Preexpression of TM4SF1 improves cell metastasis and growth by activating ERK1/2 signaling pathway in human prostate cancer

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Summary

Purpose: To explore the effects of Transmembrane-4-L-six-family-1 (TM4SF1) in prostate cancer (PCa), and the related underlying mechanisms.

Methods: PCa tissues were obtained from 78 patients. PCa cell lines DU145 and RWPE-2 were purchased from American Type Culture Collection (ATCC). Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were conducted to analyze the expression of TM4SF1 in PCa tissues and DU145 cells. Plasmid containing over-expressed TM4SF1 was achieved by plasmid transfection. Transwell assay and wound-healing assay were designed to examine the invasion and migration of DU145 cells, whereas colony formation assay and 5-Ethynyl-2'-deoxyuridine (EdU) staining assay were performed to study the proliferation ability of DU145 cells.

Results: TM4SF1 was found over-expressed in PCa tissues and DU145 cells. Over-expression of TM4SF1 significantly activated the extracellular regulated protein kinases (ERK)1/2 signaling pathway, increased the epithelial-mesenchymal transition (EMT) expression, and enhanced the invasion, migration and proliferation of DU145 cells. Further studies revealed that suppression of ERK1/2 signaling pathway nearly resisted the positive effects on DU145 cells induced by TM4SF1 over-expression.

Conclusions: The present study demonstrated that TM4SF1 enhanced the invasion, migration and proliferation of DU145 cells by activating ERK1/2 signaling pathway.

Key words: prostate cancer, Transmembrane-4-L-six-family-1, ERK1/2

Introduction

Prostate cancer (PCa) is one of the most common malignant tumors among elderly men worldwide. Developing countries have seen an increasing incidence rate year by year [1]. At present, local radical prostatectomy has emerged as the predominant treatment resulting in a postoperative 5-year survival rate of about 90%. That being said, over 60% of patients with PCa may have no possibility to be radically cured due to aging and the decline in body functions [2,3]. In China, about 80% of PCa patients have been exposed to androgen deprivation therapy but the results were not satisfying [4]. To date, though there are specific and clear treatment strategies, PCa still shows a considerable recurrence rate [5]. In this connection, exploring new biomarkers and therapeutic targets which are conducive to the diagnosis and prognosis of PCa becomes a hot spot in current studies.

Transmembrane-4-L-six-family-1 (TM4SF1), initially found as an antigen, belongs to the TM4SF family including TM4SF4, TM4SF5, TM4SF18, TM4SF19, TM4SF20 [6]. Over the past years,
TM4SF1 was reported to highly expressed in many epithelial tumors such as breast cancer [7], pancreatic cancer [8,9] and ovarian cancer [10], and could promote their metastasis and invasion. Up to now, there have been few reports on PCa and TM4SF1. Therefore, it is particularly important to explore the molecular role of TM4SF1 in PCa and to elucidate its underlying mechanisms.

In the present study, we detected TM4SF1 expression in PCa tissues and adjacent tissues, as well as DU145 and RWPE-2 cell lines. Furthermore, the role of TM4SF1 in PCa cell invasion and proliferation was evaluated. The purpose of this study was to investigate the effects of TM4SF1 on PCa cell invasion and proliferation, and to illustrate the molecular mechanism of TM4SF1 in PCa metastasis.

**Methods**

**Tissues**

All tissue specimens were obtained from patients diagnosed with PCa at our hospital. No patient was treated with endocrine therapy, radiotherapy or chemotherapy before the operation. Performing of tissue specimens is approved by the Ethics Committee, and the relevant agreement was signed with each patient.

**Cells and culture**

PCa cell lines DU145 and normal prostate cell lines RWPE-2 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). DU145 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Hyclone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). 100 U/mL penicillin and 50 µg/mL streptomycin. RWPE-2 was cultured in a keratinocyte serum-free medium with 50 mg/mL bovine pituitary extract and 5 ng/mL recombinant human epithelial growth factor in an incubator with 5% CO₂ at 37°C. When the cell confluence reached 80-90%, the original medium was removed, and cells were washed with phosphate buffer saline (PBS) and completely digested with 1 mL of 0.25% trypsin (Beyotime, Shanghai, China) for 2-3 min. Then, the complete culture solution was added to stop the digestion process as soon as the cells turned around and fell off. The cell suspension was shaken up and aspirated into a centrifuge tube for centrifugation at 1,000 rpm for 5 min. The supernatant was discarded, and complete RPMI-1640 medium was added to resuspend the cells. After that, the cells were planted on a new culture dish, and the culture was continued.

**Cell transfection**

DU145 cells were seeded into 6-well plates and incubated overnight. Then, plasmid containing TM4SF1, together with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were added into the plates based on the manufacturer's instructions. After 48-h incubation, the transwell assay, colony formation assay and 5-Ethynyl-2’-deoxyuridine (EdU) staining assay were performed.

**Transwell assay**

A total of 100 µL of Matrigel (diluted with serum-free culture solution at 1:8 and stored at 4°C after subpackage) was added to the upper chamber of the transwell chambers and incubated at 37°C for 1 h. Then, 600 µL of the RPMI-1640 medium containing 10% FBS were added to each well, and the cells were resuspended using serum-free RPMI-1640 medium. Three wells (5,000 cells/well) were set for each group. The transwell plate was taken out 24 h later, washed with PBS 3 times, then dropped with paraformaldehyde and fixed at room temperature for 20 min. Next, 1% crystal violet solution was added and kept for 5 min. The chamber was taken out and the cells attached to the filter membrane were wiped off with cotton swabs. Finally, these cells were observed under a microscope.

**Wound healing assay**

After transfected for 48 h, DU145 cells were digested and seeded into 24-well plates, and then incubated at 37°C, 5% CO₂ incubator overnight. Twenty-four h later, a 10 µL tip was used for scratching. Thereafter, DU145 cells were washed with PBS and cultured with complete medium. Finally, the plate was observed and photographed after 24 h under a microscope.

**Colony formation assay**

The proliferation potential of PCa cell lines DU145 was measured using the colony formation assay, and the number of clones formed by DU145 cells was measured as the proliferation ability. Briefly, a total of 1×10³ cells were seeded in a 6-well plate and incubated at a humidified incubator containing 37°C, 5% CO₂, with the medium changed every 3 days. Two weeks later, the number of clones was counted under a microscope.

**EdU staining assay**

A total of 1×10³ DU145 cells were planted into 6-well plates. After removing the medium, the EdU solution was added into the plates and incubated for 4 h. Subsequently, cells were fixed with 1% paraformaldehyde for 15 min and washed with PBS three times. Then, cells on the plates were stained with 4’,6-diamidino-2-phenylindole (DAPI) for 5 min, and finally observed under an inverted fluorescence microscope.

**Quantitative real-time polymerase chain reaction (qRT-PCR) analysis**

RNA in cell samples were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) agent. After measuring the concentration of Nanodrop, 1,000 ng of RNA was reversely transcribed into complementary DNAs (cDNAs). Later, 1 µL of cDNA was used for detection of mRNA expression level on Step OnePlus instrument with the following reaction conditions: pre-denaturation at 95°C for 30 s and then 95°C for 5 s and 60°C for 30 s.
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for 40 cycles, followed by dissociation stage. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference and relative quantity value was applied for analysis based on the Cycle threshold (Ct) value of the samples. The $2^{-\Delta\Delta C_t}$ method was utilized to quantify target genes expression relative to control group.

**Western blot analysis**

After washing with pre-cooled PBS three times, the cells were lysed with 300 μL of radio immunoprecipitation assay (RIPA) buffer containing 1% phenylmethylsulfonfyl fluoride (PMSF) for 50 min and centrifuged at 12,000 rpm for 5 min (Beyotime, Shanghai, China). The supernatant was collected into a 1.5 mL centrifuge tube, and the protein concentration was detected via the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. A total of 40 μg of protein was taken for dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) for 2 h and then transferred onto a membrane. After that, the membrane was rinsed with Tris-buffered saline-Tween 20 (TBST) for 5 min and sealed with 5% skim milk powder at 4°C overnight. The primary and secondary antibodies were diluted with TBST respectively. The primary antibody was incubated at 4°C overnight. The secondary antibody was incubated at room temperature for 1 h. The membrane was washed with TBST three times after incubation. Finally, a proper amount of enhanced chemiluminescence (ECL) solution A and B with equal volume were added to the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and imaged by the chemi-luminescent gel imaging system.

**Statistics**

The data in this study were analyzed using GraphPad Prism (La Jolla, CA, USA) 7.03 software. The t-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student’s t-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). P<0.05 showed statistical significance.

**Results**

**TM4SF1 expression in PCa cell lines was higher than normal prostate cell line RWPE-2**

To validate the relationship between TM4SF1 and PCa, we performed qRT-PCR and Western blot to detect the mRNA and protein levels in PCa tissues and adjacent tissues, as well as cell lines DU145 and RWPE-2. RT-PCR results showed that the mRNA expression of TM4SF1 in PCa tissues was significantly higher than that in adjacent tissues (Figure 1A). In addition, both the mRNA and protein expression levels of DU145 cells were significantly increased compared to RWPE-2 cells (Figure 1B,1C).

**Figure 1.** TM4SF1 was up-regulated in PCa tissues and cells. **(A):** Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of TM4SF1 expression in PCa tissues and corresponding adjacent normal tissues. **(B):** QRT-PCR analysis of TM4SF1 expression in DU145 cells and RWPE-2 cells. **(C):** Western blot analysis of TM4SF1 expression in DU145 cells and RWPE-2 cells (**p<0.01).
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**Figure 2.** Over-expression of TM4SF1 activated the ERK1/2 signaling pathway. (A): Western blot analysis of p-ERK1/2 and ERK1/2 expression in normal DU145 cells and TM4SF1 plasmid transfected DU145 cells. (B): Qualification of the ratio of p-ERK1/2 / ERK1/2 protein expression level. (C): Western blot analysis of E-cadherin, V-cadherin and Vimentin expression in NC group cells and NC+TM4SF1 group cells. (D): Qualification of the related protein expression level (*p<0.05, **p<0.01).

**Figure 3.** Inhibition of ERK1/2 signaling pathway resist the enhanced invasion and migration ability of DU145 cells. (A): Number of invasive cells among NC group, NC+TM4SF1 group and NC+TM4SF1+U0126 group (magnification: 10×). (B): The wound healing rate among NC group, NC+TM4SF1 group and NC+TM4SF1+U0126 group (magnification: 4×) (*p<0.05, **p<0.01, ns: no statistical differences).
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ERK1/2 signaling pathway was confirmed to play an important role in certain cancers by regulating cell proliferation, cell physiology, and biochemical status, for example ovarian cancer [11,12], testicular cancer [13] and gastric cancer [14]. We therefore detected the influence of TM4SF1 on the ERK1/2 signaling pathway. Two groups were set, negative control (NC) group (DU145 cells) and NC+TM4SF1 group (DU145 cells treated with TM4SF1 plasmid). After successfully over-expressed TM4SF1 in DU145 cells (data not shown), we observed an increased p-ERK1/2 / ERK1/2 ratio in NC+TM4SF1 group compared to NC group (Figure 2A,2B). Besides, the expression of epithelial-mesenchymal transition (EMT)-related proteins, which responded to tumor invasion and migration, including E-cadherin, V-cadherin and Vimentin were also up-regulated in DU145 cells with TM4SF1 treatment than normal DU145 cells (Figure 2C, 2D).

Effects of TM4SF1 on the invasion and migration of DU145 cells

Combining the results in Figure 2B in order to further investigate the effects of TM4SF1 on the migration and invasion of DU148 cells, we performed transwell assay and wound-healing assay. DU145 cells were divided into three groups: normal DU145 cells (NC group), DU145 cells treated with TM4SF1 plasmid (NC+TM4SF1 group), DU145 cells treated with TM4SF1 plasmid and U0126 (NC+TM4SF1+U0126 group). Results in Figure 3A showed that the number of invasive cells in the NC+TM4SF1 group was about 155.5±5.173, which was statistically higher compared to 95.66±6.288 invasive cells in the NC group. However, when TM4SF1-overexpressing DU145 cells were added to U0126, an ERK1/2 signaling pathway inhibitor, the enhanced invasion ability was significantly reduced, with 102.3±3.527 invasive cells in NC+TM4SF1+U0126 group in contrast to 155.5±5.173 invasive cells in NC+TM4SF1 group (p<0.01). No differences were found between NC group and NC+TM4SF1+U0126 group. In addition, the wound healing assay showed that the improved migration of DU145 cells induced by TM4SF1 was also decreased by co-treatment with U0126, with 29.84±2.722% in normal DU145 cells, 49.83±2.186% in DU145 cells treated with TM4SF1 plasmid and 31.22±2.349% in DU145 cells treated with TM4SF1 plasmid and U0126 (Figure 3B).

Effects of TM4SF1 on the proliferation of DU145 cells

To investigate the effects of TM4SF1 on the proliferation of DU145 cells, the colony formation assay and EdU staining assay were conducted based on the above three groups. Figure 4A shows that the colony numbers in NC+TM4SF1 group was about 140.2±8.492, dramatically elevated...
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The former. At the same time, we detected that the results showed that TM4SF1 was highly expressed in and the corresponding adjacent tissues, and the regression and metastasis of PCa. First, we compared the expression levels of TM4SF1 in PCa tissues and the corresponding adjacent tissues, and the results showed that TM4SF1 was highly expressed in the former. At the same time, we detected that the mRNA and protein expression levels of TM4SF1 were significantly higher in DU145 cells than in RWPE-2 cells, indicating a strong correlation between TM4SF1 and PCa. Subsequently, to further explore the role of TM4SF1 in PCa, we transfected plasmid containing TM4SF1 in DU145 cells. The results showed that after the high expression of TM4SF1 in DU145 cells, not only the ERK1/2 signaling pathway was activated, but also the expression level of EMT was up-regulated.

It is worth noting that the ERK1/2 signaling pathway has been reported to be involved in many tumors and was widely involved in their pathological and physiological processes. For example, Xue et al [23] have found that knockdown of suppressor of zest 12 (SUZ12) could activate the ERK1/2 pathway and significantly accelerate the invasion and metastasis of HCC cells. Jafari et al [24] have confirmed that A2B adenosine receptor (A2BAR) could induce breast cancer stem cells cycle arrest and apoptosis through inhibiting the ERK1/2 signaling pathway. In addition, it has been reported that the expression of EMT plays an important role as a marker molecule in tumor invasion and migration, and the up-regulation of EMT can be detected in many tissue samples of patients with poor prognosis [25-27].

Taking these into consideration, we then examined the invasion and migration ability of DU145 cells under different treatments through transwell assay and wound-healing assay. Consistent with previous studies [7,28], over-expression TM4SF1 in DU145 cells not only enhanced the invasion ability, but also the migration ability was improved. Since TM4SF1 could activate the ERK1/2 signaling pathway, we thus co-treated DU145 cells with U0126, an ERK1/2 signaling pathway inhibitor [29], to illustrate the underlying mechanisms. We found that the enhanced invasion and migration in DU145 cells induced by TM4SF1 was almost inhibited by U0126, suggesting that TM4SF1 enhanced the invasion and migration ability of DU145 cells by activating the ERK1/2 signaling pathway.

Furthermore, we performed colony formation assay and EdU staining assay to study the proliferation ability of DU145 cells in different groups. Similar to what we found in transwell assay and wound-healing assay, DU145 cells in NC + TM4SF1 group showed significant higher colony numbers and EdU staining positive cells compared to NC group, respectively. However, no statistically differences were found between DU145 cells in NC group and NC + TM4SF1 + U0126 group, indicating the positive effects of TM4SF1 on DU145 cells were nearly inhibited by suppression of ERK1/2 signaling pathway.

Discussion

Currently, the treatment methods of PCa mainly include surgery, endocrinotherapy, chemotherapy and radiotherapy [15]. As the onset of PCa is insidious and slow, a large proportion of patients show no obvious symptoms in the early stage and thus tend to be diagnosed in a late stage [16]. The diagnosis and treatment of PCa have been remarkably advanced in recent years, but the long-term prognosis of patients with PCa remains unfavorable due to relapse, metastasis and androgen resistance [2,3]. Studies have found that various genes and proteins are involved in the pathogenesis, progression and metastasis of PCa at the molecular level, but the mechanisms are not clear yet [17,18]. Therefore, further exploration and study of new sensitive tumor markers to enhance the detection rate of PCa and the survival rate of the patients has raised great concern.

TM4SF1 was originally found in many human epithelial malignant tumors as a tumor-associated antigen, which is located on chromosome 3 (3q21-3q25) [19]. It was confirmed that TM4SF1 could participate in different life activities of cells, as well as the occurrence and development of tumors. For instance, related functional studies have found that TM4SF1 could promote tumor cell migration through recruitment to TERM region and relevant ubiquitination modification [20]. Besides, TM4SF1 could affect cell movement and invasion by interacting with the cluster of differentiation 13 (CD13) and regulating expression of transmembrane proteins CD63, CD82 and so on [21]. Studies on tumors have pointed out that TM4SF1 was highly expressed in various tumors and closely related to the invasion and metastasis of tumor cells as well as the prognosis and survival of patients [8,22].

In this article, we mainly explored the relationship between TM4SF1 and PCa. First, we compared the expression levels of TM4SF1 in PCa tissues and the corresponding adjacent tissues, and the results showed that TM4SF1 was highly expressed in the former. At the same time, we detected that the number of colony formation in NC group was 129.5 ± 3.95, while the colony numbers reduced to 89.59 ± 3.79, showing much fewer than NC + TM4SF1 group but no differences compared to NC group, respectively. Moreover, as shown in Figure 4B, the percent of EdU staining positive cells in the NC + TM4SF1 group was also significantly higher than in the NC group, while the ERK1/2 signaling pathway inhibitor U0126 could counteract the favorable effects induced by TM4SF1.

Over-expression of TM4SF1 improves prostate cancer than 85.45 ± 3.261 colony numbers in NC group. However, after treatment with U0126, the colony numbers reduced to 89.59 ± 3.79, showing much fewer than NC + TM4SF1 group but no differences compared to NC group, respectively. Moreover, as shown in Figure 4B, the percent of EdU staining positive cells in the NC + TM4SF1 group was also significantly higher than in the NC group, while the ERK1/2 signaling pathway inhibitor U0126 could counteract the favorable effects induced by TM4SF1.
Conclusions

To sum up, TM4SF1 was significantly over-expressed both in PCa tissues and cell lines. Further study revealed that TM4SF1 enhanced the invasion, migration and proliferation by activating ERK1/2 signaling pathway. Thus, our results indicated that TM4SF1 might serve as a potential biomarker for the diagnosis and treatment of PCa.

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Conflict of interests

The authors declare no conflict of interests.

References

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