Type II transmembrane serine proteases 4 (TMPRSS4) promotes proliferation, invasion and epithelial–mesenchymal transition in endometrial carcinoma cells (HEC1A and Ishikawa) via activation of MAPK and AKT

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Introduction

Endometrial cancer is the most common gynecological cancer in the developed countries. Type II transmembrane serine proteases 4 (TMPRSS4) is a newly discovered transmembrane protein, which may be related to the invasion, metastasis of the tumor and the poor prognosis. This study aims to investigate the role of TMPRSS4 in endometrial cancer and the detailed molecular mechanism. The results showed that TMPRSS4 was highly expressed in human endometrial cancer cells (HEC1A and Ishikawa). TMPRSS4 knockdown inhibited proliferation of endometrial cancer cells. In TMPRSS4 knockdown cells, the invasion of cells was significantly suppressed. The expression of E-cadherin was significantly enhanced, while the levels of fibronectin and vimentin decreased in TMPRSS4 knockdown cells, which indicated that TMPRSS4 knockdown attenuated the EMT of cancer cells. TMPRSS4 positively regulated the activation of MAPK and AKT signaling pathways in endometrial cancer. In conclusion, this study indicated that TMPRSS4 may be associated with the progression of endometrial cancer through promoting proliferation, invasion and EMT via activation of MAPK and AKT in endometrial cancer cells. TMPRSS4 may be a new and more effective target or therapeutic strategy for treating endometrial cancer.
associated with tumor progression and prognosis (Cheng et al. 2013). Semi et al. reviewed that TMPRSS4 and TM4SF5, two cell surface proteins, were novel regulators of the molecular networks in EMT and cancer progression (Kim and Lee 2014). However, the role of TMPRSS4 in endometrial cancer has not been studied yet, and this study mainly focused on the role of TMPRSS4 in endometrial cancer and the detailed molecular mechanism.

Materials and methods

Cell culture

Human normal endometrial stromal cell (ESC) line, human endometrial cancer cell lines AN3CA (HTB-111), HEC1A (HTB-112), HEC1-B (HTB-113) and Ishikawa were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Eagle’s Minimum Essential Medium (for AN3CA and HEC1-B), McCoy’s 5a Medium (HEC-1A) and DMEM (for Ishikawa) supplemented with 10% FBS.

Transfection with short hairpin RNA (shRNA)

To construct the TMPRSS4-knockdown cells, shRNAs were used. Cells were transfected with expression plasmid vector (pGPU6) and shRNA specific to TMPRSS4 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation. The construction and sequences for the TMPRSS4 was referred to previously report (Larzabal et al. 2011; Cheng et al. 2019). The shRNAs were synthesized at Shanghai GenePharma (Shanghai, China). The transfection efficiency of TMPRSS4 was determined by RT-PCR and western blot (Li et al. 2011). The construction of TMPRSS4-overexpressed cells was referred as previous report (Jung et al. 2008b). Cells were harvested at 24 h after transfection and subjected to cell proliferation, invasion, wound healing and Colony formation assay.

Cell proliferation

Cells were seeded in 96-well plates for 24 h and transfected with vectors for TMPRSS4 knockdown. The proliferation was measured by the CCK-8 kit according to the manufacturer’s protocol.

Cell invasion assay

For cell invasion assay, HEC1A or Ishikawa cells were added into the top chamber of a transwell insert precoated with matrigel and cultured with serum-free medium. In the lower chamber, medium supplemented with 10% FBS was used as an attractant. After 24 h, cells migrated to the underside of the membrane were stained with crystal violet and counted.

Wound healing assay

In vitro cell migration was analyzed by wound healing assay. Briefly, Ishikawa cells were grown to 90% confluence, and linear wounds were made on the cell monolayer and let the wounds to heal for 24 h. Cell migration images were taken by an inverted microscope. The width of the scratch was determined using imageJ software.

Colony formation assay

Cells transfected with plasmid vectors or TMPRSS4 over-expression plasmid were seeded in 6-well plates (100 cells per well). After 16 hours, cells were cultured in fresh medium to allow colony formation for 14 days. Finally, colonies were fixed and stained with crystal violet, and counted. Each treatment was carried out in triplicate.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent according to the manufacturer’s instructions. mRNA were reverse transcribed into cDNA using Reverse Transcription System (Promega Corporation, Madison, WI., USA). Quantitative RT-PCR was performed using SYBR Green PCR Master Mix reagent kits (Promega Corporation, Madison, WI, USA). The primers used in the RT-PCR were synthesized at Sangon Biotech (Shanghai, China) and the sequences were referred as previous reports (Fan et al. 2018).

Western blot

Cells were lysed in RIPA lysis containing protease and phosphatase inhibitor cocktail. Protein concentrations were quantified by BCA protein assay. Equal amount of proteins were resolved by SDS-PAGE and transferred to PVDF membranes. After blocking, the membranes were incubated with primary antibodies (dilution 1:1000) and secondary antibodies (dilution 1:10000). Primary antibodies against TMPRSS4 (Abcam, ab82176, Cambridge, MA, USA), ERK1/2 (CST, #4695, Beverly, MA, USA), p-ERK1/2(CST, #4370S), AKT (CST, #4691), p-AKT (P-308) (CST, #2965), p-AKT (S473) (CST, #4058), E-cadherin (CST, #3195), Fibronectin (CST, #26836), Vimentin (CST, #5741), were used according to manufacturer’s
instructions. Band intensity was normalized to β-actin and quantified by ImageJ software.

**Statistical analysis**
Data were presented as mean ± SD and p < 0.05 was considered statistically significant. Data were analyzed using one-way ANOVA with Tukey’s tests.

**Results**

**TMPRSS4 knockdown inhibited proliferation of endometrial cancer cells**
To investigate the expression of TMPRSS4 in endometrial cancer cells, a human normal endometrial stromal cell line (ESC) and four human endometrial cancer cell lines (AN3CA, HEC1A, HEC1-B and Ishikawa) were selected as *in vitro* tools. The level of TMPRSS4 gene was analyzed by qPCR and the results (Figure 1(A)) showed that the relative expression levels of TMPRSS4 in human endometrial cancer cells were significantly upregulated compared with ESCs. Besides, the expression of TMPRSS4 mRNA was higher in HEC1A and Ishikawa cells compared with another two cell lines, and thus HEC1A and Ishikawa were selected for the subsequent studies based on their endogenous TMPRSS4 levels. We further validated the mRNA expression level through the GEPIA (Gene Expression Profiling Interactive Analysis) database. TMPRSS4 was highly expressed in tumor tissues of uterine corpus endometrial carcinoma (UCEC) (Figure S1(B)). To assess the biological function of TMPRSS4 in endometrial cancer, we constructed the small interfering RNA (shRNA) (si#1 and #2) targeting TMPRSS4, and infected HEC1A and Ishikawa cells. Knockdown efficiency was verified by qRT-PCR (Figure 1(B)) and Western blot (Figure 1(C)). Both shRNAs could down-regulate TMPRSS4 mRNA and protein level in HEC1A and Ishikawa cells, and Sh#1 had higher efficiency for TMPRSS4 knockdown. Furthermore, the viability and colony formation of infected cells was examined by CCK8 and colony formation assay. The cellular activity of TMPRSS4-knockdown cells was significantly decreased compared with shRNA negative control cells (Figure 1(D)) (p < 0.01) in both cell lines. The colony formation results were in accordance with that of cellular activity (Figure 1(E)). The colony spots number in sh#1 knockdown cell lines was decreased over 75% compared with the shNC group, which indicated that cancer cell growth is positively related to TMPRSS4 expression. These results demonstrated that TMPRSS4 knockdown inhibited proliferation of endometrial cancer cells.

**TMPRSS4 regulated the migration and invasion of endometrial cancer cells**
The effects of TMPRSS4 on endometrial cancer cell migration and invasion were studied by wound scratch healing and transwell assays. The wound healing results (Figure 2(A)) showed that the wound width was increased after TMPRSS4 knockdown compared with negative control (p < 0.01), which indicated that the migration of Ishikawa cells was significantly reduced after TMPRSS4 knockdown. The transwell results (Figure 2(B)) showed that the invasion of cells was significantly decreased in TMPRSS4 knockdown cells (p < 0.01), and sh#1 sequence was more efficient for migration and invasion inhibition in HEC1A cells.

**TMPRSS4 regulated epithelial–mesenchymal transition (EMT) in endometrial cancer cells**
In addition, the effect of TMPRSS4 on EMT was investigated. The expression of EMT associated proteins (E-cadherin, fibronectin and vimentin) after TMPRSS4 knockdown was analyzed by Western blot. As shown in Figure 3, in HEC1A cells, the expression of E-cadherin was significantly enhanced after TMPRSS4 knockdown, while the levels of fibronectin and vimentin were decreased (p < 0.01). The effects were more obvious in sh#1 knockdown cells. The tendency of Ishikawa cells was in accordance with that of HEC1A. These results showed that TMPRSS4 knockdown attenuated the EMT of cancer cells.

**TMPRSS4 regulated the activation MAPK and AKT signaling pathways**
Given that mitogen-activated protein kinases (MAPK) and protein kinase B (PKB, i.e. Akt) are important protein kinases in EMT and endometrial cancer process, we investigated the effect of TMPRSS4 on MAPK and AKT signaling pathways in TMPRSS4-knockdown cancer cells. The results (Figure 4) showed that p38, ERK and AKT phosphorylation (P-308) were decreased after TMPRSS4 knockdown in both HEC1A and Ishikawa cells (p < 0.01). In addition, the levels of p-p38/p38, p-ERK/ERK and p-AKT (P308 and S473)/AKT were all increased in TMPRSS4-overexpressed cancer cells (Ishikawa) (p < 0.01 vs. vector) (Figure S1(A)). These results indicated that TMPRSS4 positively regulated the activation of MAPK and AKT signaling pathways in endometrial cancer.

**Discussion**

Transmembrane II transmembrane serine proteases 4 (TMPRSS4) is a newly discovered transmembrane
Recent studies have reported that TMPRSS4 is highly upregulated in several cancer types, including NSCLC, pancreas, breast, and esophageal cancer, and the upregulation is associated with poor prognosis (Villalba et al. 2019). However, the role of TMPRSS4 in endometrial cancer has not been reported yet, and this study mainly investigated the effects of TMPRSS4 on endometrial cancer in vitro. Our results showed that TMPRSS4 was highly expressed in HEC1A and Ishikawa cells among four human endometrial cancer cell lines (AN3CA, HEC1A, HEC1-B and Ishikawa). TMPRSS4 knockdown inhibited the proliferation of endometrial cancer cells. The similar results were found in human lung adenocarcinoma cells, where TMPRSS4 silencing induced the apoptosis of lung adenocarcinoma cells (NCI-H358 and A549) (Fan et al. 2018).

The migration and invasion of malignant cancer cells is a complicated process in the metastatic process (Ghasemi et al. 2019). The primary tumor cells infiltrate the adjacent tissues, invade the systemic
circulation, move into the distal blood capillaries and may finally permeate into the soft tissues to form a secondary tumor (Friedl and Wolf 2003). Previous reports showed that TMPRSS4 played an important role in invasion, migration and metastasis of human tumor cells. Jung et al. found that the cell invasion and proliferation were reduced in TMPRSS4-knockdown cells of lung and colon cancer in vitro. In contrast, the invasiveness and adhesiveness were significantly enhanced by TMPRSS4 overexpression (Jung et al. 2008a). The similar results were found in our research. Our results indicated that the invasion and migration of endometrial cancer cells significantly decreased in the TMPRSS4 knockdown cells, which

Figure 2. TMPRSS4 regulated the migration and invasion of endometrial cancer cells. (A) Wound healing assays showed the migration ability of cancer cells after TMPRSS4 knockdown. (B) The invasion of cancer cells after TMPRSS4 knockdown measured by transwell assays. **p < 0.01.
confirmed the role of TMPRSS4 in endometrial process. Epithelial–mesenchymal transition (EMT), an important step in the invasion and metastasis of cancer, is an important biological process in which epithelial cells loss polarity and gain mesenchymal features (Zhang and Weinberg 2018). EMT was characterized by the decreased expression of E-cadherin and increased expression of vimentin. TMPRSS4 has been found to be a positive regulator of EMT in HCC (Wang et al. 2015). In our present research, TMPRSS4 knockdown...
inhibited the EMT, indicated by increased E-cadherin and decreased fibronectin and vimentin. In the lung cancer cells, Jung et al. reported that overexpression of TMPRSS4 induced the loss of E-cadherin-mediated adhesion, and led to EMT events (Jung et al. 2008a). More importantly, in clinical hepatocellular carcinoma specimens, TMPRSS4 was significantly correlated with tumor staging and was negatively correlated with E-cadherin (Wang et al. 2015). Growing evidence has suggested that TMPRSS4 regulated EMT program in endometrial cancer. 

The MAPK and AKT pathways are two vital signaling in the EMT process. They are uncontrollably activated, leading to cell proliferation, migration, invasion in tumor cells, and finally inducing EMT. And there is crosstalk between MAPK and AKT signaling pathways. In human ovarian cancer cells, Galectin-1 could modulate EMT via the MAPK JNK/p38 signaling pathway. And treatment with the MAPK antagonists could reduce cancer metastasis (Zhu et al. 2019). AKT, a Serine/Threonine kinase, is overexpressed in many human tumors and induces EMT. Liang et al. found that TMPRSS4 and PI3K/AKT/mTOR mediated the STAT3-inhibited autophagy, which partly contributed to the metastasis and EMT in ovarian cancer cells (Liang et al. 2019). Kim et al. reported that TMPRSS4 potently upregulated integrin α5 expression thereby affecting the activation of FAK, Rac1 and ERK, which are the major downstream effectors of integrin (Kim et al. 2010). In the present research, we found that the phosphorylation of ERK, p38 and AKT was reduced significantly in TMPRSS4-knockdown cells, which was consistent with previous reports. These results confirmed that TMPRSS4 may promote EMT through MAPK and AKT signaling pathway.

Conclusion

In conclusion, this study demonstrated that TMPRSS4 may be associated with the progression of endometrial cancer through promoting proliferation, invasion and EMT via activation of MAPK and AKT. Our research indicates an important role of TMPRSS4 in the pathogenesis and development of endometrial cancer. TMPRSS4 may be a new and more effective target or therapeutic strategy for treating endometrial cancer.

Acknowledgements

Huan Xiao and Zhian Zhang designed the study, supervised the data collection, Dan Peng analyzed the data, interpreted the data, Chunqing Wei and Benling Ma prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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