 500 μ l corresponding medium supplemented with 10% FBS was added into the bottom chamber. Cells were cultured in the incubator for 48 h at 37°C. The cells on the upper side of the membrane were removed and the cells that invaded through the membrane were fixed with methanol for 30 min at room temperature and stained with 1% crystal violet for 30 min at room temperature. Five random fields from each membrane were counted under a Zeiss inverted fluorescence microscope at x200 magnification.

The protocol for migration assay was identical to the invasion assay; however, Transwell chambers were not coated with Matrigel.

Statistical analysis. All data are expressed as the means \pm standard deviation. Differences between the two groups were analyzed using a Student t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

UBE2T is upregulated in pancreatic cancer cells and tissues. The expression of UBE2T was detected in the three pancreatic cancer cell lines (MIApaca-2, Bxpc-3 and Panc-1) and the normal pancreatic cell line (hTERT-HPNE cells). The

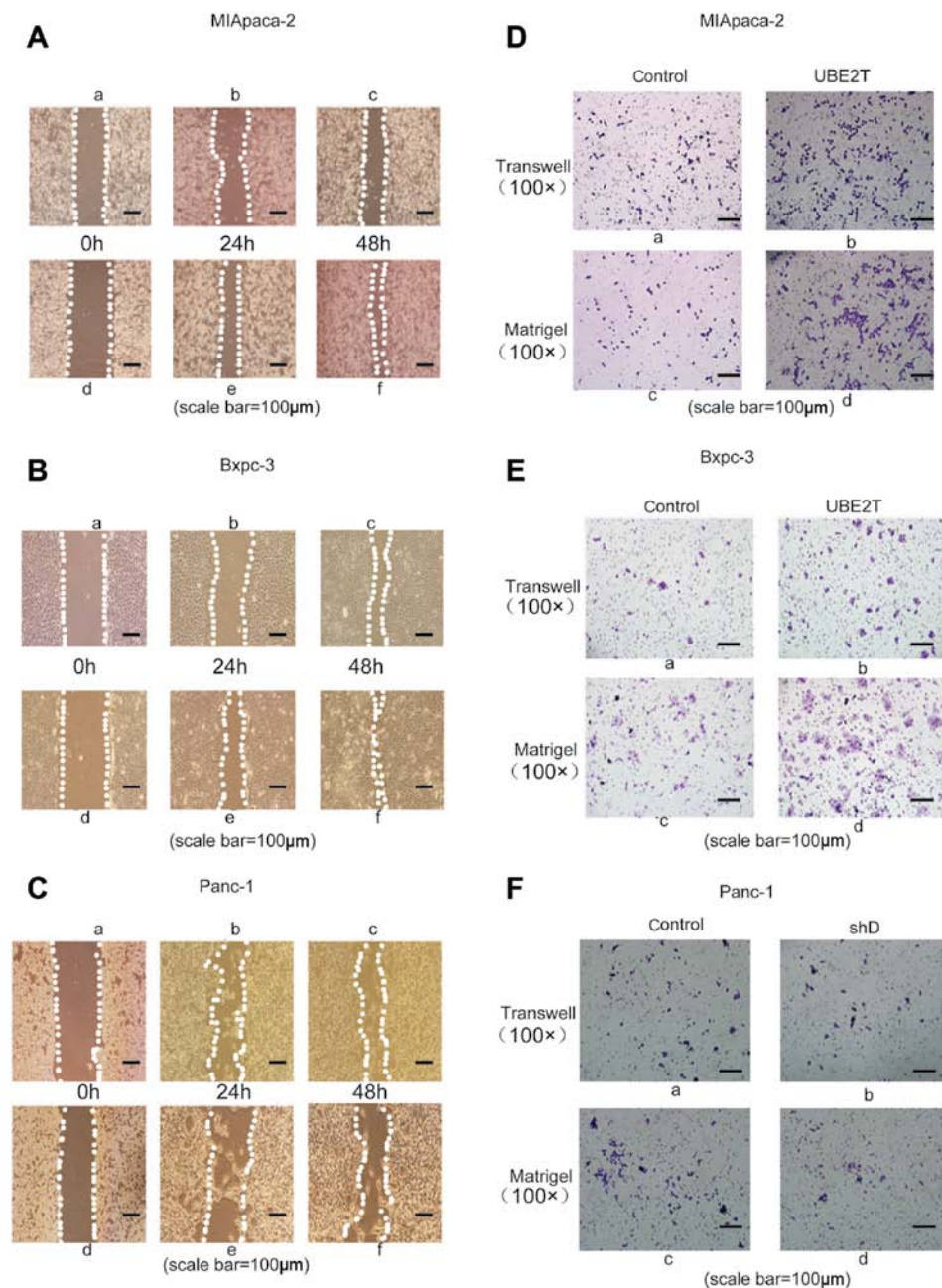


Figure 3. UBE2T promoted pancreatic cancer cell invasion and migration. (A) UBE2T overexpression improved the migratory capability of MIApaca2 cells compared with control cells. (B) UBE2T overexpression improved the migratory capability of Bxpc-3 cells compared with control cells. (C) The migratory capability of Panc1 transfected with shRNA-UBE2T was inhibited compared with control group. (D) UBE2T overexpression promoted the migration (a and b) and invasion (c and d) of MIApaca2 cells. (E) UBE2T overexpression promoted the migration (a and b) and invasion (c and d) of Bxpc-3 cells. (F) Panc1 cells transfected with shRNA-UBE2T exhibited decreased migratory (a and b) and invasive (c and d) abilities compared with the control group. UBE2T, ubiquitin-conjugating enzyme E2T.

results from western blotting demonstrated that UBE2T was upregulated in the three pancreatic cancer cell lines compared with the normal pancreatic cell line (Fig. 1A). Furthermore, the expression of UBE2T was higher in 15 pancreatic cancer tissues compared with the adjacent normal tissues (Fig. 1B). In addition, results from IHC revealed that UBE2T expression was increased in pancreatic cancer tissues compared with adjacent normal tissues (Fig. 1C).

UBE2T promotes pancreatic cancer cell proliferation. To further explore the role of UBE2T in pancreatic cancer cells,

cells were transfected with psuper, psuper-UBE2T, HA and HA-UBE2T. The results from western blotting demonstrated that UBE2T was overexpressed in pancreatic cell lines compared with control group (Fig. 2A).

Pancreatic cancer cell lines Panc-1, MIApaca-2 and Bxpc-3 were cultured in a 6-cm dish with penicillin-free medium, until the confluence was ~80%. The target plasmid and opti-MEM medium, as well as Lipofectamine™ 2000 and opti-MEM medium, was added in a ratio of 1:36.5 respectively into two tubes and mixed at room temperature for 5 min. Subsequently, the two tubes were mixed and placed on the

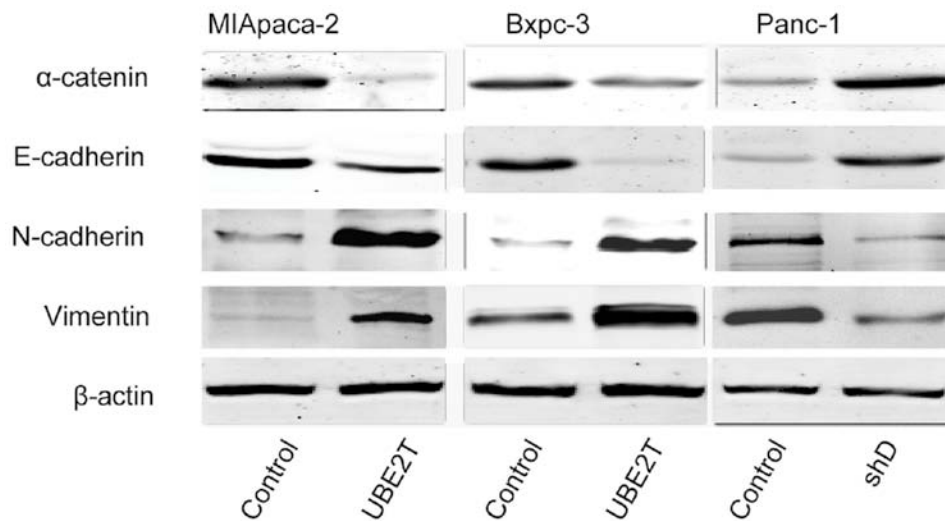


Figure 4. UBE2T overexpression promoted the epithelial-mesenchymal transition in pancreatic cancer cells. Results demonstrated that the protein expression of the epithelial markers E-cadherin and α -catenin was decreased, and that the protein expression of the mesenchymal markers vimentin and N-cadherin was increased in cells overexpressing UBE2T. In Panc1 transfected with shRNA-UBE2T, the protein expression of vimentin and N-cadherin was decreased and the protein expression of E-cadherin and α -catenin was increased. UBE2T, ubiquitin-conjugating enzyme E2T; shD, pSuper-shUBE2T.D (shRNA silence UBE2T pSuper).

shelf for 25 min. The mixture in the tube was added to the petri dish, which was replaced with serum-containing medium after 6 h. A 10-ml disposable syringe was used to aspirate the old culture medium, which was filtered with a disposable filter, and dispensed into a 15-ml disposable centrifuge tube.

The MTT assay was then used to detect the effect of UBE2T on pancreatic cancer cell proliferation. The results demonstrated that the proliferation of MIApaca-2 and Bxpc-3 cell lines overexpressing UBE2T was increased compared with normal cells (Fig. 2B and D). Conversely, the proliferation of Panc-1 cells transfected with shRNA-UBE2T was decreased compared with control cells.

UBE2T promotes pancreatic cancer cell invasion and migration. The results demonstrated that UBE2T overexpression could promote pancreatic cell proliferation. Subsequently, wound healing and Transwell assays were performed to measure the effect of UBE2T on pancreatic cancer cell invasion and migration.

The results from the wound healing assay demonstrated that UBE2T overexpression improved the migratory ability of MIApaca2 (Fig. 3A-d-f) and Bxpc3 (Fig. 3 B-d-f) cells compared with corresponding control cells (non-transfected cells) (Fig. 3A-a-c and B-a-c). However, the migratory ability of Panc-1 cells transfected with shRNA-UBE2T (Fig. 3C-d-f) was inhibited compared with control group (Fig. 3Ca-c). In order to further confirm these results, Transwell assays were performed. The results demonstrated that UBE2T overexpression (Fig. 3D-d; Fig. 3E-d) promoted pancreatic cancer cell invasion compared with control groups (Fig. 3D-c; Fig. 3E-c). The results demonstrated that UBE2T overexpression (Fig. 3D-b; Fig. 3E-b) promoted pancreatic cancer cell migration compared with control groups (Fig. 3D-a; Fig. 3E-a). However, the invasive ability of Panc-1 transfected with shRNA-UBE2T (Fig. 3F-d) was inhibited compared with the control group (Fig. 3F-c). Besides, the migration ability

of Panc-1 transfected with shRNA-UBE2T (Fig. 3F-b) was inhibited compared with the control group (Fig. 3F-a). These findings indicated that UBE2T may promote pancreatic cancer cell invasion and migration.

UBE2T promotes EMT in pancreatic cancer cells. As EMT serves a crucial role in tumor development (9), the present study investigated the effect of UBE2T on the expression of epithelial and mesenchymal marker proteins via western blotting. The results demonstrated that the expression of the epithelial markers E-cadherin and α -catenin was decreased whereas the expression of the mesenchymal markers vimentin and N-cadherin was increased in pancreatic cells overexpressing UBE2T compared with the control group. Furthermore, in Panc-1 cells transfected with shRNA-UBE2T, the expression of mesenchymal markers was decreased whereas the expression of epithelial markers was increased (Fig. 4).

Discussion

Pancreatic cancer represents a type of solid tumor that is highly malignant and invasive (7). Furthermore, little is known about the pathogenesis of pancreatic cancer. It is therefore crucial to determine some genes that would be associated with the invasive and metastatic abilities of pancreatic cancer cells and to further investigate the mechanisms involved in the occurrence and development of this cancer.

Previous studies reported that UBE2T is highly expressed in bladder cancer, prostate cancer, gastric cancer and nasopharyngeal carcinoma (7-9), and that UBE2T is elevated in the majority of tumors of the digestive system. The present study demonstrated that UBE2T was upregulated in pancreatic cancer tissues and that UBE2T overexpression promoted cancer cell proliferation and invasion *in vitro*. The underlying mechanism of UBE2T on the promotion of malignant biological behavior in pancreatic cancer requires further investigation.

EMT and mesenchymal-epithelial transformation are considered as key steps in cancer cell metastasis initiation or endpoint (10). Previous studies on prostate cancer reported that UBE2T overexpression induces the upregulation of mesenchymal markers, including vimentin, fibronectin and α -smooth muscle actin, and the downregulation of epithelial markers, including E-cadherin (9). It has been reported that UBE2T can inhibit the degradation of vimentin caused by F-box and WD repeat domain containing 7 (FBXW7), which is a tumor suppressor (7). The molecular mechanisms underlying the inhibition of vimentin degradation by UBE2T may be due to the influence from the interconnection between FBXW7 and vimentin; and that the 95 amino acid site in UBE2T protein is the binding site between UBE2T and vimentin (8). However, to the best of our knowledge, the role of UBE2T in EMT regulation in pancreatic cancer has not yet been reported. The results from the present study reported that UBE2T overexpression could promote EMT in pancreatic cancer cells, which was similar to findings from previous studies on prostate cancer (8,10).

In conclusion, results from this study suggested that UBE2T may be considered as a potential therapeutic target in pancreatic cancer progression. In addition, UBE2T was demonstrated to promote pancreatic cancer cell invasion, which may be mediated through EMT regulation and other mechanisms. Although UBE2T may be considered as a potential diagnostic biomarker and therapeutic target in pancreatic cancer, further investigation is required to determine the underlying mechanisms involved in the regulation of tumor biological behavior by UBE2T.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and CL conceived the project and designed the experiments. PG and MM performed experiments and analyzed

data. YC contributed to the conception and design of the work, as well as to the analysis and interpretation of data. YZ and CL wrote the manuscript. All authors accepted the revised version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shandong Provincial Hospital and all patients provided signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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