ABBS

Original Article

Human tetraspanin transmembrane 4 superfamily member 4 or intestinal and liver tetraspan membrane protein is overexpressed in hepatocellular carcinoma and accelerates tumor cell growth

Ying Li[1†], Leiming Wang[1†], Jie Qiu[1], Liang Da[1], Pierre Tiollais[2], Zaiping Li[1], and Mujun Zhao[1*]

[1]State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[2]Unite' d'Organisation Nucleaire et Oncogenese, INSERM U.579, Institut Pasteur, Paris, France
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-21-54921115; Fax: +86-21-54921011; E-mail: mjzhao@sibs.ac.cn

The human transmembrane 4 superfamily member 4 or intestinal and liver tetraspan membrane protein (TM4SF4/il-TMP) was originally cloned as an intestinal and liver tetraspan membrane protein and mediates density-dependent cell proliferation. The rat homolog of TM4SF4 was found to be up-regulated in regenerating liver after two-thirds hepatectomy and overexpression of TM4SF4 could enhance liver injury induced by CCl_4. However, the expression and significance of TM4SF4/il-TMP in liver cancer remain unknown. Here, we report that TM4SF4/il-TMP is frequently and significantly overexpressed in hepatocellular carcinoma (HCC). Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis showed that TM4SF4/il-TMP mRNA and protein levels were up-regulated in ~80% of HCC tissues. Immunohistochemical analysis of a 75 paired HCC tissue microarray revealed that TM4SF4/il-TMP was significantly overexpressed in HCC tissues ($P < 0.001$), and high immunointensity of TM4SF4/il-TMP tended to be in well-to-moderately differentiated HCC compared with poorly differentiated tumors. Functional studies showed that overexpression of TM4SF4/il-TMP in QGY-7701 and BEL-7404 HCC cell lines through stable transfection of TM4SF4 expression plasmid significantly promoted both cell growth and colony formation of HCC cells. Reduction of TM4SF4/il-TMP expression in QGY-7701 and BEL-7404 cells by stably transfecting TM4SF4 antisense plasmid caused great inhibition of cell proliferation. Our findings suggest that TM4SF4/il-TMP has the potential to be biomarker in HCC and plays a crucial role in promotion of cancer cell proliferation.

Keywords   TM4SF4/il-TMP; overexpression; hepatocellular carcinima; proliferation; colony formation

Received: October 10, 2011   Accepted: October 31, 2011

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and its incidence is increasing in association with viral (hepatitis C virus) and non-viral (non-alcoholic steatohepatitis) chronic liver diseases [1]. Despite advances in technology and currently available treatments, there has been little improvement in the overall survival, which is likely due to the fact that most patients are diagnosed at advanced stages. Early diagnosis of HCC should greatly improve its prognosis [2]. However, it is often difficult to distinguish between benign and malignant tumors and thus novel immunohistochemical markers are necessary.

Transmembrane 4 superfamily proteins (TM4SFs, also known as tetraspanins) are a group of small hydrophobic proteins with four transmembrane domains, two short cytoplasmic domains at the NH_2 and COOH termini, and two relatively divergent extracelluar loops, the larger of which contains several conserved CCG amino acid motifs [3,4]. Tetraspanins can interact with a variety of transmembrane and cytosolic proteins, including integrins, growth factor receptors, G-protein-coupled receptors, and their intracellular proteins to form lager complexes termed tetraspanin webs or tetraspanin-enriched microdomains on the cell surface, and play their roles associated with cell adhesion, proliferation, and motility [5–7]. Approximately half of human tetraspanins have been experimentally studied and several such as CD9, CD63, CD82, CD151, and Tspan8 have been shown to correlate with tumor prognosis and regulate tumor progression and metastasis [8].

Human TM4SF4 (also termed as il-TMP) is a member of tetraspanin protein family that is originally identified as a four transmembrane glycoprotein existed in human intestinal epithelium and liver [9], and forms a distinct L6

tetraspanin family with the tumor antigen L6 [10], the pancreatic tumor molecule TM4SF5 [11], and L6D [12]. These four L6 family proteins are structurally similar to tetraspanins, but lack a characteristic pattern of extracellular CCG motif. The L6 family is referred as a branch of the tetraspanin superfamily. Although there is much biochemical and biological information about the tetraspanins, little information is available for L6 family. Both L6 and TM4SF5 have been found to be overexpressed in some cancers including gastric, prostate, breast, liver, and pancreatic cancers, and TM4SF5 mediates uncontrolled growth of HCC cells through epithelial-mesenchymal transition [13,14]. However, the potential oncogenic function of TM4SF4 has not yet been explored.

In this study, we investigated the expression patterns of human TM4SF4 in HCC tissue samples and HCC cell lines, and found that TM4SF4 was overexpressed in HCC cells. Enforced expression of TM4SF4 in liver cancer cell lines could promote cell growth and colony formation. Reduction of TM4SF4 expression resulted in inhibition of cell proliferation. Our findings suggest that TM4SF4 has the potential to be a biomarker for HCC and plays some roles in promotion of cancer cell proliferation.

Materials and Methods

HCC specimens and cell lines

HCC specimens including adjacent non-tumor liver tissues were obtained from Eastern Hepatobiliary Surgery Hospital (Shanghai, China). The specimens were obtained immediately after surgical resection, snap-frozen in liquid nitrogen, and stored in liquid nitrogen until use. All procedures followed in this study were in accordance with the guidelines of the Human Ethics Committee of the Second Military Medical University (Shanghai, China). All cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human HCC cell lines QGY-7701 and BEL-7404 were maintained in RPMI-1640 and DMEM media (Invitrogen, Carlsbad, USA) respectively, supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cell culture was conducted at $37^{\circ}C$ in a humidified 5% CO_2 incubator.

Construction of TM4SF4 expression plasmid

The cDNA fragment containing the entire open reading frame of *TM4SF4* gene (GenBank accession number: NM 004617.3) was amplified from human liver cDNA library (Invitrogen) by polymerase chain reaction (PCR) with primers P1 (5′-GGA ATT CAG AAT GTG CAC TGG GGG CT-3′) and P2 (5′-CCG CTC GAG TAA AAC GGG TCC ATC TCC C-3′). The PCR was performed for 35 cycles with $94^{\circ}C$ for 30 s, $55^{\circ}C$ for 30 s, and $72^{\circ}C$ for

1 min with 0.25 µl ExTaq (Takara, Dalian, China). The PCR product was inserted into pcDNA3 vector (Invitrogen) in two opposite orientations to generate sense pcDNA3–TM4SF4 and antisense pcDNA3–TM4SF4-AS expression plasmids. The constructs were confirmed by DNA sequencing.

Preparation of antibodies against TM4SF4

The cDNA encoding for the secondary extracellular domain (EC2) of TM4SF4 (residues 114–165) was cloned into pGEX-4T1 vector (Amersham Pharmacia Biotech Inc., Quebec, Canada) to express the GST-TM4SF4-EC2 fusion protein in *Escherichia coli* BL21 (DE3) bacterial cells. The GST-TM4SF4-EC2 fusion protein was purified by glutathione agarose column (GE Healthcare, Uppsala, Sweden) and used to immunize rabbit. The antisera were purified with Hybond-C membrane (Amersham).

Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from the tissue samples by using TRizol reagent (Invitrogen) and reverse transcribed into cDNA by using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using a 7500 Real-time PCR System with a SYBR Green kit (Applied Biosystems, Foster City, USA). The following primer sets were used: (i) for *TM4SF4:* TM4SF4-F, 5′-CTTCCACGACGGGGATTAT-3′ and TM4SF4-R, 5′-ATTGTAGTCGTCATGCTGTAGAGTC-3′; (ii) for *B2M* (β2-microglobulin): B2M-F, 5′-CCAGCAGAGAATGG AAAGTC-3′ and B2M-R, 5′-GATGCTGCTTACATG TCTCG-3′. The B2M levels were quantified as an internal control. The mRNA levels were determined by using 7500 Fast System SDS software (Applied Biosystems). Each reaction was repeated independently at least three times in triplicate.

Western blot analysis

The human tissue samples were lysed in RIPA lysis buffer (Thermo, Rockford, USA) containing 1% protease inhibitor cocktail (Sigma-Aldrich, St Louis, USA) and solubilized in SDS loading buffer. The cultured cells were directly solubilized in SDS loading buffer. Equal amount of protein extracts was separated by SDS–PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). Western blot analysis was performed with the standard method with anti-TM4SF4 polyclonal antibody (prepared by our laboratory) or an anti-tubulin monoclonal antibody (T5168; Sigma-Aldrich). After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA), membranes were detected with enhanced chemiluminescence reagent

(Thermo scientific, Dreieich, Germany) and exposed to X-ray film (Kodak, Rochester, USA).

Tissue microarray and immunohistochemical staining

The tissue microarray (catalog no. HLiv-HCC150CS-01) containing 75 pairs of human HCC samples were purchased from Outdo Biotech (Shanghai, China). Two samples, for each pair, one from the HCC (T) and another from adjacent normal liver tissue (N), were identified. TM4SF4 expression was assessed by immunohistochemical staining using an anti-TM4SF4 antibody (prepared by our laboratory). The staining was performed according to the commercial protocol (Outdo Biotech). The protein signal was evaluated by assessing staining intensity using a BX51 microscope (Olympus, Tokyo, Japan) and quantified by evaluating staining intensity with Image-pro plus 6.0 software (Media Cybernetics, Bethesda, USA).

Selection of stable-transfected clones

The QGY-7701 and BEL-7404 HCC cells were transfected with pcDNA3–TM4SF4, pcDNA3–TM4SF4-AS, or pcDNA3 (used as a control) plasmid with Lipofectamine (Invitrogen), respectively. Forty-eight hours after transfection, the aminoglycoside antibiotic G418 (Invitrogen) was added to the medium (300 µg/ml for QGY-7701, 500 µg/ml for BEL-7404) for 3 weeks to select the stable transfected cells. The stable clones were used for subsequent experiments.

Cell proliferation assay

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in a 96-well plate at a density of 2.0×10^3 cells for QGY-7701 stable transfectants and 1.3×10^3 cells for BEL-7404 stable transfectants per well. Twenty microliters of MTT solution (5 mg/ml) (Sigma) was incubated with cells at 37°C for 4 h. The absorbance values were determined using a microplate reader (Pharmacia Biotech, Uppsala, Sweden) at a wavelength of 570 nm. Experiments were performed three times. The growth curves were generated by SigmaPlot graphing software (San Jose, USA).

Colony formation assay

The QGY-7701 and BEL-7404 HCC cell lines were stably transfected with pcDNA3–TM4SF4, pcDNA3–TM4SF4-AS, or pcDNA3 (used as a control) plasmid, respectively. One day after transfection, G418 was added to the culture for screening the stable transfected cells. Following 3 weeks of G418 selection (300 µg/ml for QGY-7701 and 500 µg/ml for BEL-7404), cells were fixed in methanol, stained with 0.1% (w/v) crystal violet and washed with water. The cell colonies were photographed using a microscope (DXM1200F; Nikon, Tokyo, Japan),

and the number of colonies >100 µm in diameter were counted.

Statistical analysis

Statistical analysis was accomplished using SPSS software. Differences between the TM4SF4 expression in tumor tissues and adjacent non-tumor tissues were analyzed by the Wilcoxon matched pairs test. The relationship between TM4SF4 expression and the clinicopathological grade was assessed by the χ^2 test. Quantitative data were presented as the mean ± standard deviation, and statistical significance was determined by Student's t-test. Differences were considered significant when $P < 0.05$.

Results

TM4SF4 mRNA and protein levels are increased in HCC tissue

To measure the expression of TM4SF4 in HCC tissues, we identified *TM4SF4* mRNA levels in 22 pairs of HCC specimens by quantitative real-time RT-PCR (qRT-PCR). Compared with the paired non-tumor tissues (N), *TM4SF4* mRNA was significantly up-regulated in HCC tissues (T) ($P < 0.05$). Among the 22 pairs of samples, 16 (72.7%) showed significant TM4SF4 up-regulation (T/N > 2), 2 showed slight up-regulation (1 < T/N < 2), and 4 showed down-regulation of TM4SF4 (1 < T/N < 0) (**Fig. 1**). In total, the frequency of up-regulated expression of *TM4SF4* mRNA among the HCC tissues was ~80%.

Next, we probed the amount of TM4SF4 protein in 31 pairs of HCC tissues by western blot. The results clearly showed that TM4SF4 protein was expressed much higher in 25 of 31 (80.6%) of the tested HCC samples (**Fig. 2**). While, TM4SF4 protein was undetected or slightly
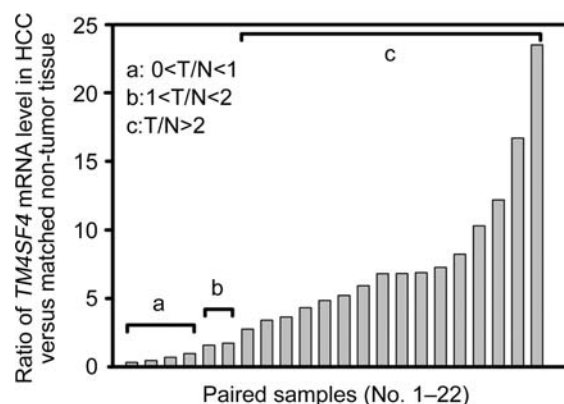

Figure 1 Expression of *TM4SF4* mRNA in pairs of human HCC (T) and adjacent non-tumor (N) tissues The *TM4SF4* mRNA levels in 22 cases were analyzed by quantitative RT-PCR. The ratio of *TM4SF4* mRNA level in HCC tissue to that in non-tumor tissue (T/N) in each case is indicated by a column. The *TM4SF4* mRNA levels were significantly up-regulated in HCC as determined by the Wilcoxon matched pairs test.
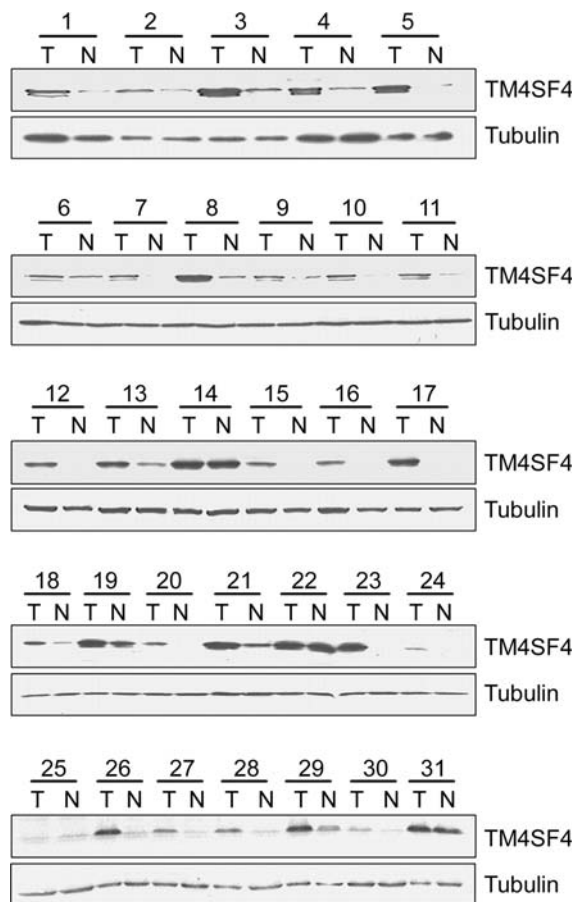
Figure 2 Expression of TM4SF4 protein in pairs of human HCC (T) and adjacent non-tumor (N) tissues TM4SF4 proteins in 31 cases were analyzed by western blot. β-Tubulin was used as an internal control.

expressed in samples of adjacent non-tumor tissue. These results indicate that TM4SF4 is overexpressed in HCC samples.

Systematic tissue microarray analysis reveals that the TM4SF4 protein immunointensity is increased in HCC tissue

We further examined TM4SF4 expression in HCC by immunohistochemistry on a tissue microarray which contained paired HCC tissues from 75 patients. In this screening, the immunointensity of TM4SF4 was significantly increased in HCC tissues (T) compared with the paired non-tumor tissues (N) ($P < 0.001$). Among the 75 pairs of samples, 47 (62.7%) of the HCC samples showed strong TM4SF4 up-regulation (T/N > 2), 12 (16%) showed slight TM4SF4 up-regulation (1 < T/N < 2), and 16 (21.3%) showed no up-regulation of TM4SF4 (0 < T/N < 1) [**Fig. 3(A)**]. In total, the frequency of up-regulated expression of TM4SF4 among the HCC tissues was ~78%. Representative staining data indicating TM4SF4 up-regulation in HCC are shown in **Fig. 3(B)**. The detailed information including the immunointensity of TM4SF4

and immunostaining data of all 75 pairs of samples were showed in **Supplementary Table S1** and **Supplementary Figure S1**. Taken together, our results demonstrate that TM4SF4 is overexpressed in HCC with statistical significance.

Overexpression of TM4SF4 correlates with HCC progression

We further analyzed the correlation between the increased TM4SF4 expression and the clinicopathological parameters among the 75 patients with HCC based on the results from the tissue microarray analysis. There were 64 men and 11 women included with age ranging from 14 to 73 years old (the median, 53 years). Based on size, 74 HCC tumors were subdivided into two categories: <5 cm ($n = 43$; 58.1%) and ≥5 cm ($n = 31$; 41.9%) in maximum diameter. The results did not reveal any significant association between TM4SF4 expression and the parameters including age, gender, and tumor size (**Table 1**). We also subdivided tumors in terms of histological grades and TNM (tumor-lymph node-metastasis) stages. All the 75 HCC samples were classified into three histological grades including well differentiated grade I, moderately differentiated grade II, and poorly differentiated grade III according to Edmondson–Steiner grading methods. Among these cases, 52 (69.3%) were well-to-moderately differentiated (grades I, I–II, and II), 23 were poorly differentiated (grades II–III and III) (**Supplementary Figure S2**). The results indicated that the well and moderately differentiated tumors tended to have stronger TM4SF4 immunointensity than poorly differentiated tumors ($P = 0.038$, **Table 1**). According to TNM cancer staging system (tumors with lymph node invasion or distant metastasis), the 75 HCC cases were classified into TNM stage I ($n = 23$; 30.7%), II ($n = 26$; 34.7%), III ($n = 23$; 30.7%), and IV ($n = 3$; 4%), the later stage should be with more severe metastasis. The results showed that strong immunointensity of TM4SF4 were in early stages rather than in late stages ($P = 0.033$, **Table 1**). Combined all together, it suggests that overexpression of TM4SF4 is significantly correlated with HCC differentiation grades and stages. The higher expression of TM4SF4 occurred in the early period of HCC progression. It implies that expression level of TM4SF4 may serve for early diagnosis and prognostic prediction of HCC.

Analysis of TM4SF4 protein level in human cell lines

Human TM4SF4 (il-TMP) was originally identified as a gene product expressed in adult human liver and jejunum tissues and was undetectable in other tissues including stomach, kidney, lung, skeletal muscle, and heart [9]. Here we examined the expression of TM4SF4 in 24 human cell lines by western blot (**Fig. 4**). The human embryonic kidney cell line 293T expressing TM4SF4 after transfection
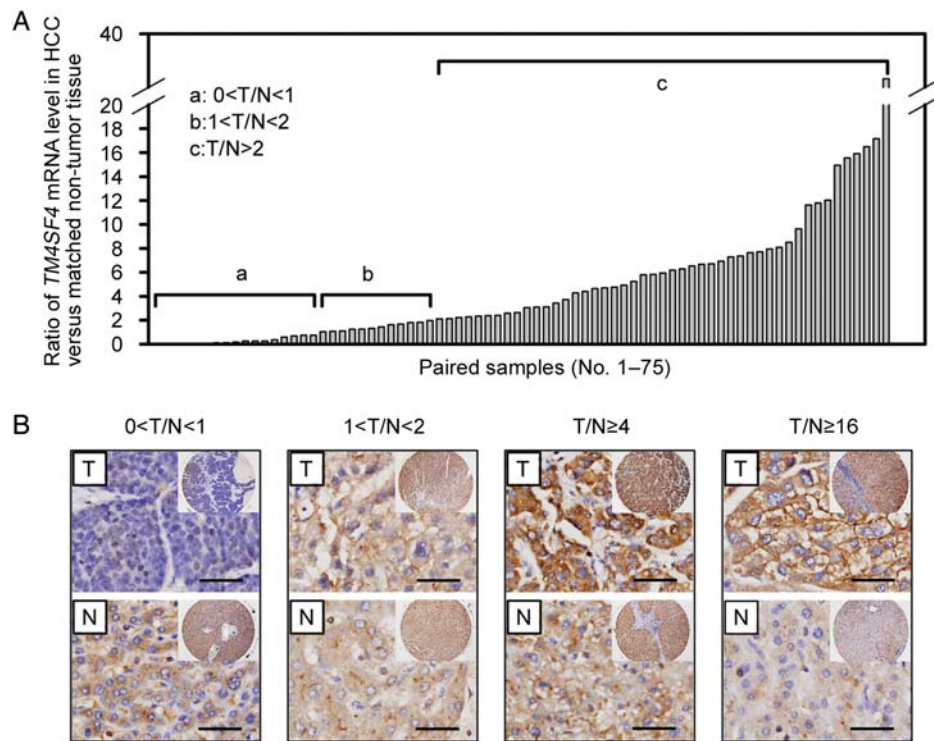
Figure 3 TM4SF4 protein levels in human HCC tissue microarray (A) A human HCC tissue microarray containing 75 pairs of HCC (T) and adjacent non-tumor (N) tissues was analyzed by immunohistochemical staining with the purified anti-TM4SF4 antibody. The ratio of TM4SF4 protein level in HCC tissue to that in non-tumor tissue (T/N) in each case is indicated by a column. The TM4SF4 protein level was significantly increased in HCC as determined by the Wilcoxon matched pairs test. (B) Representative immunostaining of TM4SF4 from the tissue microarray showed the increased expression of TM4SF4 in paired HCC tissues. Scale bar = 200 μM.

Table 1 Statistical correlation between the immunointensity of TM4SF4 and each clinicopathological parameter in 75 patients with HCC

		TM4SF4 immunointensity			
	Total	Weak N (%)	Moderate N (%)	Strong N (%)	P value
Age (years)					0.270
≤53	36	10 (27.8)	15 (41.7)	11 (30.6)	
>53	39	11 (28.2)	10 (25.6)	18 (46.2)	
Gender					0.372
Male	64	16 (25.0)	22 (34.4)	26 (40.6)	
Female	11	5 (45.5)	3 (27.3)	3 (27.3)	
Maximal tumor size (cm)					0.376
<5	43	9 (20.9)	16 (37.2)	18 (41.9)	
≥5	31	11 (35.5)	9 (29.0)	11 (35.5)	
Differentiation					0.038*
Well to moderate	52	10 (19.2)	19 (36.5	23 (44.2)	
Poor	23	11 (47.8)	6 (26.1)	6 (26.1)	
TNM stage					0.033*
I	23	5 (21.7)	5 (21.7)	13 (56.5)	
II	26	5 (19.2)	11 (42.3)	10 (38.5)	
III	23	8 (34.8)	9 (39.1)	6 (26.1)	
IV	3	3 (100.0)	0 (0.0)	0 (0.0)	

TNM, tumor-lymph node-metastasis. P values were derived using the χ^2 test.
*$P < 0.05$.

of pcDNA3-Flag-TM4SF4 plasmid was used as positive control. The TM4SF4 protein was abundantly expressed in liver cancer cell lines (BEL-7404, HepG2, Huh-7, and Hep3B) and stomach adenocarcinoma cell BGC-823, moderately expressed in hepatoma cell lines QGY-7701 and BEL-7402, gastric adenocarcinoma cell lines SGC-7901. The TM4SF4 was low in normal liver cell line Chang, gastric adenocarcinoma cell line MKN45, and neuroblastoma cell line SK-N-SH. However, TM4SF4 was undetectable in other examined cell lines (**Fig. 4**). The results indicated that TM4SF4 was overexpressed in gastric and liver cancer cell lines. Interestingly, among the nine liver cell lines, TM4SF4 was highly expressed in six hepatoma cell lines (BEL-7404, BEL-7402, QGY-7701, Huh-7, HepG2, and Hep3B) and was lower or unexpressed in three normal liver (QSG-7701, Chang, and L02) and one hepatoma (SMMC7721) cell lines. These data support the findings of that TM4SF4 is up-regulated in liver cancer cells.

Overexpression of TM4SF4 promotes the proliferation of HCC cells and reduction of TM4SF4 leads to inhibition of cell growth

To evaluate the influence of TM4SF4 expression on HCC cell proliferation, the QGY-7701 and BEL-7404 HCC cell lines were stably transfected with pTM4SF4 to overexpress TM4SF4, with antisense plasmid pTM4SF4-AS to knockdown TM4SF4 expression, or with pcDNA3 as control, respectively. QGY-7701 and BEL-7404 cell lines were chosen for moderate and high expression of endogenous TM4SF4. Expression level of TM4SF4 in the mixed stable transfectants was examined by western blot. The results demonstrated that TM4SF4 protein level was greatly increased in pTM4SF4 transfected cells (QGY-7701/

pTM4SF4 and BEL-7404/pTM4SF4) and reduced in pTM4SF4-AS transfected cells (QGY-7701/pTM4SF4-AS and BEL-7404/pTM4SF4-AS) [**Fig. 5(A)**].

The cell proliferation was measured using MTT assay. The results showed that the growth rate of TM4SF4-overexpressed QGY-7701 cells (QGY-7701/pTM4SF4) increased during culture at 5th and 6th day compared with QGY-7701/pcDNA3 control cells ($P < 0.01$). TM4SF4-overexpressed BEL-7404 cells (BEL-7404/pTM4SF4) also showed higher growth rate compared with control BEL-7404/pcDNA3 cells during culture at 5th and 6th days ($P < 0.05$, $P < 0.01$) [**Fig. 5(B)**]. The results indicated that overexpression of TM4SF4 significantly promoted HCC cell proliferation.

In the mean time, as TM4SF4 expression was inhibited by pTM4SF4-AS, the growth rate of QGY-7701/pTM4SF4-AS cells decreased at 5th and 6th days culture compared with QGY-7701/pcDNA3 control cells ($P < 0.001$), the growth rate of BEL-7404/pTM4SF4-AS cells was also reduced significantly compared with control cells at 5th and 6th days culture ($P < 0.001$) [**Fig. 5(B)**]. These results demonstrated that reduction of TM4SF4 expression resulted in significant inhibition of cell proliferation, suggesting that TM4SF4 may be a target for inhibition of HCC cell proliferation.

Effects of TM4SF4 expression on colony formation of HCC cell lines

To further identify the role of TM4SF4 in regulation of cell proliferation, the stable transfectants with TM4SF4 overexpression or down-regulation were analyzed by colony formation assay (**Fig. 6**). In QGY-7701 cells, TM4SF4 overexpression (QGY-7701/pTM4SF4 cells) produced a 2-fold increase of colony number ($P < 0.05$,
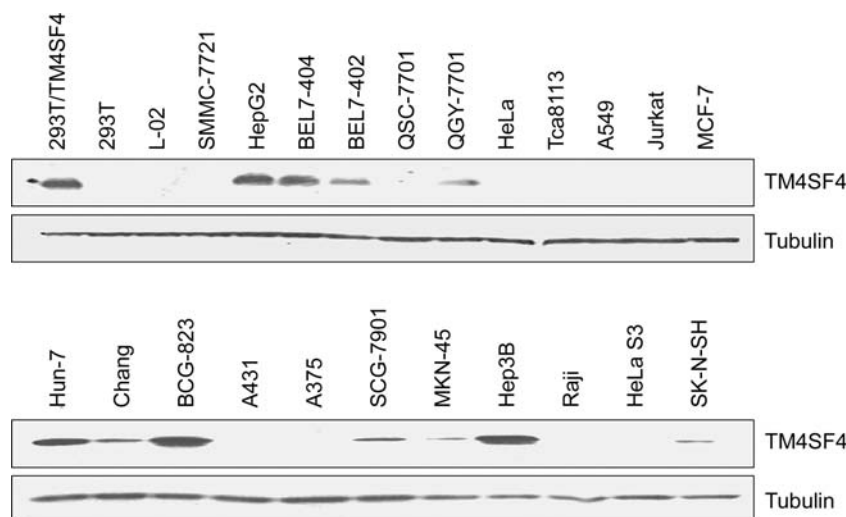
Figure 4 Analysis of TM4SF4 protein levels in human cell lines TM4SF4 protein expression in 24 of human cell lines was measured by western blot. β-Tubulin was used as an internal control. 293T cell line transfected with TM4SF4 expression plasmid was used as positive control.
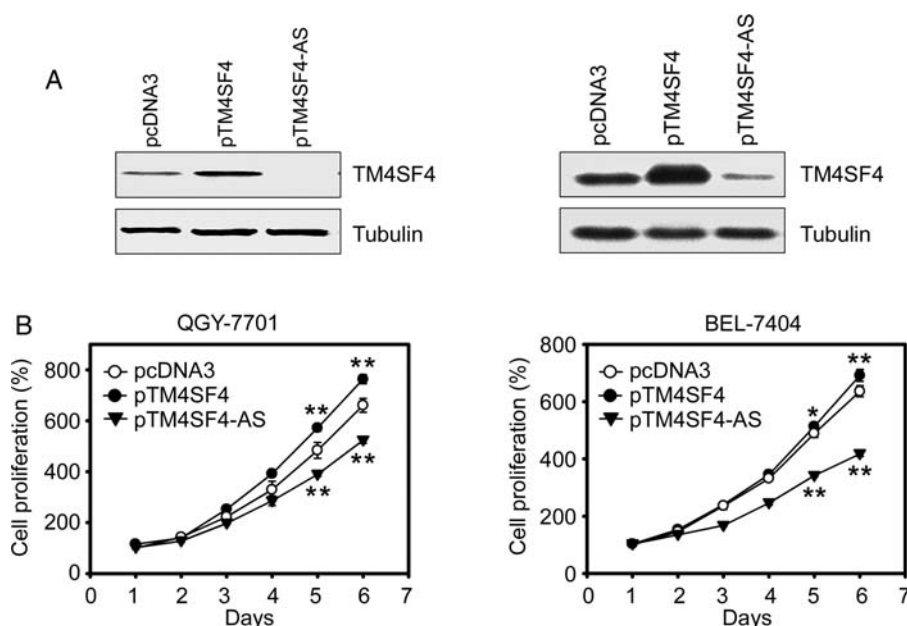
Figure 5 Effects of TM4SF4 on the proliferation of human QGY-7701 and BEL-7404 HCC cells (A) Overexpression of TM4SF4 in cells by stable transfection of pTM4SF4 and knockdown of TM4SF4 expression by stable transfection of pTM4SF4-AS were determined by western blot analysis. The cells transfected with pcDNA3 vector was used as control. (B) Cell proliferation was determined by MTT assay. Values are given as mean $\pm$ standard deviation of three independent experiments. The difference in cell growth during culture on day 5 and 6 was analyzed. $*P < 0.05$, $**P < 0.01$ vs. pcDNA3 transfectants (Student's t-test).
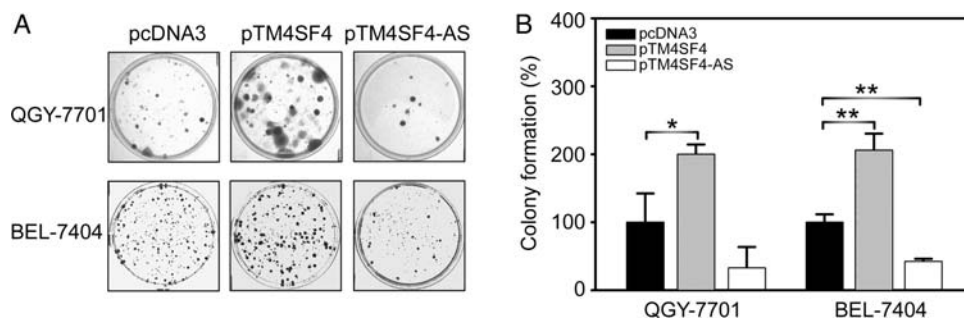

Figure 6 Effects of TM4SF4 on colony formation of human QGY-7701 and BEL-7404 HCC cells (A) QGY-7701 and BEL-7404 cells were transfected with pTM4SF4, pTM4SF4-AS, or the control vector pcDNA3. After 3 weeks of selection with G418, the colonies were visualized by staining with Giemsa. (B) Quantitative analysis of the relative colony numbers. Data are presented as percentage of colony numbers compared with pcDNA3 transfected control cells. Vertical bars represent mean $\pm$ standard deviation of three independent experiments. $*P < 0.05$ and $**P < 0.01$ vs. pcDNA3 transfected cells (Student's t-test).

vs. QGY-7701/pcDNA3). However, reduction of TM4SF4 expression (QGY-7701/pTM4SF4-AS) resulted in decreased colony number, but with no significance ($P = 0.089$, vs. QGY-7701/pcDNA3). In BEL-7404 cells, TM4SF4 overexpression (BEL-7404/pTM4SF4) lead to 2-fold increase of colony numbers ($P = 0.002$, vs. BEL-7404/pcDNA3), and knockdown of TM4SF4 (BEL-7404/pTM4SF4-AS) resulted in significant decrease of colony numbers ($P = 0.001$, vs. BEL-7404/pcDNA3). Our data clearly demonstrated that overexpression of TM4SF4 in cancer cell lines increased the colony formation and reduction of TM4SF4 inhibited the clonogenic

ability of HCC cells, suggesting a role of TM4SF4 in accelerating malignant cell growth. TM4SF4 may be able to serve as a potential target for inhibition of HCC.

Discussion

The human *TM4SF4* was originally cloned as an intestinal and liver tetraspan membrane protein (il-TMP). The il-TMP protein can mediate density-dependent cell proliferation [9]. The rat homolog of TM4SF4 was identified as a gene up-regulated in regenerating liver after two-thirds hepatectomy [15]. Further study indicated that rat TM4SF4

was overexpressed in acutely injured liver induced by CCl$_4$ and played a crucial role in accelerating liver injury [16]. Although the evidence provided implied that TM4SF4 associates with cell proliferation, there are few reports on the functions of TM4SF4. Here, we showed for the first time that TM4SF4 was overexpressed in HCC. The overexpression of TM4SF4 occurred in at least 70% of HCC tissues comparing with their adjacent non-tumor tissues. The immunohistochemistry survey of HCC tissue microarray further demonstrated that expression level of TM4SF4 protein in HCC correlated with the tumor progression. Our findings suggest an important function of human TM4SF4 in malignant cells.

Recently, tetraspanins have received attention as both metastasis suppressors and promoters [7]. For instance, the tetraspanins CD82 and CD9 are down-regulated in tumors and mostly suppress tumor progression, and CD151 and Tspan8 are overexpressed in several tumors and seem to support tumor progression [8]. CD82 was found to directly bind to Duffy antigen receptor for chemokines on vascular endothelium, thereby leading to metastasis suppression [17]. CD9 level was also reported to correlate inversely with the progression of several hematopoietic malignancies [18], and transduction of CD9 and CD82 suppressed lymph node metastasis in lung cancer mouse model [19]. Tspan8 or CO-029 was originally identified as a tumor-associated antigen [20]. CD151 is known to be functionally linked to cancer metastasis. Increased expression of CD151 is indicative of a poor prognosis in different cancer types. CD151 has emerged as a diagnostic and prognostic marker and a therapeutic target for cancers [21,22]. We demonstrated that the mRNA and protein levels of human TM4SF4 were overexpressed significantly in HCC, implying that TM4SF4 might be served as a biomarker for HCC. Further studies of TM4SF4 expression in extensive clinical cancer samples or cancer types are helpful to value the importance of TM4SF4 as a diagnostic marker for cancer.

The sequence analysis has revealed that TM4SF4 has highly significant sequence similarity to L6 and TM4SF5. The L6, as a tumor associated antigen, is expressed on most of human tumors such as lung, colon, breast, ovarian and prostate carcinomas, and modulates tumor cell migration and metastasis. L6 has generated interest as a target for antibody-mediated therapy [23,24]. TM4SF5 is overexpressed in soft tissue sarcoma, gastric cancer and pancreatic tumor, and supposed to be useful in a clinical setting for tumor diagnosis and/or therapy [13]. Similarly, TM4SF4 was overexpressed in liver cancer, and overexpression of TM4SF4 in liver cancer cell lines by transfection led to stimulation of cell proliferation. Inhibition of TM4SF4 expression resulted in the cell growth suppression. Our data indicate that TM4SF4 might play an important role in tumor cell growth and serves as therapeutic target.

However, the precise functions and functional mechanisms of TM4SF4 remain to be further elucidated.

Supplementary Data

Supplementary data are available at *ABBS* online.

Funding

References

1 Sherman M. Hepatocellular carcinoma: new and emerging risks. Dig Liver Dis 2010, 42(Suppl 3): S215–S222.

2 Marrero JA and Welling T. Modern diagnosis and management of hepatocellular carcinoma. Clin Liver Dis 2009, 13: 233–247.

3 Stipp CS, Kolesnikova TV and Hemler ME. Functional domains in tetraspanin proteins. Trends Biochem Sci 2003, 28: 106–112.

4 Hemler ME. Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol 2005, 6: 801–811.

5 Lazo PA. Functional implications of tetraspanin proteins in cancer biology. Cancer Sci 2007, 98: 1666–1677.

6 Levy S and Shoham T. Protein–protein interactions in the tetraspanin web. Physiology (Bethesda) 2005, 20: 218–224.

7 Zoller M. Tetraspanins: push and pull in suppressing and promoting metastasis. Nat Rev Cancer 2009, 9: 40–55.

8 Richardson MM, Jennings LK and Zhang XA. Tetraspanins and tumor progression. Clin Exp Metastasis 2011, 28: 261–270.

9 Wice BM and Gordon JI. A tetraspan membrane glycoprotein produced in the human intestinal epithelium and liver that can regulate cell density-dependent proliferation. J Biol Chem 1995, 270: 21907–21918.

10 Marken JS, Schieven GL, Hellstrom I, Hellstrom KE and Aruffo A. Cloning and expression of the tumor-associated antigen L6. Proc Natl Acad Sci USA 1992, 89: 3503–3507.

11 Gress TM, Wallrapp C, Frohme M, Muller-Pillasch F, Lacher U, Friess H and Buchler M, et al. Identification of genes with specific expression in pancreatic cancer by cDNA representational difference analysis. Genes Chromosomes Cancer 1997, 19: 97–103.

12 Wright MD, Ni J and Rudy GB. The L6 membrane proteins – a new four-transmembrane superfamily. Protein Sci 2000, 9: 1594–1600.

13 Muller-Pillasch F, Wallrapp C, Lacher U, Friess H, Buchler M, Adler G and Gress TM. Identification of a new tumour-associated antigen TM4SF5 and its expression in human cancer. Gene 1998, 208: 25–30.

14 Lee SA, Lee SY, Cho IH, Oh MA, Kang ES, Kim YB and Seo WD, et al. Tetraspanin TM4SF5 mediates loss of contact inhibition through epithelial-mesenchymal transition in human hepatocarcinoma. J Clin Invest 2008, 118: 1354–1366.

15 Liu Z, Zhao M, Yokoyama KK and Li T. Molecular cloning of a cDNA for rat TM4SF4, a homolog of human il-TMP (TM4SF4), and enhanced expression of the corresponding gene in regenerating rat liver. Biochim Biophys Acta 2001, 1518: 183–189.

16 Qiu J, Liu Z, Da L, Li Y, Xuan H, Lin Q and Li F, et al. Overexpression of the gene for transmembrane 4 superfamily member 4 accelerates liver damage in rats treated with CCl4. J Hepatol 2007, 46: 266–275.

17 Bandyopadhyay S, Zhan R, Chaudhuri A, Watabe M, Pai SK, Hirota S and Hosobe S, et al. Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. Nat Med 2006, 12: 933–938.

18 Yoon SO, Zhang X, Freedman AS, Zahrieh D, Lossos IS, Li L and Choi YS. Down-regulation of CD9 expression and its correlation to tumor progression in B lymphomas. Am J Pathol 2010, 177: 377–386.

19 Takeda T, Hattori N, Tokuhara T, Nishimura Y, Yokoyama M and Miyake M. Adenoviral transduction of MRP-1/CD9 and KAI1/CD82 inhibits lymph node metastasis in orthotopic lung cancer model. Cancer Res 2007, 67: 1744–1749.

20 Szala S, Kasai Y, Steplewski Z, Rodeck U, Koprowski H and Linnenbach AJ. Molecular cloning of cDNA for the human tumor-associated antigen CO-029 and identification of related transmembrane antigens. Proc Natl Acad Sci USA 1990, 87: 6833–6837.

21 Voss MA, Gordon N, Maloney S, Ganesan R, Ludeman L, McCarthy K and Gornall R, et al. Tetraspanin CD151 is a novel prognostic marker in poor outcome endometrial cancer. Br J Cancer 2011, 104: 1611–1618.

22 Haeuw JF, Goetsch L, Bailly C and Corvaia N. Tetraspanin CD151 as a target for antibody-based cancer immunotherapy. Biochem Soc Trans 2011, 39: 553–558.

23 Goodman GE, Hellstrom I, Brodzinsky L, Nicaise C, Kulander B, Hummel D and Hellstrom KE. Phase I trial of murine monoclonal antibody L6 in breast, colon, ovarian, and lung cancer. J Clin Oncol 1990, 8: 1083–1092.

24 Kao YR, Shih JY, Wen WC, Ko YP, Chen BM, Chan YL and Chu YW, et al. Tumor-associated antigen L6 and the invasion of human lung cancer cells. Clin Cancer Res 2003, 9: 2807–2816.