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Basic Study

TM9SF1 promotes bladder cancer cell growth and infiltration

Long Wei *et al*. TM9FS1 Tumor Promotion In BLCA

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Abstract

BACKGROUND

Bladder cancer (BC) is the most common urological tumor. It has a high recurrence rate, tumor heterogeneity, and resistance to chemotherapy. Furthermore, the long-term survival rate of BC patients has remained unchanged for decades, which seriously affects the quality of patient survival. To improve the survival rate and prognosis of BC patients, it is necessary to explore the molecular mechanisms of BC development and progression and identify targets for treatment and intervention. TM9SF1, also known as MP70 and HMP70, is a member of a family of nine transmembrane superfamily proteins that was first cloned in 1997. TM9SF1 has been demonstrated to be expressed in BC, but its biological function and mechanism in BC are not clear.

AIM

To investigate the biological function and mechanism of TM9SF1 in BC.

METHODS

Cells were transfected with lentivirus at 60%–80% confluence for 48–72 h. Then, stable transfections of overexpression and silencing in three BC cell lines (5637, T24, and UM-UC-3) were constructed to investigate the effect of TM9SF1 on the biological function in BC through the CCK8, wound-healing, and Transwell assays and flow cytometry technique.

RESULTS

Overexpression of TM9SF1 increased the *in vitro* proliferation, migration, and invasion of BC cells by promoting the entry of BC cells into the G2/M phase. The silencing of TM9SF1 inhibited *in vitro* proliferation, migration, and invasion of BC cells and blocked BC cells in the G1 phase.

CONCLUSION

TM9SF1 may be an oncogene in BC.

Key Words: TM9SF1; bladder cancer; biological function

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Core Tip: This study was the first to attempt to construct a stable Bladder cancer (BC) cell line to investigate the overexpression and silencing of TM9SF1 using *in vitro* experiments for the purpose of exploring the pro-cancer effect of TM9SF1 in BC. We verified that overexpressed TM9SF1 could enhance the growth, migration, and invasion of BC cells and promotes the G2/M phase of the BC cell cycle. This information not only provides a new target in developing treatments for BC but is also expected to be a source of hope for BC patients.

INTRODUCTION

Bladder cancer (BC) is the most common urological tumor, ranking tenth among global malignant tumors and fourth among male malignant tumors. It has a high recurrence rate, tumor heterogeneity, and resistance to chemotherapy, which impose a huge cost burden on the healthcare system and affect the prognosis and quality of survival in patients[1-4]. Age-standardized prevalence rates show considerable variation across geographic regions and are expected to continue to rise over the next two decades[5]. Several risk factors for BC have been identified; in addition to geography and age, gender and exposure to a variety of carcinogens, of which smoking is the most prevalent, greatly influence the risk[6, 7]. Moreover, age-standardized mortality rates have begun to decline in developed countries and are on the rise in low-income regions worldwide[8].

The most prominent symptom of BC is microscopic or gross hematuria. Seventy-five percent of bladder tumors are uroepithelial carcinomas confined to the mucosa, that is, non-muscle-invasive BC (NMIBC)[9]. Muscle-invasive BC (MIBC) is BC that has invaded the deeper layers of the bladder wall or metastasized[10-13]. For patients with NMIBC, transurethral resection of the bladder tumor (TURBT) is the standard treatment, while radical cystectomy (RC) is indicated for patients with MIBC. To prevent BC recurrence or worsening, TURBT in selected patients is supplemented with an intravesical drip[10, 14].

Despite some improvements in surgery and anesthesia techniques and the widespread adoption of perioperative chemotherapy, the long-term survival of BC patients has remained unchanged for decades[15]. However, advanced molecular studies have greatly increased the understanding of disease biology. To find better treatments for this disease, we investigated transmembrane 9 superfamily member 1 (TM9SF1) to determine its biological function and mechanism in BC and to assess whether it could be a therapeutic target.

TM9SF1, with DNA localization at NC_000014 and mRNA localization at NM_006405.7, is also known as MP70 and HMP70. It was first cloned in 1997[16].

TM9SF1 is involved in the localization of proteins to membranes and is an integral part of the membranes in which it is active, including autophagosome and lysosomal membranes and cytoplasmic vesicles. TM9SF1 is ubiquitously expressed in human tissues, widely expressed in yeast, plants, and mammals, and is highly conserved[17]. Studies have identified TM9SF1 in BC, and through genome-wide microarray analyses of tissue sections, TM9SF1 has been determined to be a common differentially expressed (DE) BC gene[18]. However, the biological function of TM9SF1 in BC cells is still unclear, and more in-depth exploration of its biological behavior is needed.

This study explored the effect of TM9SF1 on BC cells' biological phenotype by constructing stable cell transplants that overexpressed and silenced TM9SF1. The biological relationship between TM9SF1 and BC at the *in vitro* level was validated, thereby providing a new direction for and new way of thinking about targeted BC therapy.

MATERIALS AND METHODS

Cell Cultures

We purchased 5637 human BC cells, tool cells, and 293T human embryonic kidney cells from the Cell Bank at the Committee of Typical Culture Conservation at the Chinese Academy of Sciences as well as T24 human bladder transitional cell cancer cells (Guangzhou Genio Biotechnology Co., Ltd.) and UM-UC-3 human bladder transitional cell cancer cells (Wuhan Punose Life Technology Co., Ltd.).The conditions for incubating the three cells were a CO_2 incubator set to 37 C and 5% CO_2.

Stable Transplant Construction for TM9SF1 Overexpression

The lentivirus used for constructing the TM9SF1 overexpression stable transient strain was provided by Hanheng Biologicals; its vector system consisted of pSPAX2, pMD2G, and a shuttle plasmid carrying the target genes. The three plasmids were co-transfected into the 293T packaging cell *via* a transfection reagent; thus, the RNA of the target genes, which was translated by the transcribed RNA and proteins that were translated

by the pSPAX2 and pMD2G genes, could be loaded into lentiviruses carrying G418 resistance. After lentiviral infection, qRT-PCR which is an experimental method for detecting RNA or DNA molecules and quantifying their content verified the efficiency of TM9SF1 overexpression in BC cells.

Stable Transplantation Strain Construction for TM9SF1 Silencing

Lentiviral vectors are gene therapy vectors that have been developed based on human immunodeficiency virus-1 (HIV-1). The TM9SF1 silencing plasmid used for constructing short hairpin RNA (shRNA) was provided by GeneCopoeia. The vector used was psi-LVRU6H, had BamHI (5') and EcoRI (3') cloning sites, was 7451 bp in length, was ampicillin resistant, and had a thaumatin screening marker. The lentiviral infection was followed by qRT-PCR to validate the silencing efficiency of TM9SF1 in BC cells.

Cell Proliferation

The CCK8 cell counting kit used was mainly used to detect cell proliferation. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5(2,4-disulfophenyl)-2H-tetrazole monosodium salt], the main component of the kit, reacts with intracellular mitochondria to generate orange-yellow carapace in proportion to the number of viable cells. This indirectly detects the number of viable cells and shows cell proliferation. The optical density (OD) value was measured at 450 nm using an enzyme-labeling instrument; the higher the OD value, the more live cells are represented.

Cell Migration and Invasion

The migration of the stably transfected strains demonstrating silenced or overexpressed TM9SF1 was verified by cell wound healing and Transwell assays. First, the cells were incubated until they spread over the bottom of the plate. Next, a 1000 µL pipette tip was used to trace a line across the cell plane along a straight ruler held perpendicular to the bottom of the plate. The culture was then continued by adding medium containing the

appropriate percentage of Fetal sovine serum（FBS）which is the natural culture medium in the cell culture (5637: 1%; T24: 1%; UM-UC-3: 4%). Starting at 0 h, scratches at the same location were photographed under an inverted fluorescence microscope at intervals of 12 h or 48 h. The area of the scratches was calculated, and the healing rate was determined using ImageJ software.

For the Transwell migration and invasion assays, 500 µL of complete medium was added to the 24-well plates first. Logarithmically grown 5637, T24, and UM-UC-3 BC cells were digested, centrifuged, and counted using resuspension with a cell culture medium containing 2% FBS. To measure invasion, 100 µL of suspended cells was added on top of the solidified matrix gel in the upper chamber of each Transwell and incubated for 24 h. After 24 h, the chambers were removed, fixed, and stained with methanol and crystal violet. All photography was performed using a Leica DMi8 inverted fluorescence microscope and calculated using ImageJ software.

Cell Cycle

The original medium was aspirated, and fresh serum-free medium was added based on starvation treatment in the incubator for 12 h. Then, the serum-free medium was aspirated, and a complete medium containing 10% FBS and 1% double antibody was added. Incubation continued for 12 h to synchronize the cell status of the experimental and control groups. Finally, 5×10^5 cells were removed and processed using a cell cycle staining kit (Lianke Bio). A flow cytometer was used to detect the cells.

Statistical analysis

Student's t test in SPSS 25.0 was employed to analyze the statistical signifcance of the experiments, and $p < 0.05$ was defned as statistically signifcant.

RESULTS

Effects of TM9SF1 Overexpression on BC Cell Proliferation, Migration, Invasion, and the Cell Cycle

Stable Transplant Construction for TM9SF1 Overexpression

The relative expression of the TM9SF1 gene in BC cells transfected with overexpressed lentivirus was detected by qRT-PCR testing in three cell lines (5637, T24, and UM-UC-3) using cells transfected with empty lentivirus (control group). The expression of TM9SF1 was 27.4-fold higher in the TM9SF1 overexpression group （OE group） of 5637 cells than in the vector group, 3.5-fold higher in overexpressing T24 cells than in the empty cells, and 12.5-fold higher in the UM-UC-3 cells. All of these values were statistically significant ($p<0.05$), indicating that stable transfections of overexpressing TM9SF1 were successfully constructed in all three BC cell lines (Figure 1).

TM9SF1 Overexpression Promotes BC Cell Proliferation in Vitro

The cell activity was tested by a CCK8 assay. The results showed that, compared with the control vector group transfected with airborne lentivirus, the proliferation rate of BC cells in the TM9SF1 overexpression group was higher and statistically significant at 72 h in the 5637 cells, 96 h in the T24 cells, and 48 h and 72 h in UM-UC-3 cells ($p<0.05$) (Figure 2). This indicates that TM9SF1 overexpression plays a role in promoting BC cell proliferation in vitro.

TM9SF1 Overexpression Promotes BC Cell Migration in Vitro

In this study, a cell wound-healing assay was utilized to detect the migration ability of cells. After calculating the change of scratch area, it was found that in the 5637 cells, the wound healing rate of the TM9SF1 overexpression group was significantly higher than that of the control group at 12 h, 24 h, and 36 h ($p<0.01$). The scratch healing rate of the cells in the T24 overexpression group was also significantly higher than that of the unloaded control group at 24 h and 36 h ($p <0.01$). Although UM-UC-3 cells were less likely to migrate toward the scratches, the rate of scratch closure in the OE group was still slightly higher than that in the control group and was statistically significant at 96 h ($p<0.01$) (Figure 3).

A Transswell migration assay was performed to verify the changes in cell migration ability. After 24 h of incubation, the number of cells crossing the membrane of the Transwell was photographed and counted. As shown in Figure 4, the number of cells in the three BC cell overexpression groups crossing the membrane was significantly higher than the number of cells in the control group (p<0.05).

By combining the above two migration experiments, it can be hypothesized that the overexpression of TM9SF1 can significantly improve the migration ability of BC cells.

TM9SF1 Overexpression Promotes BC Cell Invasion in Vitro

Matrigel matrix gel was used to mimic the extracellular matrix. The high-nutrient culture medium in the lower chamber of the Transwell was separated from the low-nutrient culture medium in the upper chamber, which caused the BC cells to secrete hydrolases and pass through a deformation movement to cross the filter membrane lined with matrix gel. This achieved a response to the invasive ability of the BC cells. After analyzing the fixed staining of cells, it was found that the number of cells in the OE group of the three BC cell lines was more than 1.3 times that in the vector group (p<0.05), indicating that the overexpression of TM9SF1 has a significant effect on promoting the invasive ability of BC cells (Figure 5).

TM9SF1 Overexpression Promotes Cell Entry into the G2/M Phase

Flow cytometry was used to investigate the effect of TM9SF1 overexpression to detect the percentage of cells distributed in different cell cycles. The results showed that the proportion of cells distributed in the G1 phase in all three BC cell lines was smaller in the OE group than in the vector group, while the proportion of cells in the G2/M phase was higher in the OE group than in the vector group. However, the OE group's 5637 and UM-UC-3 cell lines had more cells in the S phase than the vector group's, while the opposite was true regarding the T24 cell line. Although the statistical results of S and G2/M and G1 of 5637, G1, S and G2/M phases of T24 were not statistically significant, the trend combined with the statistical results of G1, S and G2/M phases of UM-UC-3

can suggest that TM9SF1 overexpression had a certain effect on BC cell cycle, reducing the proportion of cells in G1 phase and promoting the entry of cells into G2/M phase (Figure 6).

Effects of TM9SF1 Silencing on BC Cell Proliferation, Migration, Invasion, and the Cell Cycle

Stable Transplant Construction for TM9SF1 Silencing

shRNA was used to construct TM9SF1-silenced stable transplants in three cell lines: 5637, T24, and UM-UC-3. The TM9SF1-silenced stable transplants and the cells transfected with empty lentivirus were used as the Lentiviral null control group of silencing TM9SF1 （shVector control group）. qRT-PCR was used to detect the TM9SF1 silencing efficiency in BC cells transfected with silenced lentivirus in the silenced TM9SF1 group （shTM9SF1 group）. The quantitative results showed that the silencing efficiency was 88.09%, 90.39%, and 92.04% in the 5637, UM-UC-3, and T24 cells, respectively. All of these values were statistically significant (p<0.01), suggesting that the TM9SF1-silencing stable transplant were successfully constructed in all three BC cell lines (Figure 7).

TM9SF1 Silencing Inhibits BC Cell Proliferation in Vitro

To test the effect of silencing TM9SF1 on cell proliferation in BC cells, we examined cell viability using a CCK8 assay. Compared with the shVector control group transfected with airborne lentivirus, the proliferation rate of BC cells in the silenced TM9SF1 group was statistically significantly reduced in the 5637 cells at 24 h, 48 h, 72 h, and 96 h; in the T24 cells at 48 h, 72 h, and 96 h; and in the UM-UC-3 cells at 48 h (p<0.05). This indicates that silencing TM9SF1 had an inhibitory effect on BC cell proliferation *in vitro* (Figure 8).

TM9SF1 Silencing Inhibits BC Cell Migration in Vitro

Cell scratch and Transwell migration assays were used to detect the migration ability of the cells. Of the three BC cell lines, the 5637 and T24 cells were selected for the cell

scratch assay (Figure 9). The closure rate of the TM9SF1-silenced 5637 cells was higher than that of the control group at 12 h and 24 h, but was statistically significant only at 24 h (p<0.01). The closure rate of the TM9SF1-silenced T24 cells compared to the empty control group was statistically significant at 12 h, 24 h, and 36 h. Moreover, the T24 cells' closure rate statistically significantly decreased at 24 h and 36 h (p<0.001).

All three BC cell lines were subjected to Transwell migration assays to validate the inhibitory effect of silenced TM9SF1 on the migratory ability of BC cells. After spreading the cells in the Transwell chambers, they were incubated for 24 h. The chambers were then removed and washed with phosphate buffered solution （PBS）, and the cells that passed through the chambers' filter membranes were fixed with methanol. The cells under the filter membranes were then stained purple with crystal violet (the excess dye was removed with PBS). Next, the chambers were air dried at room temperature. Once dried, images of the cells were captured *via* microscope, and the cells were counted using ImageJ software. This information was statistically analyzed using SPSS and graphed using GraphPad Prism 8.0. As can be seen in Figure 10, the number of cells that crossed the filter membrane was significantly lower in all three BC cell strains than in the shVector group (p<0.01). By comprehensively analyzing both assays, it can be concluded that silencing the expression of TM9SF1 in BC cells can significantly reduce their migration ability.

TM9SF1 Silencing Inhibited BC Cell Invasion in Vitro

Invasion occurs when malignant tumor cells enter adjacent host tissue by secreting proteins that digest the tissue cells' extracellular matrix. Detecting a tumor's invasion ability can help determine the rate of metastasis, as cell invasion is the first step: tumor cells break through the basement membrane in situ, infiltrate blood and lymphatic vessels, colonize other tissues, and proliferate. The Transwell invasion assay mimics the *in vivo* environment of the human body and can therefore determine the invasive ability of tumor cells.

Therefore, in this study, BC cells were induced to secrete hydrolytic enzymes to pass through a layer of Matrigel matrix gel (which mimics the extracellular matrix in vivo) on a Transwell filter membrane through deformation. After that, the BC cells that had passed through the matrix gel and reached the bottom of the membrane were washed, fixed, stained, dried, photographed, and counted to determine their invasive ability.

The number of 5637 cells in the shTM9SF1 group with silenced TM9SF1 was 1/1.42 of that in the control shVector group. The number of T24 cells in the TM9SF1-silenced group was 1/2.46 of that in the control group. The number of UM-UC-3 cells in the shTM9SF1 group was 1/2.6 of that in the shVector group. In all groups, p<0.05. The results suggest that the invasion ability of BC cells is significantly reduced after TM9SF1 is silenced (Figure 11).

TM9SF1 Silencing Blocks Cells in the G1 Phase

To investigate the effect of silenced TM9SF1 on the BC cell cycle, shTM9SF1 collected from the 5637, T24, and UM-UC-3 cell lines was stained and detected by flow cytometry. The number of cells distributed in each part of the cell cycle was analyzed and compared. In all three BC lines, the shTM9SF1 group had a higher proportion of BC cells in the G1 phase than did the shVector group (p<0.05). The number of 5637 cells in the S phase was lower in the TM9SF1-silenced group than in the shVector group, but the difference was not significant. However, the number of 5637 cells in the G2/M phase was significantly lower in the shTM9SF1 group than in the shVector group. The number of T24 cells in the S phase in the shTM9SF1 group was significantly lower than that in the shVector group (p<0.001). However, silencing TM9SF1 did not have much effect on T24 cells in the G2/M phase. The number of the shTM9SF1 group's UM-UC-3 cells in the S phase was significantly less than the same cells in the shVector group (p<0.05), but there was no significant difference found in the G2/M phase. These combined results suggest that silencing TM9SF1 expression inhibits BC cell proliferation by arresting BC cells in the G1 phase and prolonging the time between mitosis completion and DNA replication (Figure 12).

DISCUSSION

In this study, we confirmed that TM9SF1 is a pro-carcinogenic gene in BC by overexpressing and silencing it in the 5637, T24, and UM-UC-3 cell lines using cellular function assays. TM9SF1 is expressed not only in BC but also in esophageal squamous cell carcinoma and cervical cancer, and because of its pro-cancer role, it can aggravate poor prognoses in patients[18-20]. The experimental results of the present study revealed that overexpression of TM9SF1 reduces the number of cells in the G1 phase and prompts them to enter the G2/M phase and start mitosis, thereby promoting BC cell proliferation. On the other hand, the silencing of TM9SF1 blocks cells in the G1 phase and prevents them from entering the DNA replication phase, thereby inhibiting BC cell proliferation. It is hypothesized that TM9SF1 may promote BC cell proliferation by mainly affecting the G1 phase of BC cells, which in turn promotes the development of BC. Although TM9SF1 was experimentally demonstrated to be an oncogene of BC in this study, WeiZhuo *et al* found TM9SF1 to be a gastric cancer suppressor gene *via* N6,2'-O-dimethyladenosine (m6Am) sequencing, TM9SF1 is able to act as an oncogene for the phosphorylation of the CTD-interaction factor 1 (PhosphorylatedCTD-interactingfactor1, PCIF 1) for the functional mRNA targets, Reducing translation by m6Am modification, Further reversed the effect of PCIF 1 on the invasiveness of gastric cancer cells, Decreased invasive in gastric cell carcinoma[21]. This indicates that TM9SF1 has different functions and effects on different tumors.

In other studies, TM9SF1 has been shown to synergistically interact with the tumor marker gene estrogen receptor-binding fragment-associated antigen 9 (EBAG9) to regulate the epithelial–mesenchymal transition in cancer cells *via* an attenuated anti-tumor immune response, leading to malignant cells leading to immune escape, which results in tumor growth[22]. Therefore, in addition to affecting the biological phenotype of BC and promoting BC development, TM9SF1 can also affect an anti-tumor immune response to BC, the mechanism of which must be studied in future research.

Furthermore, it has been shown through a combination of suppression, subtractive, hybridization, and transmembrane trapping techniques that MYC proto-oncogene (myc)-tagged TM9SF1 has been localized on the surface of transfected COS-7L cells[23]. If the same technology can be used for BC, then researching targeted therapy may go further.

It has also been reported that TM9SF1 plays a crucial role in autophagy. High expression has been reported to worsen the prediction of related prognoses in patients with cervical cancer[24]. From this perspective, TM9SF1 may affect the occurrence and development of BC by regulating autophagy. However, this study was based on the results obtained from cell *in vitro* experiments, and expression detection in BC tissues still needs to be improved.

This study was the first to attempt to construct a stable BC cell line to investigate the overexpression and silencing of TM9SF1 using *in vitro* experiments for the purpose of exploring the pro-cancer effect of TM9SF1 in BC. We verified that overexpressed TM9SF1 enhances the growth, migration, and invasion of BC cells and promotes the G2/M phase of the BC cell cycle. This information not only provides a new target in developing treatments for BC but is also expected to be a source of hope for BC patients.

CONCLUSION
TM9SF1 may be an oncogene in BC.

ARTICLE HIGHLIGHTS
Research perspectives
In addition to influencing the biological phenotype of bladder cancer (BC) and promoting BC development, TM9SF1 may also impact the anti-tumor immune response in BC. Further research is needed to elucidate the mechanisms underlying this effect. Furthermore, genomic factor analysis and targeted therapy aimed at inhibiting carcinogenesis in cancer pathways offer promising prospects.

Research conclusions

TM9SF1 may be an oncogene in BC.

Research results

In this study, we confirmed that TM9SF1 is a pro-carcinogenic gene in BC by overexpressing and silencing it in the 5637, T24, and UM-UC-3 cell lines using cellular function assays. The experimental results of the present study revealed that overexpression of TM9SF1 reduces the number of cells in the G1 phase and prompts them to enter the G2/M phase and start mitosis, thereby promoting BC cell proliferation. On the other hand, the silencing of TM9SF1 blocks cells in the G1 phase and prevents them from entering the DNA replication phase, thereby inhibiting BC cell proliferation. It is hypothesized that TM9SF1 may promote BC cell proliferation by mainly affecting the G1 phase of BC cells, which in turn promotes the development of BC.

Research methods

Cells were transfected with lentivirus at 60%–80% confluence for 48–72 h. Then, stable transfections of overexpression and silencing in three BC cell lines (5637, T24, and UM-UC-3) were constructed to investigate the effect of TM9SF1 on the biological function in BC through the CCK8, wound-healing, and Transwell assays and flow cytometry technique.

Research objectives

This study explored the effect of TM9SF1 on BC cells' biological phenotype by constructing stable cell transplants that overexpressed and silenced TM9SF1. The biological relationship between TM9SF1 and BC at the *in vitro* level was validated, thereby providing a new direction for and new way of thinking about targeted BC therapy.

Research motivation

Previous studies have identified TM9SF1 in bladder cancer, and through genome-wide microarray analyses of tissue sections, TM9SF1 has been found to be a commonly differentially expressed (DE) gene in bladder cancer. However, the biological function of TM9SF1 in bladder cancer cells remains unclear. To discover better treatment options for this disease, it is crucial to conduct a more in-depth exploration of its biological behavior.

Research background

Bladder cancer (BC) is the most common urological tumor. It has a high recurrence rate, tumor heterogeneity, and resistance to chemotherapy. Furthermore, the long-term survival rate of BC patients has remained unchanged for decades, which seriously affects the quality of patient survival. To improve the survival rate and prognosis of BC patients, it is necessary to explore the molecular mechanisms of BC development and progression and identify targets for treatment and intervention. TM9SF1, also known as MP70 and HMP70, is a member of a family of nine transmembrane superfamily proteins that was first cloned in 1997. TM9SF1 has been demonstrated to be expressed in BC, but its biological function and mechanism in BC are not clear.

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