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ORIGINAL ARTICLE

TMPRSS4 Upregulates TWIST1 Expression through STAT3 Activation to Induce Prostate Cancer Cell Migration

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Abstract Transmembrane protease serine 4 (TMPRSS4), a type-II transmembrane serine protease, is involved in the development and progression of wide range of tumors. However, the biological role of TMPRSS4 in prostate cancer remains obscure. Here, we investigated the effect of TMPRSS4 on proliferation and migration in prostate cancer and potential mechanisms. Our findings demonstrated over-expression of TMPRSS4 promoted the PC3 prostate cancer cells migration, which could be reversed by TMPRSS4 silencing. TMPRSS4 induced TWIST1 expression and followed progression of EMT along with upregulation of N-cadherin and downregulation of E-cadherin via STAT3 phosphorylation. Silencing TWIST1 significantly attenuated TMPRSS4-induced PC3 migration. Moreover, knockdown of STAT3 effectively attenuated TMPRSS4-induced TWIST1 expression and TWIST1 promoter activity. Taken together, we demonstrated a mechanistic cascade of TMPRSS4 up-regulating STAT3 activation and subsequent TWIST1 expression, leading to prostate cancer migration.

Keywords Prostate cancer · Transmembrane protease serine 4 · Epithelial-mesenchymal transition · Migration · Signal transducer and activator of transcription 3 · TWIST1

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Introduction

Prostate cancer (PCa) is the most frequently diagnosed tumor in old men and also the second leading cause of male cancer death in the Western countries [1]. In addition, the incidence and mortality of PCa have been increasing recently in China. Although radical prostatectomy and radiation therapy remain the primary choice for localized PCa, there is no effective treatment for patients who develop into metastatic disease. Due to the growth and metastasis of prostate cancer are associated with molecular genetic mutations, identification of critical genes and therapeutic targets is urgently needed for treatment modalities.

Transmembrane protease serine 4 (TMPRSS4), a type-II transmembrane serine protease, is frequently overexpressed in various human cancers, such as non-small-cell lung cancer, breast cancer, cervical cancer, gastric cancer, and colon cancer [2, 3]. Increased TMPRSS4 expression plays a pivotal role in processes of cancer progression, including angiogenesis, invasion and metastasis [4], which indicates TMPRSS4 could act as a potential therapeutic target in human malignancies [5]. In human gallbladder cancer, high expression of TMPRSS4 is correlated with poor prognosis [6]. Similarly, increased TMPRSS4 expression has been found to be associated with colorectal cancer stage progression [7]. It has been reported that TMPRSS4 silencing in lung cancer cells lead to a significant impairment of metastasis in animal models [8]. In-vitro cellular studies demonstrated that TMPRSS4 overexpression promoted the invasiveness, motility and adhesiveness of colon cancer cells, whereas a knockdown strategy to reduce TMPRSS4 levels resulted in inhibited cell invasion and proliferation in lung and colon cancer cells [9]. Studies showed that TMPRSS4-induced invasion of colon and lung cancer cells involved activation of multiple signaling pathways including focal adhesion kinase (FAK), extracellular signal-

regulated kinase (ERK) and Protein kinase B (PKB), resulting in epithelial-to-mesenchymal transition (EMT) of cancer cells [10]. A recent study showed that prostate cancer patients with tumors that highly expressed TMPRSS4 had a significantly worse disease-free survival than the remaining patients [11, 12]. However, the biological roles of TMPRSS4 in prostate cancer progression remain unclear.

TWIST1, a highly conserved basic helix-loop-helix transcription factor, is responsible for many cancer cells migration [13]. It has been found TWIST1 plays a critical role in promoting the development and progression of malignant tumor by inducing epithelial mesenchymal transition (EMT) [14]. Congruently, TWIST1 is highly expressed in prostate cancer cells and regulates PCa cell metastasis [15]. Signal transducer and activator of transcription 3 (STAT3) has been detected in a variety of human tumors, including PCa. Activated STAT3 is often correlated with tumor invasion and metastasis [16]. In this study, we demonstrated TMPRSS4 increased TWIST1 expression through STAT3 activation to induce prostate cancer cell migration.

Materials and Methods

Cells Culture

PC3 cells were obtained from China Center for Type Culture Collection (Wuhan, China) and cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 mg/L penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Expression vector, small interfering RNA (siRNA) and transfection.

A cDNA encoding full-length human TMPRSS4 (GenBank accession number: NM_019894.3) was purchased from Sino Biological Inc. (Beijing, China) and subcloned into the expression vector pcDNA3.1 (p-TMPRSS4) (Invitrogen, shanghai, China). A TWIST1 luciferase reporter vector pGL3-TWIST1 was kindly provided by Dr. Hung MC (Molecular and Cellular Oncology, Anderson Cancer Center, Houston, TX). A pRL-TK plasmid expressing Renilla luciferase was purchased from Promega Corporation (Promega, WI, USA). TMPRSS4-targeting siRNA (si-TMPRSS4), TWIST1-targeting siRNA (si-TMPRSS4), STST3-targeting siRNA (si-STST3) and negative control siRNA (c-siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cells were transfected with indicated expression vector or siRNA using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. 24 h after transfection, cells were collected and subjected to analysis.

RT-PCR (Real-Time Quantitative RT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen, CA, USA), and single-stranded cDNA was generated with the cDNA synthesis kit (Takara, Kyoto, Japan) according to standard protocols. Real-time PCR was performed using SYBR Green master mix kit (Applied Biosystems, CA, USA). Human GAPDH gene was used as endogenous control. The PCR primer sequences were as follows: TMPRSS4, 5′-CCGA TGTGTTCAACTGGAAG-3′ and 5′- CCCATCCAATGATC CAGAGT-3′; GAPDH, 5′-TCAACGACCACTTTGTCAAG CTCA-3′ and 5′-GCTGGTGGTCCAGGGGTCTTACT-3′. All reactions were performed in triplicate. Data were analyzed using the $2^{-\Delta\Delta Ct}$.

Western Blot

Cell lysates were prepared. Total cellular proteins (50 μg) were subjected to SDS-PAGE, and transferred to nitrocellulose membranes (Amersham, USA). Specific polyclonal antibodies against TMPRSS4, STAT3, p-STAT3, TWIST1, N-cadherin and E-cadherin (Cell Signaling Technology, MA, USA) diluted in TBS-T containing 5% nonfat milk were used to detect indicated proteins. The appropriate horseradish peroxidase (HRP) conjugated IgG was used as secondary antibody. Antibody on membrane was visualized by enhanced chemiluminescence (Pierce Biotechnology, IL, USA). Western blot for β-actin was used as an internal sample.

Luciferase Assay

A TWIST1 luciferase reporter vector pGL3-TWIST1 [17] was kindly provided from Dr. Hung MC (Molecular and Cellular Oncology, Anderson Cancer Center, Houston, TX). A pRL-TK plasmid expressing Renilla luciferase (Promega, WI, USA) was used as an internal control. Cells were seeded in 24-well plates and transiently transfected with TMPRSS4-expressing plasmid, STAT3 siRNA and TWIST1 reporter plasmids pGL3-TWIST1 utilizing Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 24 h of transfection, cells were collected and tested for luciferase activity using the Dual–Luciferase reporter assay system (Promega, WI, USA).

Cell Proliferation Assay

The cellular proliferation was measured by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) assay. Briefly, cells were transplanted in 96-well-plates at a density of 1×10^5 per well. 10 μl CCK-8 resolution was added to each well of 100 μl medium. Absorbance was measured at 450 nm on automatic ELISA reader (TRITURUS). All determinations were carried out in triplicate and repeated three times.

Cell Migration Assay

The ability of cells in vitro migration assays were performed in Transwell chambers (Corning, NY, USA) following the manufacturer's instructions. Briefly, 5×10^5 cells were cultured in serum-free medium on 8 μm porous polycarbonate membranes in the upper chambers. The lower chambers were added with RPMI-1640 medium containing 10% FBS. After incubation for 24 h, non-invading cells remaining on the upper surface of the filter were removed, and the cells that migrated to the underside of the membrane were fixed with 4% paraformaldehyde and stained with Giemsa (Sigma, MO, USA). Cells were calculated in 10 randomly selected fields under 200× magnification and expressed as the average number of cells/field of view. These data were represented as the average of the three independent experiments.

Histology and Immunohistochemistry

Human samples were obtained with informed consent from the first affiliated hospital of Zhengzhou University, and the study was approved by the Institutional Review Board of first affiliated hospital of Zhengzhou University. Immunohistochemical staining was done according to the published data [18]. Formalin-fixed, paraffin-embedded tissue blocks were cut into 4 μm thick sections and mounted on glass slides. Sections were deparaffinized in xylene and rehydrated. Specific polyclonal antibody against TMPRSS4 or TWIST1 diluted in 1% phosphate buffered saline/bovine serum albumin (1% PBS-BSA) at 1:50 were used for incubation overnight. Next, sections were incubated with biotin-labeled IgG for 1 h at room temperature. Then, sections were stained by a streptavidin-peroxidase detection system (Dako, Copenhagen, Denmark). Negative control reactions replaced the primary specific antibody by PBS. Immunohistochemical evaluation was performed by two independent observers blinded as to the intensity of the samples. Staining intensity was graded according to the following scale: 0, negative; 1+, weak; 2+, moderate and 3+, strong staining. Staining extent (percentage of stained cells) was scored into four categories: 1, 0% to 10%; 2, 11% to 25%; 3, 26% to 50%; and 4, 51% to 100%. The staining score was defined as the product of staining intensity and staining extent [19].

Statistical Analysis

All data were presented as mean ± standard deviation. Differences were considered statistically significant for $P < 0.05$ as determined by ANOVA, student's t-test or spearman's rank correlation test using SPSS13.0.

Results

TMPRSS4 Silencing Reduced the Growth and Migration of Prostate Cancer Cells

PC3 prostate cancer cells were transfected with TWIST1-specific siRNA. qRT-PCR and Western blot showed TMPRSS4 expression was reduced at both mRNA and protein levels (Fig. 1a, b). Silencing TMPRSS4 suppressed PC3 growth and migration (Fig. 1c, d).

TMPRSS4 Overexpression Promoted the Progression of Prostate Cancer Cells

PC3 prostate cancer cells were transfected with TMPRSS4-indicated expression vector p-TMPRSS4. Western blot showed TMPRSS4 expression was enhanced (Fig. 2a). Enhanced expression of TMPRSS4 promoted PC3 growth and migration (Fig. 2b, c).

TWIST1 was Critical for TMPRSS4-Induced Prostate Cancer Cells Migration

Western blot showed upregulation of TMPRSS4 expression by p-TMPRSS4 efficiently increased TWIST1 expression (Fig. 3a). While silencing TWIST1 by TWIST1-specific siRNA, the PC3 migration improved by enhanced expression of TMPRSS4 was significantly attenuated (Fig. 3b). More importantly, PC3 EMT factors induced by enhancing TMPRSS4 expression were reversed with increase of E-cadherin and decrease of N-cadherin (Fig. 3c).

STAT3 was Required for TMPRSS4-Induced TWIST1 Expression and Prostate Cancer Cell Migration

To explore how TMPRSS4 induces TWIST1 expression, we tested whether STAT3 is involved in TMPRSS4-mediated signaling events. PC3 prostate cancer cells were transfected with TMPRSS4-indicated expression vector. Western blot showed enhanced expression of TMPRSS4 increased phosphorylation level of STAT3 (Fig. 4a). However, silencing STAT3 by STAT3-specific siRNA strongly attenuated TMPRSS4-induced TWIST1 expression (Fig. 4b). Luciferase reporter assay confirmed that TMPRSS4-induced TWIST1 promoter activity was dramatically reduced when the expression of STAT3 was silenced (Fig. 4c). Moreover, the PC3 migration was inhibited by si-STAT3 (Fig. 4d).

TMPRSS4 Expression Correlated with TWIST1 Expression in Human Prostate Cancer

To detect whether TMPRSS4 expression correlates with TWIST1 expression in prostate cancer, immunohistochemical

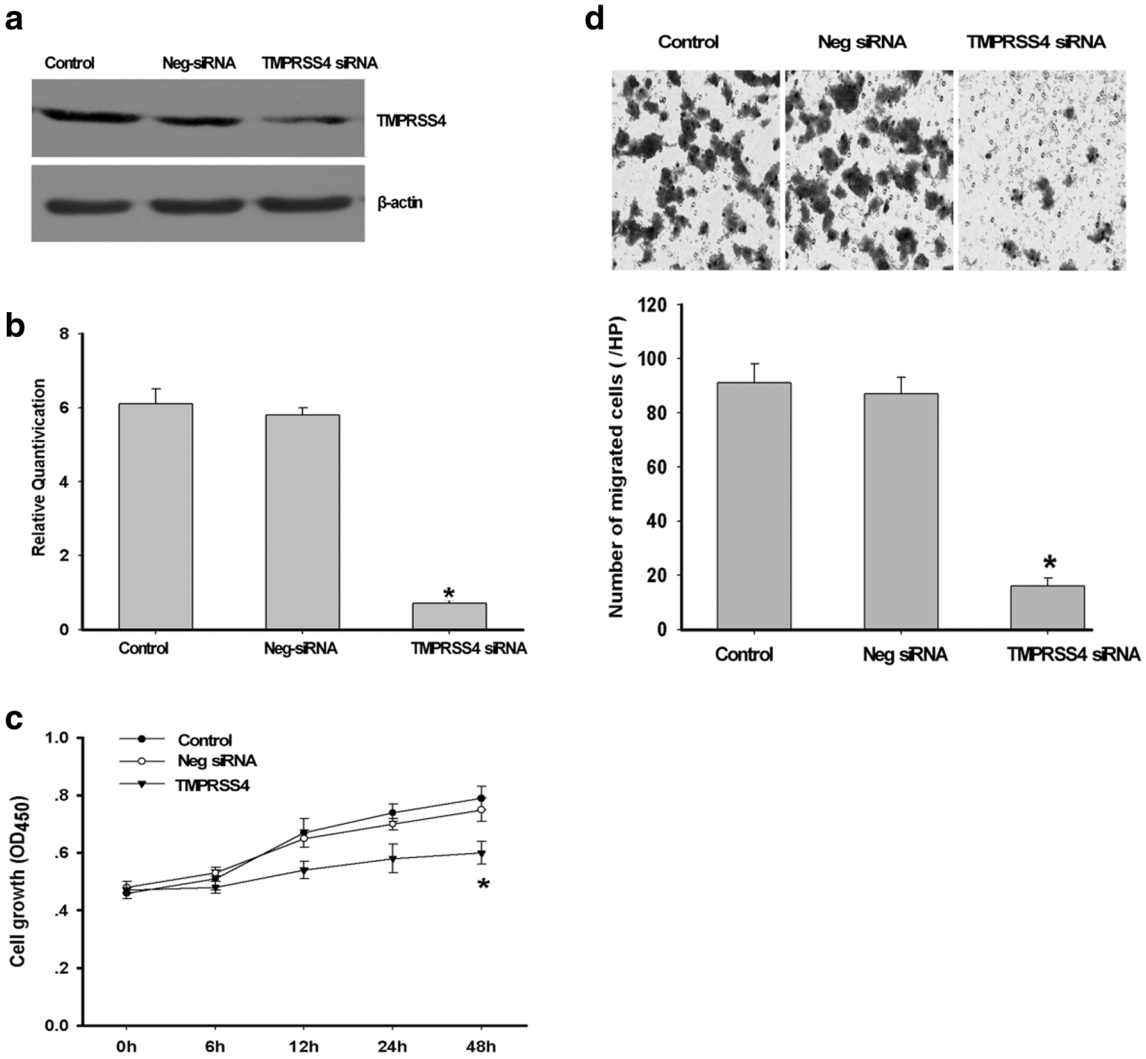

Fig. 1 TMPRSS4 silencing reduced the growth and migration of prostate cancer cells. PC3 prostate cancer cells were transfected with negative siRNA (neg siRNA) or TMPRSS4-specific siRNA (TMPRSS4 siRNA). Non-transfected cells were used as control. **a** Western blot was used to detect TMPRSS4 exptession. **b** TMPRSS4 mRNA level was analyzed by real-time quantitative PCR (*$P < 0.05$). (**c**) The cells' proliferation activity was tested by CCK-8 assay, data presented as the mean ± SD of 3 independent experiments, each performed in triplicate (*$P < 0.05$). (D) Cells migration was observed by transwell chambers (magnification 200×) and quantification of migrated cells were calculated, data presented as the mean ± SD of 3 independent experiments (*$P < 0.05$)

staining of the human prostate cancer tissues with both the anti-TMPRSS4 and TWIST1 antibodies was performed. Both TMPRSS4 and TWIST1 were highly expressed in prostate cancer, especially in those metastatic cancers (Fig. 5). Spearman's rank correlation test showed a significant association between expression of TMPRSS4 and TWIST1 (Table 1, $P < 0.01$).

Discussion

TMPRSS4 expression is closely implicated with tumorigenesis and development of multiple cancers. In human colorectal carcinoma, increased expression of TMPRSS4 is correlated with progression and poor prognosis [20]. It has been demonstrated that TMPRSS4 knockdown inhibits the proliferation of lung cancer cells, and similarly, knockdown of TMPRSS4 has been reported to decrease the proliferation of HCT116 colon cancer cells [7, 21]. In the present study, we also observed that prostate cancer tissue highly expressed TMPRSS4, which correlated with prostate cancer migration. Silencing TMPRSS4 effectively inhibited prostate cancer cells growth and migration. In contrast, ectopic expression of TMPRSS4 enhanced the growth and migration of prostate cancer cells. Moreover, enhanced expression of TMPRSS4 promoted the occurrence of EMT with downregulated E-cadherin expression and upregulated N-cadherin. These results demonstrated the importance of TMPRSS4 in the regulation of prostate cancer cell progression.

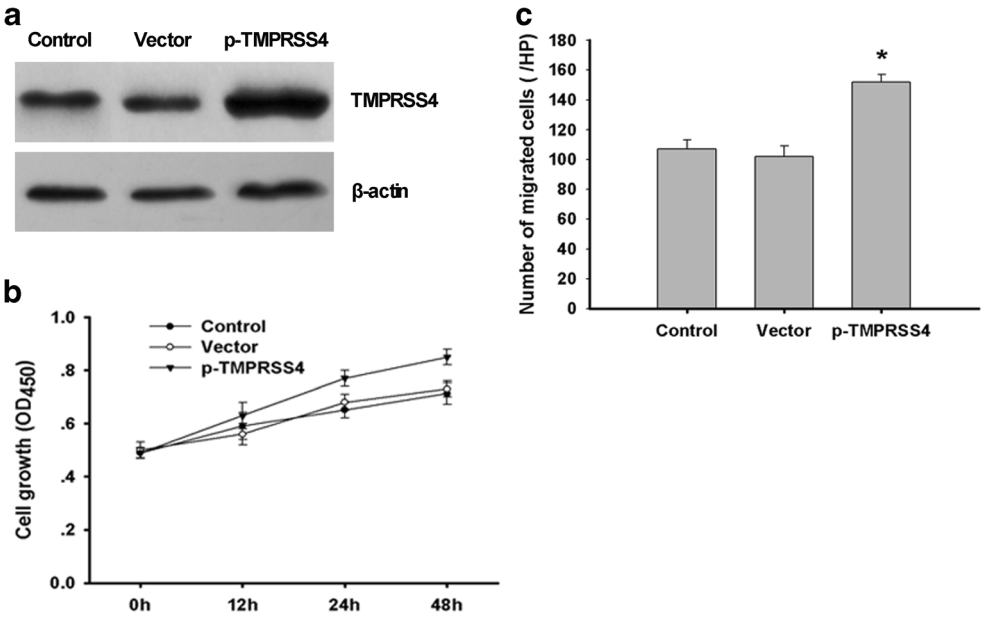

Fig. 2 TMPRSS4 overexpression promoted the progression of prostate cancer cells. PC3 prostate cancer cells were transfected with empty vector or TMPRSS4 expression vector (p-TMPRSS4). Non-transfected cells were used as control. **a** Western blot was used to detect TMPRSS4 exptession. **b** The cells' proliferation activity was tested by CCK-8 assay, data presented as the mean ± SD of 3 independent experiments, each performed in triplicate (*$P < 0.05$). **c** Cells migration was observed by transwell chambers, data presented as the mean ± SD of 3 independent experiments (*$P < 0.05$)

Previous studies have shown that TMPRSS4 downregulated E-cadherin expression by inducing the transcriptional repressor SIP1/ZEB2 in colon cancer cell lines, integrin alpha5 expression and activation of FAK and ERK signaling pathway in colorectal cancer cells [9, 10]. Here, we observed that TWIST1 was involved in TMPRSS4-mediated EMT. Our data revealed that TMPRSS4 overexpression significantly increased the expression of TWIST1 in prostate cancer cells. Upregulation of TWIST1 contributed to the EMT and metastasis of prostate cancer cells [22]. TWIST1 silencing significantly suppressed the migration of TMPRSS4-overexpressing prostate cancer cells. These data suggested that upregulation of TWIST1 might represent a novel mechanism by which TMPRSS4 facilitated prostate cancer cells EMT and migration.

STAT3 has been reported to induce a TWIST1-dependent EMT in breast and bladder cancer [23]. To understand how TMPRSS4 regulated TWIST1 on prostate cancer cells EMT and migration, we detected STAT3 further. Indeed, we observed a critical role of STAT3 in TWIST1 expression and prostate cancer cells progression. Our data showed enforced expression of TMPRSS4 activated STAT3 with increased

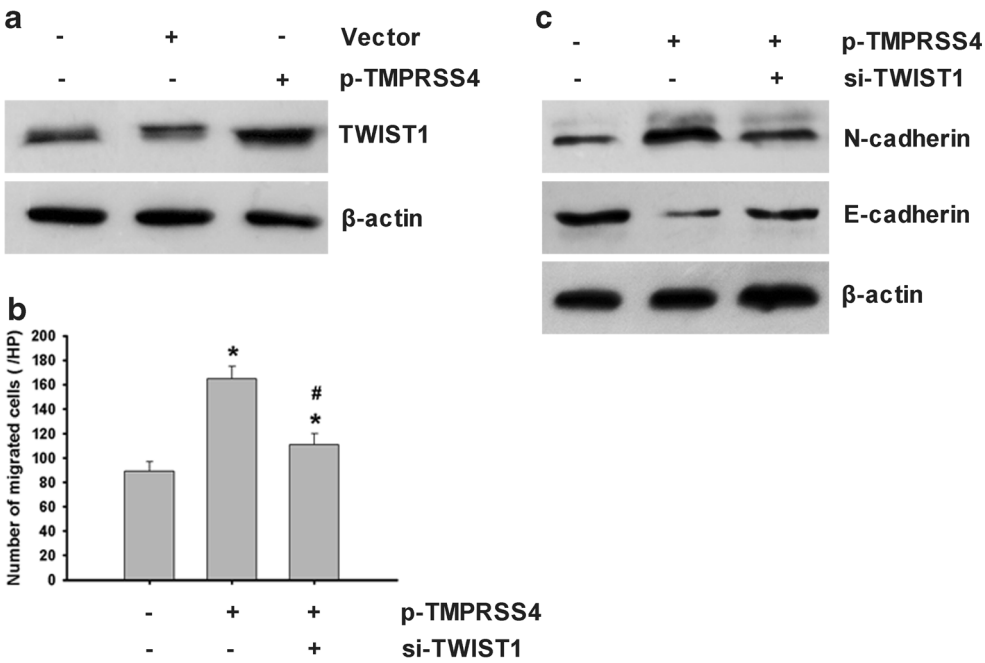

Fig. 3 TWIST1 was required for TMPRSS4-induced prostate cancer cells migration. **a** PC3 prostate cancer cells were transfected with empty vector or TMPRSS4 expression vector for 48 h. TWIST1 expression was analyzed by Western blot. **b** PC3 cells were transfected with TMPRSS4 expression vector for 48 h, and then transfected with TWIST1-specific siRNA (si-TWIST1) or not for another 24 h. Then, the cells migration was observed by transwell chambers, data presented as the mean ± SD of 3 independent experiments (*$P < 0.05$). **c** The expression of E-cadherin and N-cadherin was detected by Western blot

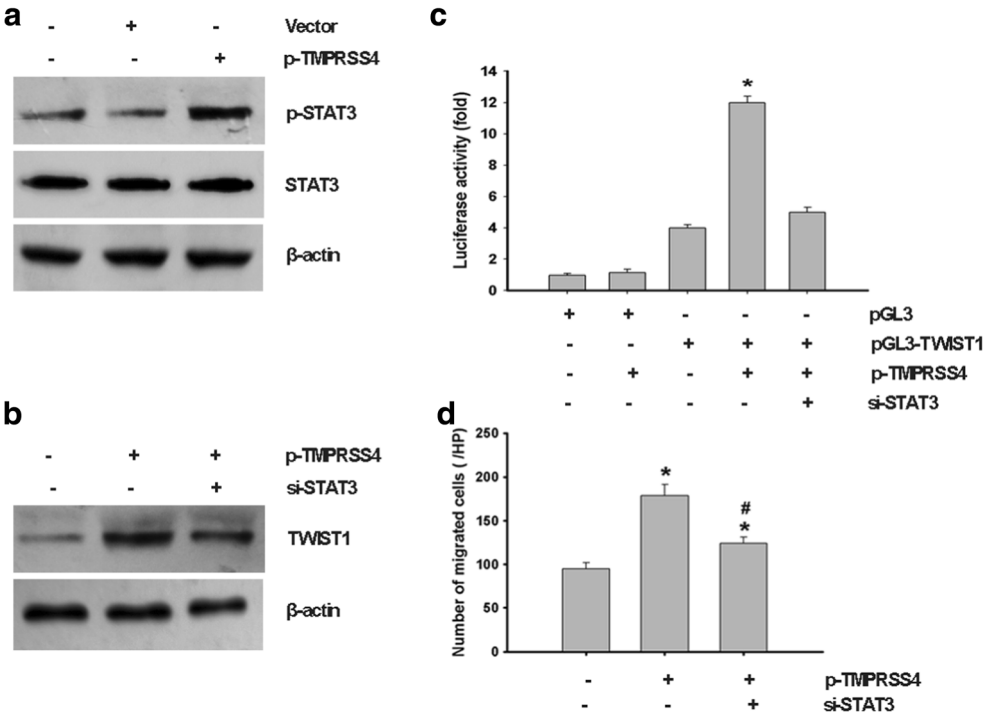

Fig. 4 STAT3 mediated TMPRSS4-induced TWIST1 expression and prostate cancer cell migration. **a** PC3 prostate cancer cells were transfected with empty vector or TMPRSS4 expression vector for 48 h. STAT3 expression and STAT3 phosphorylation were analyzed by Western blot. **b** PC3 cells were transfected with TMPRSS4 expression vector for 48 h, and then transfected with STAT3-specific siRNA (si-STAT3) or not for another 24 h. TWIST1 expression was analyzed by Western blot. **c** PC3 cells were co-transfected with the TMPRSS4 expression vector and TWIST1 promoter construct in the pGL3 basic vector, with si-STAT3 or not. After 48 h, cell extracts were analyzed by dual-luciferase reporter assay. Relative luciferase activity (RLA) was calculated after normalization to Renilla luciferase activity. Data are expressed as fold change relative to control cells without transfection of TMPRSS4-expressing vector (*$P < 0.05$). **d** Cells migration was observed by transwell chambers, data presented as the mean ± SD of 3 independent experiments (*$P < 0.05$)

phosphorylation of STAT3. Silencing STAT3 by STAT3-specific siRNA strongly attenuated TMPRSS4-induced TWIST1 expression and PC3 prostate cancer cells migration. TMPRSS4-induced TWIST1 promoter activity was dramatically reduced when STAT3 was silenced, which demonstrated that STAT3 induced TWIST1 expression directly by binding to the promoter region. Taken together, our data demonstrated that TMPRSS4 promoted prostate cancer migration could via increase of TWIST1 expression by activating STAT3.

Although, we have not demonstrated clearly the concrete mechanisms underlying the knockdown of the TMPRSS4 gene here, there has been evidence that inhibition of TMPRSS4 could inactivate urokinase-type plasminogen activator (uPA) and Slug-signaling pathways which in turn suppresses the metastasis activity of cancer cells [24, 25]. Thus, we speculate TMPRSS4 gene silencing may inhibit proliferation and metastasis of prostate cancer in multitude mechanisms, and TMPRSS4 may be a rational molecular therapeutic

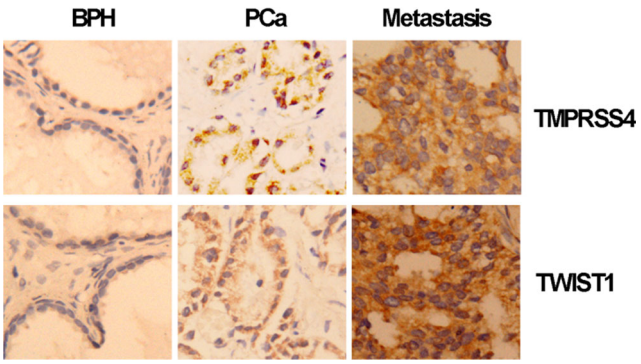

Fig. 5 TMPRSS4 expression correlated with TWIST1 expression in human prostate cancer tissue. Representative tissue sections from prostate cancer samples were immunostained with antibodies against TMPRSS4 and TWIST1

Table 1 Association of TMPRSS4 expression with TWIST1 expression in prostate cancer tissue

TMPRSS4 expression	TWIST1 expression					*rs*	*P*
	-	+	++	+++	Total		
-	1	2	0	0	3	0.574	0.001
+	1	2	2	0	5		
++	2	2	5	2	11		
+++	1	3	6	17	27		
Total	5	9	13	19	46		

target in prostate cancer. More studies should be done to explore the regulation of TMPRSS4.

Taken together, we demonstrated a mechanistic cascade of TMPRSS4 up-regulating STAT3 activation and subsequent TWIST1 expression, leading to prostate cancer migration. TMPRSS4 could be taken as a critical determinant of cancer cellular behavior and serve as a promising therapeutic target for prostate cancer.

Compliance with Ethical Standards

Conflict of Interest There are no potential conflicts of interest in this paper.

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