Clinicopathological and prognostic significance of TM4SF5 in colorectal cancer

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Abstract

Background

Transmembrane 4 L six family member 5 (TM4SF5) has been found to play a vital role in the cancer process, but the relationship between TM4SF5 and colorectal cancer remains unclear. Therefore, the present study aimed to investigate the clinical and prognostic value of TM4SF5 in colorectal cancer (CRC).

Methods

Immunohistochemistry and RT-qPCR were used to identify the clinical significance and prognostic value of TM4SF5 expression in colon cancer. The expression of TM4SF5 was knocked down using the siRNA method to evaluate the role of TM4SF5 in regulating the biological behavior of colon cancer cell lines.

Results

The immunohistochemistry study revealed that the expression of TM4SF5 in human colorectal cancer tissues was significantly higher than that in adjacent normal tissues ($P < 0.0001$). However, there was no correlation between the expression of TM4SF5 and the clinicopathological features of these patients. The RT-qPCR analysis revealed that the mRNA expression of TM4SF5 was higher in colon cancer cell lines than in normal cells ($P < 0.05$). The survival analysis revealed that the overall survival (OS) rate of patients with a low expression of TM4SF5 was significantly higher, when compared to patients with a high expression of TM4SF5 ($P = 0.048$). The COX model revealed that the expression of TM4SF5 ($P < 0.001$) and clinical stage ($P = 0.025$) can be used as independent prognostic factors for patients with colon cancer. In addition, the knockdown of TM4SF5 in colon cancer cell lines LoVo, SW480, RKO and HCT-116 significantly reduced the ability of cells (such as proliferation, migration and invasion).

Conclusion

The present results indicate that the increase in TM4SF5 expression in human colon cancer is associated with cancer progression and poor survival, and that its intervention can be an important therapeutic strategy against colon cancer.

Background

Colorectal cancer (CRC) is one of the most common malignancies in the world. Furthermore, it is the fourth cause of cancer death in Asia, and its incidence is increasing [1]. The 5-year survival rate after early surgical resection of CRC is approximately 85%, and the incidence of stage III CRC with lymph node metastasis significantly decreases ($< 50\%$). Distant metastasis (stage IV) is the most common cause of

treatment failure, with a 5-year survival rate of less than 5% [2, 3]. Although increasing knowledge has been mastered, the choice of treatment for patients with advanced metastatic diseases remains limited, and the prognosis is poor [4, 5]. Thus, there is an urgent need to develop reliable biomarkers and simple detection methods, which are usually suitable for early detection, progression, prognosis and treatment monitoring [6].

Transmembrane 4L six family member 5 (TM4SF5) is a transmembrane glycoprotein of the transmembrane 4L six family (a branch of the four transmembrane protein family) [7]. TM4SF5 is a homologue of tumor-related antigen L6 (TM4SF1 or L6-Ag), which is structurally similar to four transmembrane proteins [8]. TM4SF5 has been shown to be highly correlated to integrins, thereby regulating cell shape, cell migration, and signal transduction in many cell types, especially in cancer [9]. For example, TM4SF5 have been found to be overexpressed and associated with tumor cell invasion and movement in cancer [10–12]. The expression of TM4SF5 in epithelial cells leads to the loss of contact inhibition and abnormal proliferation of epithelial-mesenchymal transition (EMT) [13]. Recently, four transmembrane proteins have been associated with tumor progression and metastasis [14]. Therefore, the tetratransmembrane protein has been suggested as a diagnostic and prognostic marker, and therapeutic target for tumor therapy [15].

The present study evaluates the expression of TM4SF5 in human colorectal cancer tissues and cells by immunohistochemistry and RT-qPCR analysis, and discusses its clinical significance. In addition, cellular studies were carried out to reveal the important role of TM4SF5 in the functional regulation of human colon cancer cells.

Materials And Methods

Patients and Samples

The CRC tissue microarrays (JS W-11-01) were purchased from Shanghai Tufei Biotech Co., Ltd. A total of 100 patients, who underwent surgery between January 2011 and December 2017, were enrolled in the present study. A total of 100 patients collected during this period were randomly selected, and none of them received neoadjuvant chemotherapy. The analysis of 100 patients with colon cancer revealed that there is no correlation between the expression of TM4SF5 and the factors listed in Table 1, such as gender, age, tumor size and pathological grade. The postoperative pathology confirmed the primary colorectal adenocarcinoma. For colon cancer, when the investigators chose the best cut-off value and H-score = 210 as the critical value, patients with a H-score of > 210 were assigned to the high expression group, while patients with a H-score of $\leq$ 210 were assigned to the low expression group. Paired tumor tissues and normal tissues adjacent to cancer were selected as the experimental materials. All patients were informed of the reason for the study in detail, and provided a signed informed consent. The experimental study was approved by the ethics committee.

Table 1
The relationship between the expression of TM4SF5 and the clinicopathological parameters of patients with colorectal cancer

Clinical parameters	Cases	TM4SF5 expression level		χ^2	P
		H-score $\leq$ 210	H-score $>$ 210		
Gender				0.142	0.707
Male	45	10	35		
Female	55	14	41		
Age (years)				0.481	0.488
< 65	52	11	41		
$\geq$ 65	48	13	35		
Pathological grade				0.096	0.756
I + II	21	4	17		
III	79	20	59		
Tumor size (cm)				0.585	0.444
< 5	60	16	44		
$\geq$ 5	40	8	32		
Clinical stage				0.439	0.508
I + II	68	15	53		
III + IV	32	9	23		
T stage				1.057	0.304
T1 + T2 + T3	59	12	47		
T4	41	12	29		
N stage				0.831	0.362
N0	63	17	46		
N1 + N2 + N3	37	7	30		
M stage				0.439	0.508
M0	68	15	53		
M1	32	9	23		
^a Italics signifies $P < 0.05$.					

Antibodies And Other Reagents

The rabbit anti-human TM4SF5 polyclonal antibody (a63568-050) was purchased from Abcam (Cambridge, MA, USA). The HRP-conjugated goat anti-mouse/rabbit secondary antibody (K500711) was purchased from Dako (Glostrup, Denmark). The DMEM and fetal bovine serum (FBS) were purchased from Gibco (Cambrex, MD, USA). The Lipofectamine 2000 was purchased from Jiangsu Ruijie Biology Co., Ltd.

Immunohistochemistry And Evaluation Of Immunohistochemical Staining

The tissue chip (tissue microarray) was dewaxed in xylene, and rehydrated and graded in ethanol solution. The antigen retrieval was performed by heating the tissue sections at 100°C for 30 minutes in citrate solution. After cooling the slices, the tissue sections were immersed in 0.3% hydrogen peroxide solution for 30 minutes to block the endogenous peroxidase activity. Then, the sections were rinsed in PBS for five minutes, and blocked with 3% BSA solution for 30 minutes in an incubator at 37°C. Next, the chip was incubated with the rabbit anti-human TM4SF5 polyclonal antibody at 4°C overnight, and incubated with the HRP-labeled goat anti-mouse/rabbit secondary antibody. Diaminobenzene was used as the chromophore, and hematoxylin was used as the nuclear counterstain. Finally, the slices were dehydrated, cleaned and fixed.

All slides were blindly examined by two independent senior pathologists. The positive staining of TM4SF5 was defined as brown-yellow granules on the cell membrane and in the cytoplasm. The immunostaining intensity of TM4SF5 was assessed according to the H-score method, as previously described: $H\text{-score} = (\% \text{ unstained tumor cells} \times 0) + (\% \text{ weakly stained tumor cells} \times 1) + (\% \text{ moderately stained tumor cells} \times 2) + (\% \text{ strongly stained tumor cells} \times 3)$. The H-scores ranged from 0 (100% negative tumor cells) to 300 (100% strongly stained tumor cells) [16–18]. The scoring results provided by the two pathologists were averaged and used for the statistical analysis.

Cell Lines And Cell Culture

The colorectal cancer cell lines LoVo, SW480, HCT-116 and RKO used in the present experiment were donated by the Oncology Laboratory of the Third Clinical Hospital Affiliated to Suzhou University. The human normal colonic mucosal epithelial cell line CCD-841CON was purchased from Qingqi (Shanghai) Biotechnology Development Co., Ltd. Colorectal cancer cell lines LoVo, SW480, HCT-116 and RKO were cultured in standard DMEM supplemented with 10% fetal bovine serum under standard culture conditions (5% CO₂, 37°C).

Sirna Transfection With Lipofectamin 2000 As The Vector Transfection

At the day before the transfection, the LoVo and SW480 cell lines in the cell culture medium were digested with trypsin and centrifuged. Then, an appropriate amount of cells was inoculated in a 24-well plate, and cultured in DMEM medium overnight. These were divided into the experimental group and control group, with three holes in each group, and the number of cells in each well was 2×10^5 . On the following day, when the cells adhered to the wall and the confluence degree reached 50–70%, the culture medium was abandoned. Then, these were washed twice with PBS, and 1.5 ml of serum-free DMEM medium was added to each well. Afterwards, these were cultured at 37°C with 5% CO₂. Two groups of EP tubes were prepared. One group was diluted with 5 ul of Lipofect2000 reagent in 250 ul of medium, while the other group was added with 250 µl of culture medium and siRNA, at room temperature for five minutes. Then, these were mixed. Next, 500 µl of the above mixture was added to the cells, shaken well, and cultured in an incubator. After 12 hours, the supernatant was discarded, and the culture medium was changed. After 48 hours of culture, the transfection efficiency was detected and used in the follow-up experiment. The Recombinant TM4SF5-targeting Lipofectamin 2000 (TM4SF5-sh) or control mock Lipofectamin 2000 (NC) was transfected into LoVo and SW480 cells.

Real-time Reverse Transcriptase-polymerase Chain Reaction (Rt-pcr)

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of TM4SF5 at the mRNA level in different human CRC cell lines was assessed by RT-PCR. The total RNA was extracted from various cell lines using the TRIzol reagent (Invitrogen, USA), and the PCR reactions were performed using the ABI 7600 system (Applied Biosystems, USA), according to manufacturer's instructions. The primers were synthesized, as follows: human GAPDH forward primer: 5'-TGA CTTCAACAGCGACACC CA-3', human GAPDH reverse primer: 5'-CAC CCTGTTGCTGTAGCCAAA-3', human TM4SF5 forward primer: 5'-ACTGGCATTGCTGAACG-3', human TM4SF5 reverse primer: 5'-TCCTCCATTTCCCAATAGAC-3'. The relative expression of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Cell Viability Assay

According to manufacturer's instructions, the cell proliferation was evaluated using Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China). Briefly, the cancer cells in the TM4SF5-sh group and NC group (2×10^3 - 5×10^3) were seeded into a 96-well plate, and incubated for 24, 48, 72 and 96 hours. At two hours before the incubation end point, the CCK-8 reagent was added to each well, and the absorbance of each well was measured at 450 nm using the enzyme labeling instrument. The increase or decrease in absorbance value was determined at 450 nm. The experimental hole indicated cell growth or death, relative to the initial value, respectively. Each experiment was repeated for at least three times.

Wound Healing Assay

Before the experiment, 5–6 horizontal parallel lines were drawn on the bottom of the 6-hole plate. Then, cells of each group that grew to the logarithmic phase (up to 70–80%) were inoculated in 6-well plates, with 8×10^5 cells in each well. After the cells adhered to the wall, these were starved and cultured overnight in serum-free medium. On the following day, a 200- μ l gun was used to draw a line on the single cell layer, and the gun tip vertically, uniformly and rapidly crossed the bottom of the petri dish, ensuring that the scratch width was consistent, with 3–6 holes in the shape of nine palace grids. After washing the cells with $1 \times$ PBS for three times, the scraped cells were washed off and cultured in the serum-free DMEM medium at 37°C for 48 hours in a 5% CO₂ incubator. The width of the scratches was recorded by taking photographs at different time points (0 and 24 hours). The wound width was measured using a BX50 microscope (Olympus®) with a calibrated eyepiece grid. The data from three independent experiments were averaged, and expressed as the percentage of the original width.

Transwell Assay

As described in the previous study of the investigators, the co-culture system was used to evaluate the invasive ability of the knocked down TM4SF5 expression in LoVo and SW480 cells. The invasion was determined using invasion chambers coated with matrix glue (Corning, NY, USA). Cells in the TM4SF5-sh group and NC group (5×10^4 cells / well) were starved with serum for 24 hours, and placed in the super chamber of the serum-free medium. Then, the medium that contained 10% FBS was placed in the subscript chamber as a chemical attractant. After incubation in the incubator for 48 hours, the non-invasive cells were removed from the top of the super chamber of the matrigel using cotton swabs, and the invasive cells were fixed and stained with 0.1% crystal violet. The invasive cells were photographed under a microscope, and counted from five random visual fields (5 fields per chamber $\times$ triplicate).

Cell Cycle Assay

The LoVo and SW480 cells from the TM4SF5-sh and NC groups were seeded in 6-well plates, and cultured for 48 hours. Then, these cells were washed with ice-cold PBS and fixed in 70% cold ethanol solution overnight at 4°C. On the following day, according to the guidance of the cell cycle analysis kit (Sigma, MO, USA), these cells were analyzed by flow cytometry. The cell cycle information was analyzed using the Flowjo 10.7.0 software.

Statistical Method

The R software package and GraphPad Prism 8.0 statistical software were used to analyze and process the data. We use Mean $\pm$ SD to record and represent the processed data. Chi-square test and student's t-test were used for the analysis of the counting data and the inter-group comparison. $P < 0.05$ was considered statistically significant.

Results

Expression of TM4SF5 based on TCGA data and Encyclopedia of tumor Cell Lines at mRNA level in Human Colorectal Cancer

First, according to the TCGA data obtained from <http://gepia.cancer-pku.cn/>, the investigators compared the TM4SF5 expression at the mRNA expression level, between human CRC tissues and adjacent normal tissues, and the TM4SF5 expression in human CRC tissues was found, when compared to adjacent normal tissues (Fig. 1a). A higher expression in human CRC tissues was found, when compared to adjacent normal tissues (Fig. 1b, $P < 0.05$). Second, the image of TM4SF5 expression level in colorectal cancer cells is between several cancer cell lines. The histogram was downloaded from The Cancer Cell Line Encyclopedia. Different colors represent the expression level of TM4SF5 in different cell types (Fig. 1c). Since there is evidence that TM4SF1 is correlated to the occurrence and development of colon cancer and poor prognosis, the investigators assessed the associations between the TM4SF5 expression at the mRNA level and TM4SF1 in human CRC tissues. It was found that the TM4SF5 mRNA expression level was positively and significantly correlated with TM4SF1 (Fig. 1d, $P < 0.05$), suggesting the certain similarity of the potential biological function of TM4SF5 with TM4SF5 in human CRC.

Figure 1 Expression of TM4SF5 based on TCGA data and Encyclopedia of tumor Cell Lines at mRNA level in Human Colorectal Cancer. Based on TCGA data from <http://gepia.cancer-pku.cn/>, we compared the TM4SF5 expression at mRNA level between CRC tissues and adjacent normal tissues. **a** TM4SF5 expression in human CRC tissues was found compared with adjacent normal tissues. **b** Higher expression in human CRC tissues was found compared with adjacent normal tissues ($P < 0.05$). **c** The image of TM4SF5 expression level in colorectal cancer cells is between several cancer cell lines. The image was downloaded from The Cancer Cell Line Encyclopedia. Different colors represent the expression level of TM4SF5 in different cell types. **d** The TM4SF5 mRNA expression level was positively and significantly correlated with TM4SF1 ($P < 0.05$).

Immunostaining of the TM4SF5 expression in human CRC tissues and its clinical implications

The investigators used immunohistochemistry assay to detect the expression of TM4SF5, and determine whether that the expression of TM4SF5 in CRC tissues was high or low (Fig. 2a,2b). Similar to the expression pattern of TM4SF5 at the mRNA expression level obtained from the TCGA data, the staining intensity of TM4SF5 in human CRC tissues was significantly higher than that in adjacent normal tissues (Fig. 2c, $P < 0.0001$). The results of the survival analysis revealed the overall expression of TM4SF5 in colon cancer (Fig. 2d, $P = 0.048$). As shown in Table 1, it was found that there was no correlation between the expression of TM4SF5 and the clinical features. Furthermore, as shown in Table 2, the COX model also revealed whether the expression of TM4SF5 was high or low (univariate analysis, pooled HR = 1.857, 95% CI: [1.13–3.05], $P = 0.015$, multivariate analysis, pooled HR = 4.038, 95% CI: [2.15–7.584], $P < 0.001$), and the clinical stage (univariate analysis, pooled HR = 0.423, 95% CI: [0.258–0.695], $P < 0.001$, multivariate analysis, pooled HR = 0.218, 95% CI: [0.057–0.828], $P = 0.025$). The COX model also revealed that the TM4SF5 expression level can be used as an independent risk factor for prognostic prediction for these patients ($P < 0.05$).

Table 2
Cox model analysis for the association between the TM4SF5 expression level and patients' clinical parameters

Variables	Univariate		Multivariate	
	HR (95%CI)	P	HR (95%CI)	P
TM4SF5 expression(high/low)	1.857 (1.130–3.050)	<i>0.015</i>	4.038 (2.150–7.584)	<i>< 0.001</i>
Gender(male/female)	1.134(0.702–1.832)	0.607	1.242 (0.734–2.103)	0.419
Age(< 65/≥65 years)	1.176(0.724–1.909)	0.512	1.076 (0.625–1.852)	0.792
Pathological grade(I + II/III)	0.692(0.329–1.458)	0.333	1.134 (0.486–2.644)	0.771
Tumor size(< 5/≥5 cm)	1.022 (0.619–1.687)	0.932	1.495 (0.838–2.666)	0.173
Clinical stage (I + II/III + IV)	0.423(0.258–0.695)	<i>< 0.001</i>	0.218 (0.057–0.828)	<i>0.025</i>
T stage	0.990 (0.606–1.617)	0.968	1.302 (0.760–2.230)	0.337
N stage	0.556 (0.331–0.933)	<i>0.026</i>	1.167 (0.393–3.462)	0.781
M stage	0.457 (0.253–0.826)	<i>0.009</i>	0.751 (0.295–1.908)	0.547
^a Italics signifies <i>P</i> < 0.05.				

Figure 2 TM4SF5 expression in human CRC tissues. **a** The positive staining of TM4SF5 could be predominantly found in the cytoplasm and on the membrane of the cancer cells in CRC tissues, weak staining of TM4SF5 was also found in CRC tissues. **b** The positive and weak staining of TM4SF5 was found in adjacent normal tissues. **c** The staining intensity of TM4SF5 in human CRC tissues was significantly higher than that in adjacent normal tissues (*P* < 0.0001). **d** The overall survival rate of CRC patients with lower TM4SF5 expression was significantly higher than that of the patients with higher TM4SF5 expression (*P* = 0.048).

The Expression Of Tm4sf5 In The Cell Lines

According to the Human Protein Atlas data obtained from <https://www.proteinatlas.org/>, the investigators initially compared the TM4SF5 expression at the expression level, among the human single cell types (Figs. 3a, 3b). In order to detect the expression of TM4SF5 in CRC cells, the investigators detected the mRNA expression of TM4SF5 in CRC cell lines LoVo, SW480, RKO and HCT-116, and human normal

colonic epithelial cell line CCD-841CON by RT-qPCR. It was found that the expression level of TM4SF5 in other four CRC cell lines was higher than that in normal colonic mucosal cell line CCD-841CON. The human normal colonic mucosal epithelial cell lines and the other four kinds of CRC cell lines were compared, and the difference was statistically significant (Fig. 3c, LoVo vs. CCD-841CON, 4.394 ± 1778 vs. 1.000 ± 0.063 , $P < 0.05$, SW480 vs. CCD-841CON 10.794 ± 3.630 vs. 1.000 ± 0.063 , $P < 0.01$, RKO vs. CCD-841CON, 1.730 ± 0.366 vs. 1.000 ± 0.063 , $P < 0.05$, HCT-116 vs. CCD-841CON, 2.879 ± 1.000 vs. 1.000 ± 0.063 , $P < 0.05$). Based on the high expression level of TM4SF5 in the LoVo and SW480 cell lines, the investigators selected these two cell lines for the transfection experiment. The siRNA technique was used to knockdown the expression of TM4SF5 in LoVo and SW480 cells, and these were divided into the TM4SF5-sh group and NC group. Then, the knockdown efficiency was determined by RT-PCR analysis. The present data revealed that the mRNA expression level of TM4SF5 significantly decreased in the TM4SF5-sh group (Figs. 3d, 3e, $P < 0.01$, in LoVo cells and SW480). At the same time, the investigators ruled out the effect of Lipofectamin 2000 (Mock group) on the cell lines ($P > 0.05$).

Figure 3 Expression of TM4SF5 in cells lines. **a** We compared the TM4SF5 expression at single cell types according to The Human Protein Atlas data from <https://www.proteinatlas.org/>. **b** The Human Protein Atlas data showed that higher expression in intestinal endocrine cells was found compared with other single cell types. **c** The RT-PCR analysis showed that the mRNA expression level of TM4SF5 was significantly increased in the CRC cell compared with normal cell respectively. **d, e** The knockdown efficiency was confirmed by real-time RT-PCR analysis, and the data showed that the mRNA expression level of TM4SF5 was significantly decreased in the TM4SF5-sh ($P < 0.01$ in LoVo cells and SW480) compared with NC group cells respectively. The mRNA expression level of TM4SF5 was no significant in the Mock group ($P > 0.05$ in LoVo cells and SW480) compared with NC group cells.

The effect of TM4SF5 on the proliferation of colon cancer cells

LoVo and SW480 cells were selected for the proliferation experiment. The proliferation level in the TM4SF5-sh and NC groups was measured by CCK-8. The CCK-8 assay results revealed that the absorbance of LoVo cells was significantly lower in the TM4SF5-sh group than in the NC group (0.533 ± 0.020 vs. 1.017 ± 0.053) at the time point of 96 hours (Fig. 4a, $P < 0.0001$), and that of SW480 cells was also significantly lower in the TM4SF5-sh group than in the NC group (0.748 ± 0.085 vs. 1.812 ± 0.087) at the time point of 96 hours (Fig. 4b, $P < 0.0001$). This suggests that the knockdown of the TM4SF5 expression can significantly inhibit the proliferation of LoVo and SW480 cells.

Figure 4 CCK-8 assay was used to compare the proliferation ability of stably transfected cells in each group. **a, b** Our CCK-8 assay showed that the cell proliferation ability of CRC cell lines was significantly decreased in the TM4SF5-sh ($P < 0.0001$ in LoVo and SW480) compared with the NC group cells.

The effect of TM4SF5 on the migration ability of colon cancer cells

The scratch test was used to study the effect of TM4SF5 on the migration ability of colon cancer cells. These two kinds of cells were assigned to the TM4SF5-sh and NC group, and the migration ability of

these two groups was observed under a microscope (Fig. 5a). The results revealed that the mobility of LoVo cells was significantly lower in the TM4SF5-sh group than in the NC group (0.039 ± 0.041 vs. 0.321 ± 0.090) at the time point of 24 hours (Fig. 5b, $P < 0.01$). Furthermore, the mobility of SW480 cells was also significantly lower in the TM4SF5-sh group than in the NC group (0.100 ± 0.078 vs. 0.447 ± 0.159) at the time point of 24 hours after scrape (Fig. 5b, $P < 0.01$). This suggests that the knockdown of the TM4SF5 expression can significantly inhibit the migration ability of LoVo and SW480 cells.

Figure 5 Effect of TM4SF on migration ability of colon cancer cells LoVo and SW480. **a** Observation of scratch healing under microscope. **b, c** The migration ability of the CRC cell lines after knockdown TM4SF5 expression in the TM4SF5-sh groups was examined compared with the NC group cells. At the time point of 24 h, the relative migration ratio of TM4SF5-sh ($P < 0.01$ in LoVo and SW480) was significantly decreased compared with the NC group cells.

The effect of TM4SF5 on the invasive ability of colon cancer cells

Invasion assay was used to study the effect of TM4SF5 on the invasive ability of colon cancer cells. The invasive ability of these two groups was observed under a microscope (Fig. 6a). The results revealed that the number of LoVo cells per visual field was significantly lower in the TM4SF5-sh group than in the NC group (24.58 ± 23.39 vs. 277.33 ± 113.00) at the time point of 48 hours (Fig. 6b, $P < 0.01$). Furthermore, the number of SW480 cells per visual field was also significantly lower in the TM4SF5-sh group than in the NC group (67.86 ± 39.32 vs. 310.00 ± 195.43) at the time point of 48 hours (Fig. 6b, $P < 0.01$). This suggests that the knockdown of the TM4SF5 expression can significantly inhibit the invasive ability of LoVo and SW480 cells.

Figure 6 Effect of TM4SF on invasion and metastasis of colon cancer cells LoVo and SW480. **a** The cell membrane penetration was observed under microscope. **b, c** Transwell assay showed that the cell invasive ability of CRC cell lines was significantly decreased in the TM4SF5-sh ($P < 0.01$ in LoVo and SW480) compared with the NC group cells.

The effect of TM4SF5 on the cell cycle of colon cancer cells

Cell cycle assay was used to study the effect of TM4SF5 on the phase of colon cancer cells. The cell cycle was analyzed in the two groups by Flowjo (Fig. 7a). The cell cycle assay revealed that LoVo cells were significantly higher in the TM4SF5-sh group than in the NC group (45.80 ± 4.08 vs. 54.96 ± 0.96) at the G1 phase (Fig. 7b, $P < 0.05$), while LoVo cells were significantly lower in the TM4SF5-sh group than in the NC group (53.5 ± 4.34 vs. 42.56 ± 0.05) at the G2/M phase (Fig. 7b, $P < 0.05$), LoVo cells in the TM4SF5-sh group was not significant difference with that in the NC group (3.38 ± 1.06 vs. 2.07 ± 1.56) at the S phase (Fig. 7b, $P > 0.05$). The cell cycle assay revealed that SW480 cells were significantly higher in the TM4SF5-sh group than in the NC group (67.76 ± 1.80 vs. 71.83 ± 1.62) at the G1 phase (Fig. 7c, $P < 0.05$), and SW480 cells were significantly lower in the TM4SF5-sh group than in the NC group (36.33 ± 9.15 vs. 15.03 ± 4.30) at the G2/M phase (Fig. 7c, $P < 0.05$), while SW480 cells in the TM4SF5-sh group were no significantly difference with that in the NC group (1.85 ± 14.07 vs. 10.03 ± 8.75) at the S phase

(Fig. 7c, $P > 0.05$). This suggests that the expression of TM4SF5 in colon cancer cells can lead to G1 phase arrest.

Figure 7 Effect of TM4SF5 on cell cycle of colon cancer cells. **a** flow cytometry analysis of cell cycle distribution. **b, c** The knockdown TM4SF5 expression significantly increased the ratio of G1 phase and induced cell cycle arrest in human CRC cell lines.

Discussion

The evolution of tumor is related to the complex microenvironment, and the tumor microenvironment is related to various signal pathways. Therefore, mastering the receptor-mediated signaling pathways involved in the occurrence and development of cancer is very important for cancer-specific treatment. The carcinogenic pathways reported in the literature include epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGF / c-Met) and vascular endothelial growth factor (VEGF) pathway [19–23]. Mutations in genes associated with these receptor signal transduction pathways are usually observed in several cancers [23, 24]. In the past decade, inhibitors and antibodies against these receptors have been extensively studied to block the occurrence and development of cancer [25–29].

At present, 33 members of the four transmembrane protein superfamily (TM4SF) have been identified in mammals, including TM4SFI, TM4SF4, TM4SF5 and TM4SF20 [30, 31]. These proteins have different sequence homology, and can be widely involved in many life activities, such as cell growth, adhesion and migration. Transmembrane 4L6 family member 5 (TM4SF5) is a membrane glycoprotein with four transmembrane domains. Transmembrane 4L6 family member 5 (TM4SF5) can form tetraspanin-rich microdomain (TERMs) on the cell surface. TERM contains a protein-protein complex that comprises of four transmembrane proteins, growth factor receptors, and integrins [32]. These complexes can regulate the signal transduction between inside and outside cells, affect the interstitial change of epithelium and change in the EMT, and enhance cell migration and invasion. TM4SF5 activates transforming growth factor- β 1 (TGF- β 1) signal transduction by binding to epidermal growth factor receptor (EGFR). TM4SF5 plays a key role in tumorigenesis and metastasis by binding to different types of membrane proteins or receptors, or even to complex intracellular signal molecules. Therefore, tetra-protein has attracted increasing attention as a diagnostic and prognostic marker, and a therapeutic target for the prevention of tumor progression [15].

TM4SF5 plays a key role in tumorigenesis [33, 34]. In human hepatocytes, the ectopic expression of TM4SF5 enhances the phosphorylation of FAK, which leads to the inactivation of RhoA transforming protein [13]. In addition, TM4SF5 induced the stabilization of p27kip1, which may be an inhibitor of the RhoA signaling pathway [35]. Therefore, TM4SF5-mediated RhoA inactivation leads to the process of EMT, which in turn leads to tumor cell migration, invasion and proliferation, due to the loss of contact inhibition [36]. In the process of tumor immune escape, evidence has revealed that the interference between the TM4SF5/FAK pathway and IL-6 (IL-6)/signal transducer and transcriptional activator 3 pathway can promote metastatic potential by reducing the expression level of IL-6, and preventing its

immune function. The overexpression of TM4SF5 in gastric cancer leads to the entry of CD63 into the late lysosomal membrane, and the removal of CD63 from the cell surface, in order to prevent the inhibitory effect of CD63 on migration. The interaction between TM4SF5 and FAK can mediate the activation of FAK. This TM4SF5-mediated activation process appears to involve the conformational release of FAK after cell adhesion, which transduces the signals of the intracellular FAK, c-Src and STAB pathways, in order to regulate cell migration and possible ECM production, thereby promoting the metastasis of cancer cells [37]. TM4SF5 can act as a lysosomal membrane arginine sensor to form complexes with MTOR and SLC38A9 on the lysosomal membrane in an arginine-regulated manner, resulting in arginine efflux, the activation of mTORC1, and the subsequent phosphorylation of RPS6KB1 and EIF4EBP1 [38]. This promotes the progression of the G1/S phase of the cell cycle, and the translocation of the CDK4-CCND1 complex to the nucleus. CDKN1B and RHOA/ROCK signal transduction activities are involved in the acceleration of the TM4SF5-mediated G1/S phase [36].

However, there are few reports on the relationship between TM4SF5 and colorectal cancer. Therefore, the present study detected TM4SF5 by immunohistochemistry and RT-qPCR, and cellular functional experiments. The results revealed that the protein content of TM4SF5 was significantly higher in colorectal cancer tissues than in the corresponding adjacent normal tissues. The results of the RT-qPCR revealed that the mRNA content of TM4SF5 was higher in colorectal cancer.

Conclusion

TM4SF5 is highly expressed in colon cancer tissues, and the overall survival rate was lower in TM4SF5 with a high expression than in TM4SF5 with a low expression. The results of the cell function experiments revealed that TM4SF5 with a low expression can inhibit the proliferation, invasion and migration of colon cancer cells. TM4SF5 may be associated with the occurrence, progression and poor prognosis of colon cancer. Hence, TM4SF5 may become an important molecular marker and potential therapeutic target for colon cancer.

Declarations

Abbreviations

CRC

colorectal cancer, TM4SF5:transmembrane 4 L six family member 5, FBS:fetal bovine serum, DMEM:Dulbecco's modified Eagle's medium, PBS:phosphatebufferedsaline, siRNA:Small interfering RNA, OS:overall survival rate.

Declarations

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Authors' contributions

ZY and HW designed research, ZY, JF, YZ,XX, HH, YL, JJ and QL performed research, ZY and HH performed the statistical analysis, ZY and HW drafted the manuscript. All authors read and approved the final manuscript.

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Unsectioned Paragraphs

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