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Overexpression of TAFI promotes epithelial mesenchymal transition in endometriosis

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Abstract. – OBJECTIVE: Endometriosis is a disease that occurs in women. Thrombin-activated fibrinolytic inhibitor (TAFI) is mainly secreted by stem cells and acts as a regulatory role in the body. Epithelial leaf transition plays a leading role in cell growth and invasion. Our study focuses on the mechanism of TAFI in patients with endometriosis.

PATIENTS AND METHODS: The expression of TAFI was determined by immunohistochemistry. Reverse transcriptase-polymerase chain reaction (RT-PCR) served to detect the expression of TAFI and the effect of TAFI on overall survival (OS) and progression-free survival (PFS) levels. The changes of primary cytology in patients with endometriosis were observed under a microscope. The cell source was further determined by immunofluorescence labeling of vimentin and cytokeratin, and the expression of TAFI was detected by Western-blot. 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and cell invasion assay were utilized to detect the viability and aggressiveness of cells after epithelial mesenchymal transition (EMT).

RESULTS: TAFI was overexpressed in endometriosis tissues and no expression of TAFI was found in normal tissues, which is consistent with RT-PCR results. TAFI overexpressed endometriosis patients had low levels of overall OS and PFS. There were statistically significant differences. Cell morphology shows that endometriosis primary cells are mainly composed of epithelial cells and fibroblasts. Immunofluorescence assay showed that vimentin and cytokeratin were expressed in cells, and the expression of TAFI was detected by Western-blot. Compared with normal tissues, TAFI was considerably higher in patients with endometriosis. The results of Western-blot and RT-PCR showed that the expression of TAFI was significantly increased in patients with endometriosis and the cell proliferation and cell invasion were significantly accelerated.

CONCLUSIONS: Our results show that TAFI is highly expressed in endometriosis and causes EMT, which accelerated the cell proliferation and cell invasion. Snail is an inhibitor of E-cadherin, which may participate in metastasis and invasion of endometriosis by mediating EMT. So, we suspect that Snail controls the occurrence of the EMT and then affects the cell metastasis and invasion, which requires further verification.

Key Words:
Endometriosis, TAFI, EMT.

Introduction

Endometriosis is the implantation of endometrial cell in the abnormal position and cause of a common female gynecological disease. Endometrial cells should be grown in the uterine cavity, due to uterine cavity and pelvic interruption through the fallopian tube, so that endometrial cells can enter the pelvic cavity through the fallopian tube and ectopic growth. There are a variety of theories about the current pathogenesis of the disease; the generally recognized is the endometrial implant theory. In addition, the occurrence of endometriosis is associated with immune function, genetic factors, and environment factors. Endometriosis is a major factor associated with estrogen-related diseases, leading to 6-10% of women of childbearing age with pelvic pain and low fertility[1-3]. It is characterized by the deposition of the endometrium and the growth of the tissue outside the uterus, which increases the local secretion of estrogen due to molecular aberrations. In steroid therapy, it is also considered to result in pelvic inflammatory disease. Over the past four decades, the study of endometriosis has increased exponentially, but its exact pathogenesis and pathophysiology are not clear. Recent studies have shown that endometriosis is essentially caused by multiple tissue damage and repair (retraction), leading to epithelial mesenchymal transition (EMT), fibroblast-myofibroblast differentiation (FMT), smooth muscle metaplasia (SMM) and final fibrosis[4-6].

In recent years, EMT is thought to be a central process in embryonic development, chronic inflammation and fibrosis, and tumors. The

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most significant feature of EMT is the decrease in E-cadherin expression and the increase in the expression of mesenchymal calcium-binding proteins such as N-cadherin[7,8]. More and more studies have shown that EMT leads to the formation of ectopic lesions. In addition, hypoxia can lead to EMT procedures; studies have reported that β-catenin is a key transcription factor of endometriosis, which can be activated by hypoxia and participate in the appearance of endometriosis. β-Catenin also plays an important role in many tumor EMT processes[9,10]. The current data suggest that EMT and hypoxia may be a very important link in favor of endometriosis invasion and migration[11]. Thrombin-activated fibrinolytic inhibitor (TAFI) is secreted by hepatocytes and is present in the inactive form of plasma, which mainly regulates the production of plasmin and inhibits the activation of glutamate-plasminogen[12]. It shows that TAFI and its encoded genes are associated with gynecological diseases[13]. To take this into account, we will explore the relationship between TAFI and EMT.

Patients and Methods

Extraction of Primary Cells

The inner wall of the chocolate cysts was digested with collagenase (Beyotime, Shanghai, China) and cultured in 1640 cells containing 15% serum (Waltham, MA, USA). No adherent cells were found after 24 h. However, adherent cells appeared after 48 h, and the cells were in the form of shuttle or star. This study was approved by the Ethics Committee of Jinan Maternity and Child Care Hospital.

Hematoxylin and Eosin (HE) Staining

The tissue was fixed by conventional methods and dehydrated. The tissues were embedded in paraffin and then cooled in 4°C. A slice thickness of 4 μm was obtained and dewaxed according to the procedure. Slices were stained with hematoxylin for 1 min, and eosin stained for 10 s. Finally, they were observed under a microscope (BX-42, Olympus, Tokyo, Japan).

Immunohistochemistry

Tissue was fixed overnight with neutral formalin and it was dehydrated. 4 μm paraffin sections were dewaxed and hydrated according to the procedure. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide. Thereafter, 10% of fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA) was used to block for half an hour, then incubated with primary antibody. After 24 h, the secondary antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated at 37°C for 1 h. After diaminobenzidine (DAB) coloration was done, the reaction was terminated. Hematoxylin was utilized for re-stained for 3 min and the tablets were observed under a microscope. Phosphate-buffered saline (PBS, Nanjing Keygen Biotech, Nanjing, China) was invoked as a negative control instead of primary antibody.

Immunofluorescence Assay

The fixed tissue was dehydrated by a sucrose gradient to obtain a frozen section with a thickness of 6 μm. Sections were blocked by 10% bull serum albumin (BSA, Waltham, MA, USA) for 50 min, incubated with primary antibody followed by incubation with secondary antibody. Finally, the sections were stained with 4',6-diamidino-2-phenylindole (DAPI).

Western Blotting

For the determination of N-cadherin, E-cadherin, vimentin, and snail, tissue protein was extracted using Beyotime Protein Kit (Shanghai, China). The extracted proteins were isolated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. Then, the protein on the gel was transferred to the polyvinylidene difluoride (PVDF) membrane (EMS Millipore, Billerica, MA, USA), followed by blocking the unabsorbed protein region with skimmed milk. The PVDF membrane with the protein band was sequentially labeled with the specific antibody and Alexa Fluor-conjugated secondary antibodies. The Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA) was employed to detection.

RT-PCR

Fresh tissues were kept in liquid nitrogen. Total RNA extraction was performed using the TRIzol method. The RNA was converted into a complementary DNA (cDNA) using a reverse transcriptase or stored at -80°C.

MTT Assays

The MCF-7 cells were inoculated into 96-well plates (Corning, Corning, NY, USA). After 12 h, MTT was added and cultured for 4 h. The culture medium was discarded and 150 μL of dimethyl sulfoxide (DMSO) were added to each well. The crys-

tals were sufficiently dissolved by shaking, and the OD (optical density) value was measured at 490 nm.

Invasion Assessment

The cell invasion ability was detected using a Transwell membrane (8 μm pore size, Costar, Cambridge, MA, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). A total number of $1x10^5$ cells per well was cultured in the upper chamber, while 20% fetal bovine serum (FBS) was added to the lower compartment as a chemoattractant. Following 48 h incubation, non-invading cells were scraped by a cotton swab. The invaded cells were fixed with 100% methanol for 10 min, stained in 0.5% crystal violet (ApexBio, Shanghai, China) for 20 min, and then photographed by a light microscope (Olympus, Tokyo, Japan) at 200 x magnification. Also, invaded cells were immerged in 33% ice-cold acetic acid for 10 min and absorbance at 570 nm was assessed by a microplate reader (Bio-Rad, Hercules, CA, USA). The values of negative control were set as 100%.

Statistical Analysis

All experimental data were expressed as $\bar{x} \pm s$ and analyzed by Image-Pro-Plus 6.0 and Graph Pad Prism 5 (La Jolla, CA, USA). The one-dimensional variance analysis was used to compare the multi-group comparisons. The paired *t*-test was used to compare the two groups. $p < 0.05$ represents a significant difference.

Results

Expression of TAFI in Tissue and Its Effect on Survival Rate

The expression level of TAFI protein in endometriosis tissues is very high, mainly in the ductal cavity, especially in the matrix and cytoplasm. However, there is no expression of TAFI in normal tissues (Figure 1A). The results were consistent with RT-PCR, and the expression of TAFI was dramatically higher in patients with endometriosis than in normal tissues, indicating that mRNA did not change before transcribed

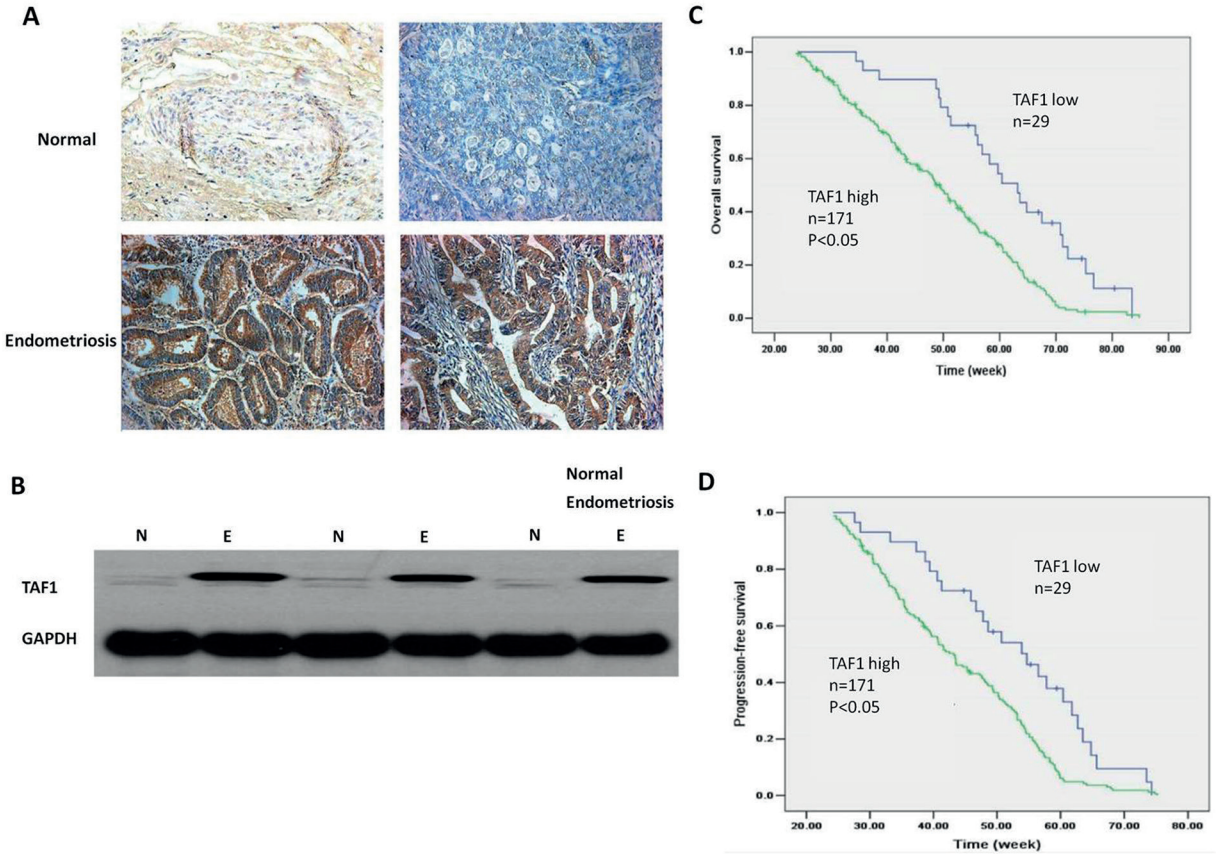

Figure 1. Expression of TAFI in tissue and the effect on survival rate. Detection of TAFI expression in patients with endometriosis by immunohistochemistry ***(A)*** and RT-PCR ***(B)***; The effect of TAFI on overall survival ***(C)*** and PFS ***(D).***

into protein (Figure 1B). The overall survival (OS) is defined as the interval between the diagnostic dates and the date of death for any reason or last visit to the patient. The study found that the survival rates of patients with overexpressed TAFI were significantly lower than those with normal TAFI expression (Figure 1C). Similarly, progression-free survival (PFS) in patients with high expression of TAFI was also statistically lower than other patients (Figure 1D).

Identification of Ectopic Primary Cells and Expression of TAFI

To further determine whether the extracted cells were endometriosis cell lines, cell observations showed that these cells were polygonal or shaped and showed clear cell boundaries. The interstitial cells were fibrous cell morphology of fusiform cells (Figure 2A). Given that endometrial ectopic primary cells are mainly comprised of epithelial cells and fibroblasts, we detected vimentin and cytokeratin for cell identification. The expression of vimentin and cytokeratin by extraction of cells showed that both of vimentin and cytokeratin were overexpressed in cells, respectively (Figure 2B). At the same time, Western-blotting detection showed that TAFI was significantly higher in patients with endometriosis compared with normal tissues, as showed in Figure 2C. These results confirmed that the primary cells of patients with endometriosis were successfully isolated and the level of TAFI was high.

Overexpression of TAFI Leads to Epithelial Mesenchymal Transition

It is well known that the EMT is associated with many diseases, especially inflammation and cancer. So, changes in EMT-related factors in endometriosis patients with high expression of TAFI were examined, and the changes of EMT-related cytokines were also examined when using si-RNA interference. Western blotting showed that EMT-related cytokines such as E-cadherin, N-cadherin, vimentin, snail, slug, and twist, were significantly increased in the primary cells with high expression of TAFI, while, the level of EMT-related cytokines was significantly lower in TAFI-lowly expressed cell lines (Figure 3A). The results of RT-PCR were in agreement with Western blotting (Figure 3B).

Overexpression of TAFI Leads to Cell Proliferation and Cell Migration

Cell proliferation and cell migration are closely associated with EMT. Cell proliferation refers to the disorderly growth of cells under

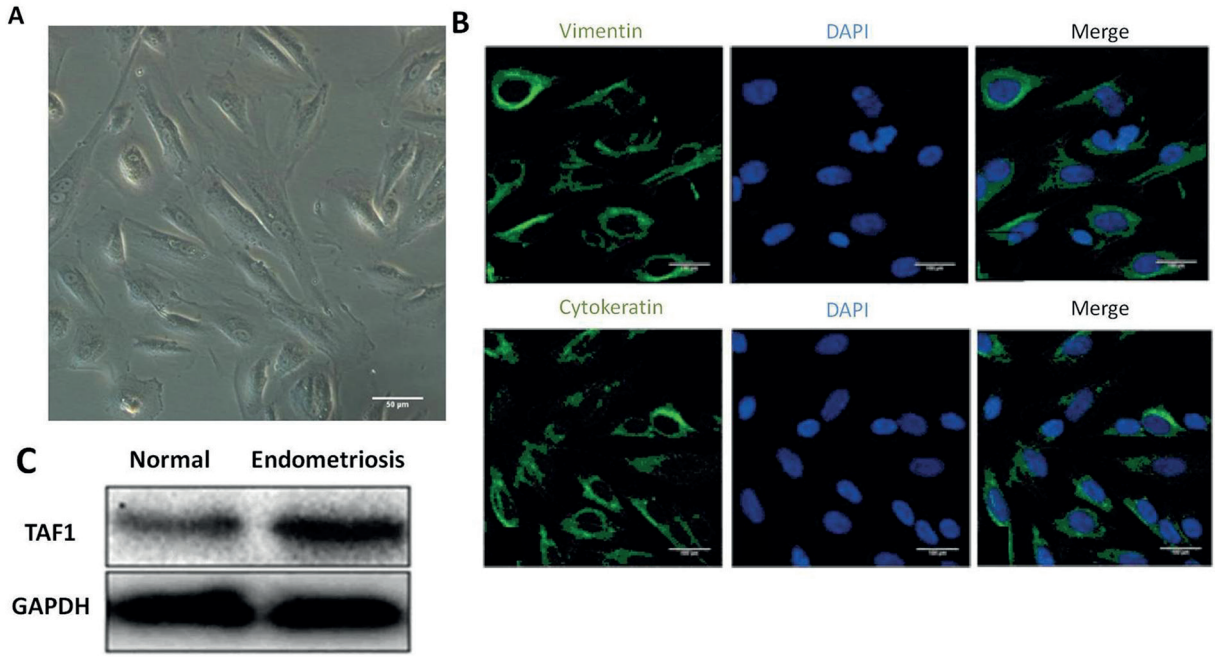

Figure 2. Identification of ectopic primary cells and level of TAFI. ***A,*** Morphological changes of primary endometriosis cells were observed under microscope (40×). ***B,*** Expression of vimentin and cytokeratin in primary endometrial cells (40×). ***C,*** The expression of TAFI in normal primary cells and endometriosis primary cells was detected by Western blotting.

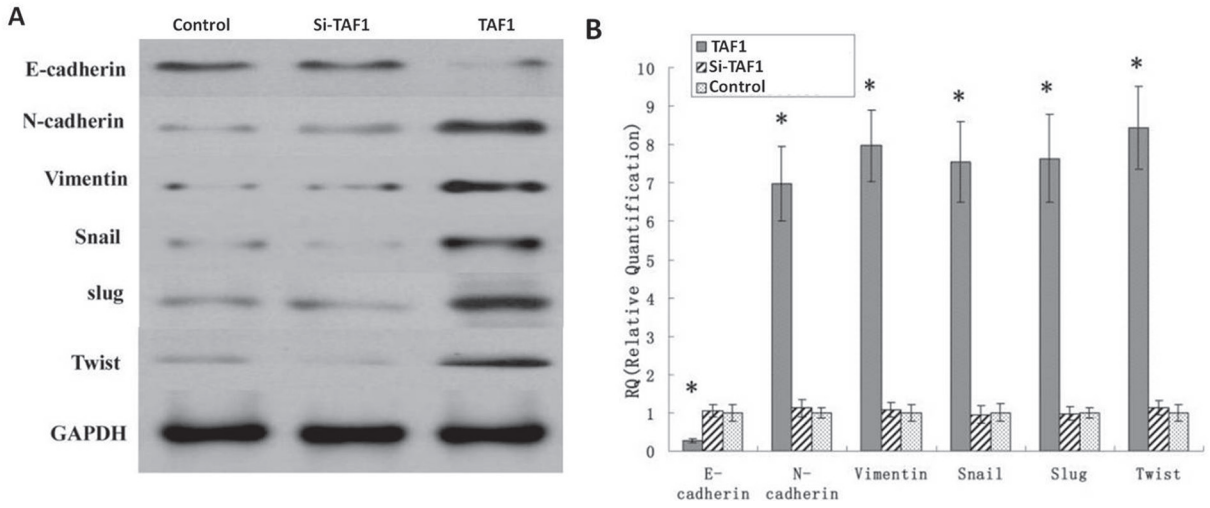

Figure 3. Overexpression of TAFI leads to epithelial mesenchymal transition. EMT-related cytokines (E-cadherin, N-cadherin, vimentin, snail, slug, twist) were detected by Western-blot ***(A)*** and RT-PCR ***(B)***.

the regulation of signaling mechanisms. The above results confirm that EMT occurred in primary cells with high expression of TAFI. Compared with the control group and the si-TAFI interference group, the cell proliferation rate of cells with overexpression of TAFI was higher than that of the other two groups (Figure 4A). Figure 4B reports the statistical results. Meanwhile, it was compared to the control group and si-TAFI interference group. Transwell assay confirmed that the cell invasion of low-expressed TAFI was stagnated after

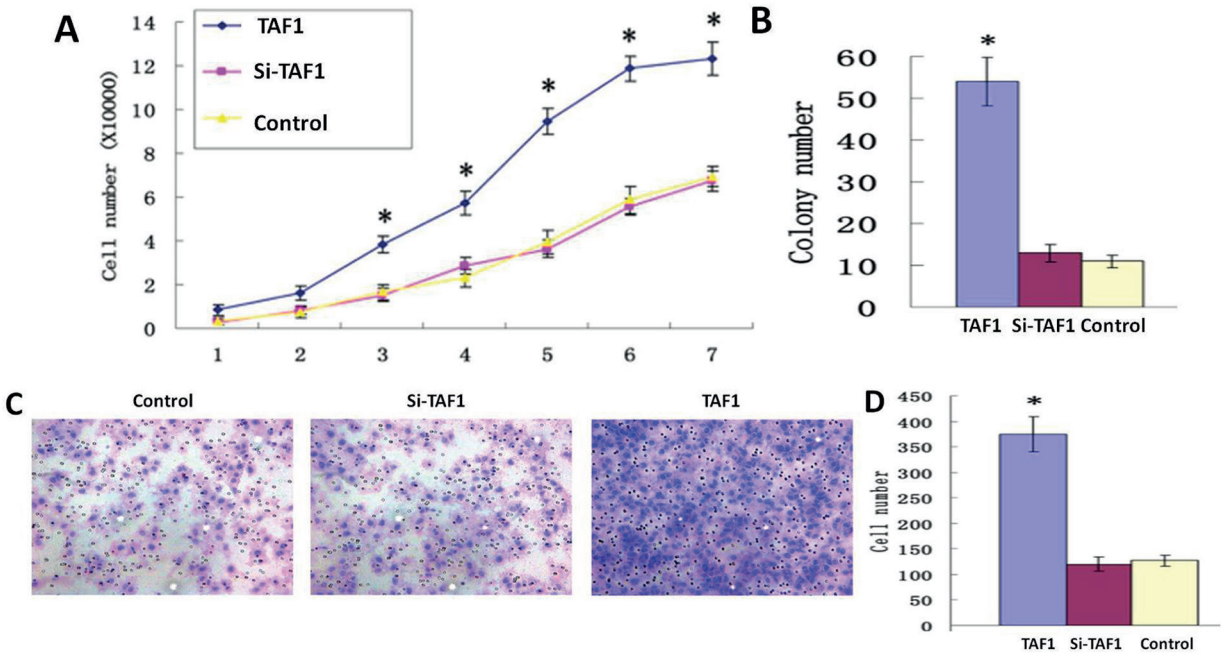

Figure 4. Overexpression of TAFI leads to cell proliferation and cell invasion. ***A,*** Changes in the number of cells in primary cells of patients with endometriosis were detected by MTT. ***B,*** The statistical results of MTT. ***C,*** The results of cell invasion experiments. ***D,*** The statistical results of invasion experiments.

48 h, and significantly accelerated when TAFI was overexpressed (Figure 4C). The statistical results are given in Figure 4D.

Discussion

Endometriosis is the active endometrial cells planted in the endometrium outside the formation of the disease. It is a benign disease in histology, but it has the potential in malignant biology such as proliferation, infiltration, planting, recurrence and malignant transformation. Nearly 90% of the endometriosis lesions are located in the pelvic cavity, especially the ovaries, uterine rectum pits, uterosacral ligament and some other positions[14-16]. According to the 2015 China Obstetrics and Gynecology published endometriosis diagnosis and treatment guidelines, endometriosis can be divided into the following types: (1) peritoneal endometriosis; (2) ovarian endometriosis; (3) deep infiltrating endometriosis (DIE); (4) other parts of the endometriosis. 1 and 2 are common types, 3 and 4 are relatively rare. Atypical symptoms and signs often lead to its misdiagnosis. Although there are early symptoms but no specificity leading to more difficult diagnosis, the literature has numerous reports[17,18].

In our study, TAFI was highly expressed in patients with endometriosis and had a negative regulatory relationship with the survival rate of patients. Similarly, epithelial cells from patients with endometriosis were found to have high expression of TAFI. In previous studies, TAFI had proven to be superior to normal epithelial cells in prostate cancer epithelial cells, consistent with our findings. At the same time, the expression of TAFI in osteosarcoma and glioma was remarkably higher than that in the normal group. However, whether the expression of TAFI regulates the conversion of epithelial mesenchyme still remains unclear. Further, the cells of endometriosis were extracted for epithelial and mesenchymal identification. Mesenchymal cells were identified by vimentin; the results showed that vimentin was expressed on the cell surface. The epithelial cells were marked with cytokeratin and the results showed that the cells of the patients with endometriosis were normal. A large number of papers indicate that cell adhesion molecules are closely associated to the pathogenesis of endometriosis, and the lack of E-cadherin expression is a critical event in epithelial EMT. Snail could promote EMT in normal epithelial cells[19,20]. The expression of EMT-related cytokines in patients with endometriosis was determined by Western-blotting and RT-PCR, respectively. The results showed that the EMT was obvious in overexpressed TAFI cells and the results are consistent with previously reports[21,22]. The expression of E-cadherin, N-cadherin, vimentin, snail, slug and twist in endometriosis was higher than that in control group and si-TAFI interference group. Cell proliferation and migration are closely related to EMT. Our results showed that cell proliferation and cell migration were much higher than those in the control and invasive groups.

Conclusions

Our results show that TAFI is highly expressed in endometriosis and causes EMT, which accelerated the cell proliferation and cell invasion. Snail is an inhibitor of E-cadherin, which may participate in metastasis and invasion of endometriosis by mediating EMT. So, we suggest that snail controls the occurrence of the EMT and then affects the cell metastasis and invasion, which requires further verification.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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