

Original Article

UBE2T silencing inhibits TGF- β 1-induced epithelial-to-mesenchymal transition in colon cancer cells

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Abstract: Epithelial-mesenchymal transition (EMT) is considered as the first and key step for the migration and invasion of cancers including colon cancer. Ubiquitin-conjugating enzyme E2T (UBE2T), a member of the E2 family, plays an important role in tumorigenesis and progression. However, its role in colon cancer has not been investigated. In this study, we investigated the effect of UBE2T on transforming growth factor- β 1 (TGF- β 1)-mediated EMT in colon cancer cells and its underlying mechanisms. Our results showed that UBE2T was highly expressed in human colon cancer tissues and cell lines, and TGF- β 1 treatment obviously induced the expression of UBE2T in colon cancer cells. In addition, knockdown of UBE2T inhibited TGF- β 1-induced EMT process, as well as migration and invasion in colon cancer cells. Furthermore, knockdown of UBE2T inhibits phosphorylation of PI3K and Akt in TGF- β 1-stimulated colon cancer cells. In conclusion, we demonstrated that UBE2T silencing prevented TGF- β 1-induced EMT in colon cancer cells by inhibiting the PI3K/Akt signaling pathway. Therefore, UBE2T may be a potential therapeutic target for the treatment of colon cancer.

Keywords: Ubiquitin-conjugating enzyme E2T (UBE2T), colon cancer, epithelial-mesenchymal transition (EMT), transforming growth factor- β 1 (TGF- β 1)

Introduction

Colon cancer is one of the most commonly diagnosed cancers and the fourth leading cause of cancer mortality in the world, and the incidence is increasing steadily every year [1, 2]. Despite very aggressive treatment including surgery and combined radio and chemotherapy, about half of newly diagnosed colon cancer patients will still die of this disease due to tumor recurrence and metastasis [3-5]. Therefore, it is urgent to identify new therapeutic molecular targets and therapeutic strategies for the prevention and treatment of colon cancer.

Epithelial-mesenchymal transition (EMT) is a critical cellular process in tumor metastasis, during which epithelial polarized cells become motile mesenchymal cells. During the process of EMT, epithelial marker (E-cadherin) is down-regulated, while mesenchymal markers, such as N-cadherin and vimentin, are up-regulated

[6, 7]. Transforming growth factor- β 1 (TGF- β 1), a cytokine with a variety of biological activities, can induce and maintain EMT in tumor cells, including colon cancer cells [8-11]. Therefore, inhibition of TGF- β 1-induced EMT may be a therapeutic approach for the treatment of colon cancer.

Ubiquitin-conjugating enzyme E2T (UBE2T) is a member of the E2 family that mediates the ubiquitin-proteasome system. UBE2T has been shown to be necessary for the efficient DNA damage-induced monoubiquitination of FANCD2 [12]. Increasing evidence has shown that UBE2T plays an important role in tumorigenesis and progression [13-15]. For example, Wen *et al.* reported that UBE2T is highly expressed in human prostate cancer tissues, and while UBE2T depletion by shRNA significantly inhibits prostate cancer cell proliferation, motility and invasion [16]. However, its role in colon cancer has not been investigated. In this study, we investigated the effect of UBE2T on TGF- β -

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mediated EMT in colon cancer cells and its underlying mechanisms. In general, our findings showed that UBE2T silencing inhibits TGF- β 1-induced EMT in colon cancer cells through suppressing the PI3K/Akt signaling pathway.

Materials and methods

Tissue specimens

Nine pairs of matched colon cancers and normal tissues from the same patients were obtained from the Department of Digestive Medicine, Huaihe Hospital of Henan University (China), between January 2015 and May 2015. All tissues were fresh-frozen and stored at -80°C . The present study was approved and monitored by the Ethics Committee of Huaihe Hospital of Henan University, and informed consent was obtained from each patient.

Cell culture

Human colon cancer cell lines (HCT116, DLD1 and RKO) and human colon mucosa cell line (NCM460) were purchased from the American Type Cell Culture Collection (ATCC, Manassas, VA) and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 100 units/ml penicillin/streptomycin (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2 .

Small interfering RNA (siRNA) and cell transfection

Scrambled siRNA and small-interfering RNA (siRNA) targeting UBE2T (siUBE2T) were purchased from Santa Cruz Biotechnology. Their sequences were as follows: siUBE2T: GCU-GACAUUCCUCAGAAUTT; Scrambled: UUCU-CCGAACGUGUCACGUTT. For *in vitro* transfection, HCT116 cells were transfected with siUBE2T or scramble using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. Stable clones were selected with $1\ \mu\text{g}/\text{ml}$ of G418 and maintained with $200\ \text{ng}/\text{ml}$ of G418.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from colon cancer tissues or cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. Up to $5\ \mu\text{g}$ of the total RNA was reverse-transcribed into cDNA using M-MLV

reverse transcriptase (Clontech, Palo Alto, CA, USA). The specific primers for UBE2T were sense, 5'-CAA ATA TTA GGT GGA GCC AAC AC-3', and antisense, 5'-TAG ATC ACC TTG GCA AAG AAC C-3'; and for β -actin were sense, 5'-GAT CAT TGC TCC TCC TGA GC-3' and antisense, 5'-ACT CCT GCT TGCTGATCCAC-3'. Amplification cycles consisted of 94°C for 3 min, then 35 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min, followed by 72°C for 15 min. The relative expression levels were calculated by $2^{-\Delta\Delta\text{Ct}}$ method and the target gene was normalized to the internal reference gene.

Western blot

Cells were lysed in lysis buffer containing 1% NP40, 1 mM EDTA, 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl, supplemented with complete protease inhibitors mixture (Roche, Monza, Italy). Protein concentrations were measured by BCA protein assay kit. Equal amounts of protein ($30\ \mu\text{g}$ protein each lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membrane (Millipore Corporation, Billerica, MA, USA). Immunoblots were blocked with 5% non-fat dry milk in TBS/Tween 20 (0.05%, v/v) at room temperature for 1 h. Subsequently, the membrane was incubated with the following primary antibodies: UBE2T, E-cadherin, vimentin, PI3K, p-PI3K, Akt, p-Akt and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C . After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the target protein was visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

In vitro migration and invasion assays

Cell migration and invasion assays were performed using Transwell chambers ($8.0\ \mu\text{m}$ pore size; Millipore, MA), which were coated with or without Matrigel (1:5 dilution in DMEM). Briefly, HCT116 cells transfected with siUBE2T or scramble at a density of 1×10^4 cells/well were added to the upper chamber of transwells (BD Bioscience, USA), and DMEM medium with 10% FBS ($500\ \mu\text{L}$) was added to the lower chamber. After incubation of 24 h, not migrating cells on the upper side of the filter were wiped off. Then, cells on the lower surface of the filter were fixed, stained and examined under a microscope ($100\times$ magnification).

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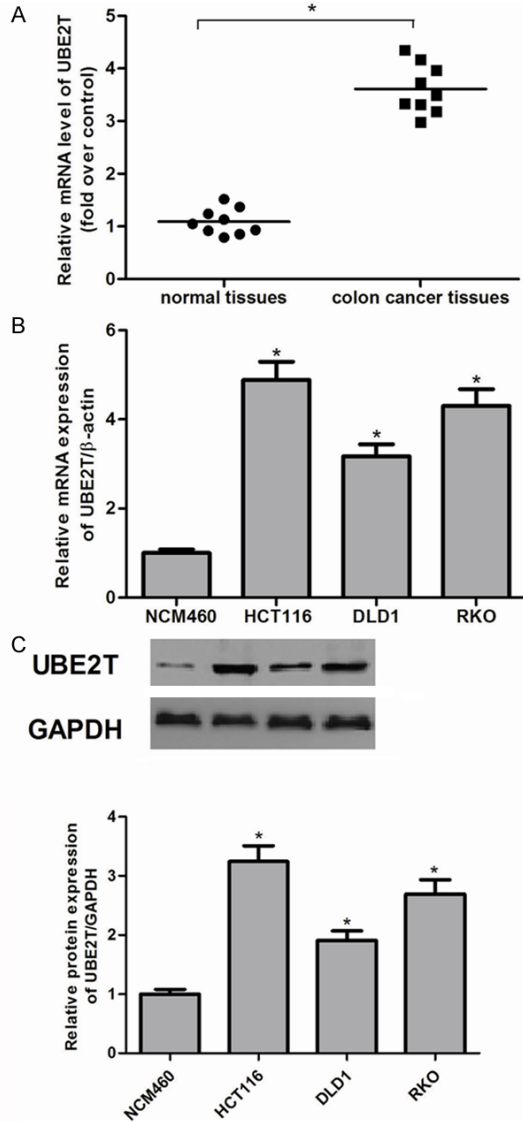


Figure 1. UBE2T is highly expressed in colon cancer tissues and cell lines. A. mRNA expression of UBE2T was analyzed by qRT-PCR. UBE2T mRNA levels in colon cancer tissues were obviously higher than that in normal colon tissues, * $P < 0.05$ compared to normal colon tissues. B. representative mRNA expression of UBE2T in colon cancer cell lines; C. representative Western image of UBE2T protein in colon cancer cell lines. * $P < 0.05$ compared with NCM460.

Statistical analysis

All data were expressed as the mean \pm SD of three independent experiments. The differences were analyzed by the Student's *t* test or one-way analysis of variance and Student's *t* test. A $P < 0.05$ was regarded as statistically significant.

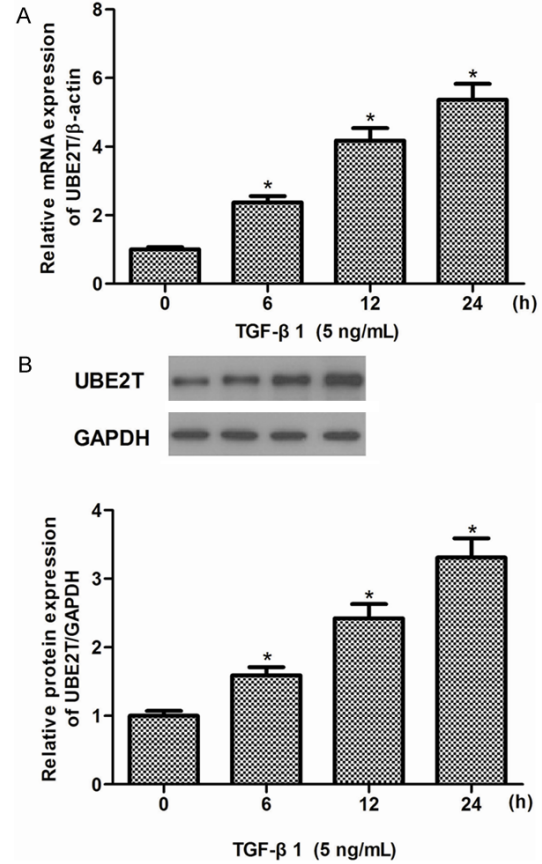


Figure 2. TGF- β 1 induces UBE2T expression in colon cancer cells. HCT116 cells were treated with TGF- β 1 (5 ng/ml) for 0, 6, 12 or 24 h. A. The mRNA expression of UBE2T was detected by qRT-PCR. B. The protein expression of UBE2T was detected by Western blotting. Results are means \pm SD from three independent experiments performed in duplicate. * $P < 0.05$ compared with control.

Results

UBE2T is highly expressed in colon cancer tissues and cell lines

We first examined the expression of UBE2T in colon cancer tissues. As compared with the normal colon samples, UBE2T mRNA levels in colon cancer tissue samples were significantly upregulated (**Figure 1A**). We examined *UBE2T* expression in three human colon cancer cell lines. The results of qRT-PCR demonstrated that *UBE2T* expression was up-regulated in all colon cancer cell lines (HCT116, DLD1 and RKO) compared with human colon mucosa cell line (NCM460) (**Figure 1B**). Western blotting showed similar results (**Figure 1C**).

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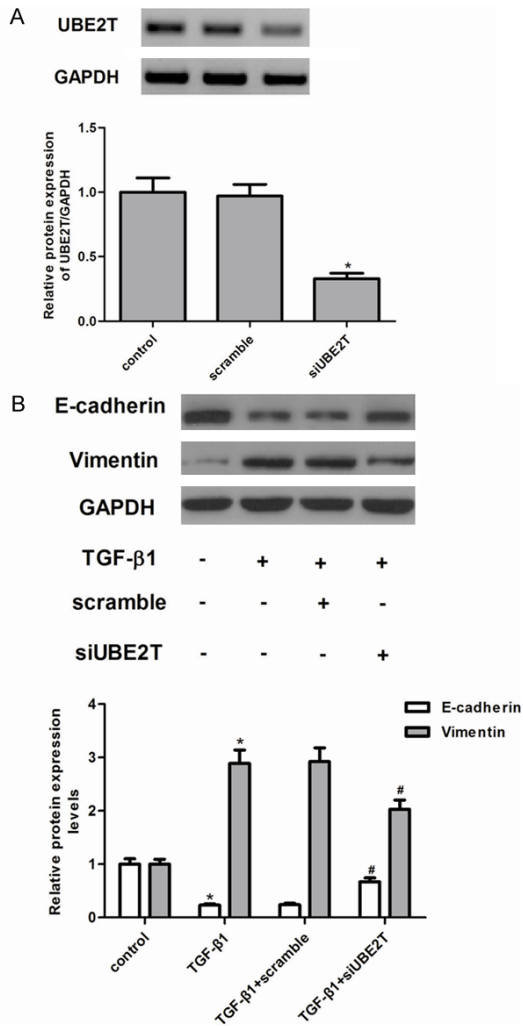


Figure 3. UBE2T silencing suppresses EMT induced by TGF-β1 in colon cancer cells. A. The transfection efficiency was confirmed by Western blot analysis in HCT116 cells after transfection with siUBE2T. B. HCT116 cells transfected with siUBE2T or scramble were treated with TGF-β1 (5 ng/ml) for 24 h. The protein expression levels of E-cadherin and vimentin were detected by western blot analysis. Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. Results are means ± SD from three independent experiments performed in duplicate. * $P < 0.05$ compared with control; # $P < 0.05$ compared with scramble + TGF-β1.

TGF-β1 induces UBE2T expression in colon cancer cells

Next, we detected the expression of UBE2T in HCT116 cells treated with TGF-β1. As shown in **Figure 2A**, the treatment of TGF-β1 significantly increased the expression of UBE2T mRNA in HCT116 cells in a time-dependent manner. In addition, consistent with the data of qRT-PCR,

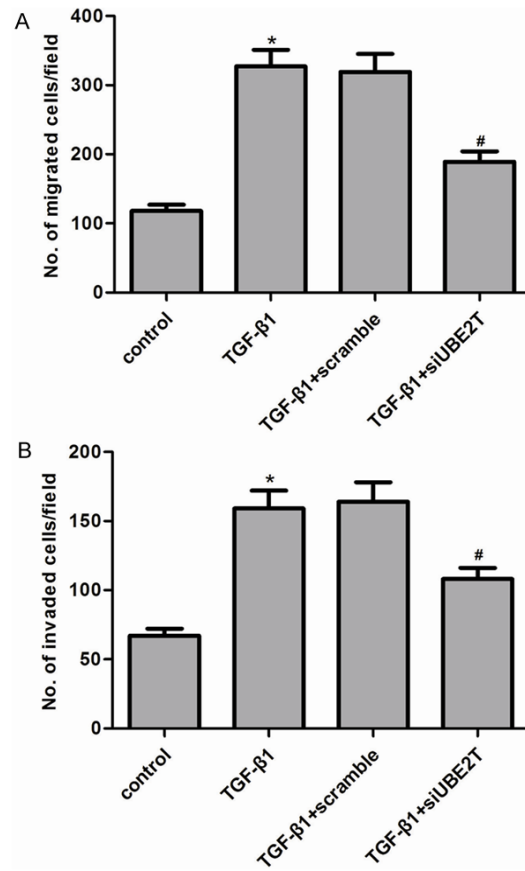


Figure 4. UBE2T silencing inhibits colon cancer cells migration and invasion induced by TGF-β1. HCT116 cells transfected with siUBE2T or scramble were treated with TGF-β1 (5 ng/ml) for 24 h. A. The migratory potential of HCT116 cells was detected by transwell assay. B. The invasive ability of HCT116 cells was detected by transwell assay with Matrigel. Results are means ± SD from three independent experiments performed in duplicate. * $P < 0.05$ compared with control; # $P < 0.05$ compared with scramble + TGF-β1.

Western blot analysis indicated that the expression of UBE2T was also increased by TGF-β1 (**Figure 2B**).

UBE2T silencing suppresses EMT induced by TGF-β1 in colon cancer cells

To investigate the effect of UBE2T on EMT process in colon cancer cells, UBE2T was down-regulated by siUBE2T in HCT116 cells. Western blot analysis showed that shUBE2T successfully downregulated UBE2T expression in HCT116 cells (**Figure 3A**). Additionally, we determined the expression of E-cadherin and vimentin. The results showed that the treatment of TGF-β1 significantly decreased the expression of

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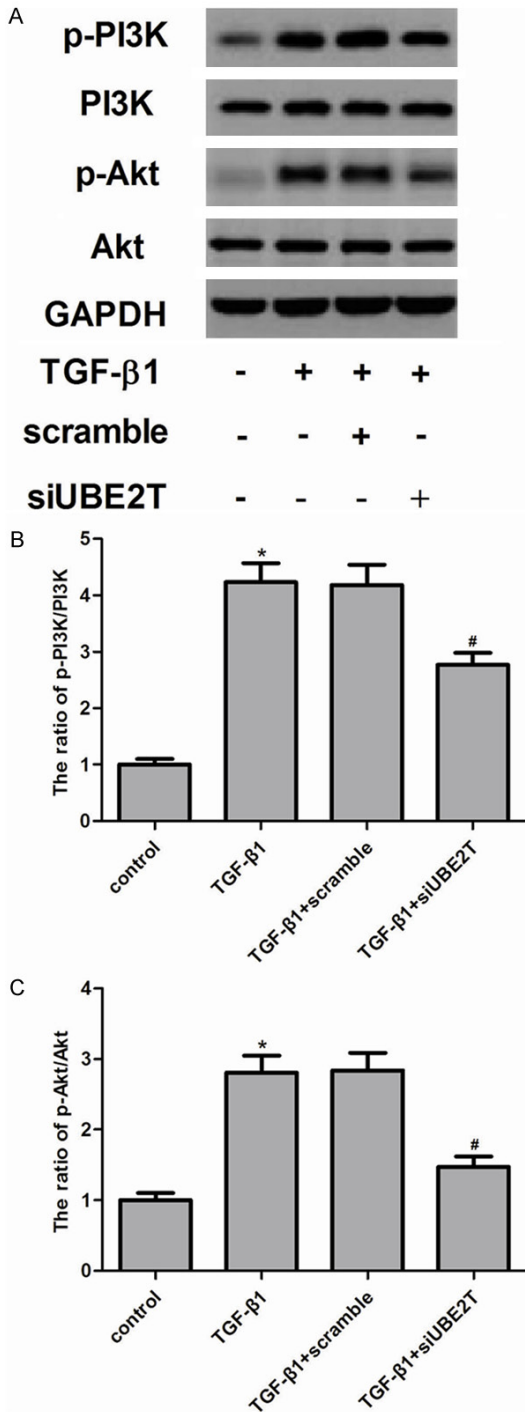


Figure 5. UBE2T silencing inhibits TGF-β1-induced EMT by suppressing the PI3K/Akt signaling pathway in colon cancer cells. A. HCT116 cells transfected with siUBE2T or scramble were treated with TGF-β1 (5 ng/ml) for 30 min. The protein expression levels of p-PI3K, PI3K, p-Akt and Akt were detected by western blot analysis. B and C. Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. Results are means ± SD from three independent experiments performed in duplicate. * $P < 0.05$ compared with control; # $P < 0.05$ compared with scramble + TGF-β1.

E-cadherin and increased the expression of vimentin in colon cancer cells. In addition, we found that knock down of UBE2T markedly increased the expression of E-cadherin, while decreased the expression of vimentin in colon cancer cells treated with TGF-β1 (**Figure 3B**).

UBE2T silencing inhibits colon cancer cells migration and invasion induced by TGF-β1

Then, we examined the effects of UBE2T on cell migration and invasion in colon cancer cells in response to TGF-β1. As shown in **Figure 4**, TGF-β1 treatment obviously increased the migration and invasion in HCT116 cells by transwell migration/invasion assays. In addition, the increased migration (**Figure 4A**) and invasion abilities (**Figure 4B**) of HCT116 cells were both significantly inhibited by the transfection with siUBE2T.

UBE2T silencing inhibits TGF-β1-induced EMT by suppressing the PI3K/Akt signaling pathway in colon cancer cells

Several studies suggest that the PI3K/Akt signaling pathway may play an important role in the EMT process in the progression of tumor. Therefore, we investigated whether siUBE2T regulates the PI3K/Akt pathway to suppress TGF-β1-mediated EMT in HCT116 cells. As shown in **Figure 5**, TGF-β1 significantly promoted the phosphorylation of PI3K and Akt. At the same time, knockdown of UBE2T dramatically reversed TGF-β1-induced the phosphorylation of PI3K and Akt in HCT116 cells.

Discussion

The main findings of the present study can be summarized as follows: (1) UBE2T was highly expressed in human colon cancer tissues and cell lines, and TGF-β1 treatment obviously induced the expression of UBE2T in HCT116 cells; (2) Knockdown of UBE2T inhibited TGF-β1-induced EMT process, as well as migration and invasion in HCT116 cells; (3) Knockdown of UBE2T inhibits phosphorylation of PI3K and Akt in TGF-β1-stimulated HCT116 cells.

Aberrant expression of UBE2T has been detected in many types of human cancers. However, the role of UBE2T in human colon cancer is unclear. In this study, we displayed that UBE2T was overexpressed in human colon cancer tissues and cell lines. Our observations are consistent with numerous reports of increased

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UBE2T expression in gastric cancer, prostate cancer and nasopharyngeal carcinoma. These results suggest that UBE2T may be an oncogene in the progression of colon cancer.

EMT plays an important role in the metastasis of colon cancer [17-19]. In the EMT process, epithelial cells acquire fibroblast-like properties and exhibit reduced intercellular adhesion and increased motility [20]. Several inflammatory mediators such as TGF- β , hypoxia and IL-6 can upregulate Snail and therefore trigger EMT [21]. A recent study showed that TGF- β 1 treatment induced morphological changes, disappearance of E-cadherin staining and formation of actin stress fiber in HT29 cells [22]. Another study reported that TGF- β 1/ β 2 significantly increased expression of EMT-related transcription factors, as well as promoted the migration and invasion in SW-480 and HT-29 cells [23]. Similarly, in this study, we found that the treatment of TGF- β 1 significantly decreased the expression of E-cadherin and increased the expression of vimentin in HCT116 cells. However, knockdown of UBE2T markedly increased the expression of E-cadherin, while decreased the expression of vimentin in colon cancer cells treated with TGF- β 1. It also inhibited colon cancer cells migration and invasion induced by TGF- β 1. These data suggest that UBE2T silencing inhibits TGF- β 1-induced EMT, consequently markedly suppresses cell migration and invasion *in vitro*.

A growing body of evidence indicates that the PI3K/Akt signaling pathway plays an important role in development and progression of cancer [24-26]. The serine/threonine kinase Akt, the most studied signaling molecule downstream of PI3K, is involved in the stimulation of EMT process. It was reported that the activation of Akt increased N-cadherin and vimentin, and decreased E-cadherin [27]. Previously, Liu *et al.* confirmed that TGF- β treatment increased the motility of HCT116 cells, and treatment with a potent PI3K inhibitor (LY294002) abrogated promotion of motility by TGF- β [28]. Interestingly, one study reported that UBE2T overexpression activated, whereas UBE2T knockdown suppressed the AKT/GSK3 β / β -catenin signaling pathway in nasopharyngeal carcinoma cells [14]. In the present study, we found that knockdown of UBE2T dramatically reversed TGF- β 1-induced the phosphorylation of PI3K and Akt in HCT116 cells. These data

suggest that knockdown of UBE2T inhibited TGF- β 1-induced EMT in colon cancer cells by suppressing the PI3K/Akt signaling pathway.

In conclusion, we demonstrated that UBE2T silencing prevented TGF- β 1-induced EMT in colon cancer cells by inhibiting PI3K/Akt signaling pathway. Therefore, UBE2T may be a potential therapeutic target for the treatment of colon cancer.

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Disclosure of conflict of interest

None.

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