Original Article MicroRNA-520f represses non-small cell lung cancer progression by inhibiting TM4SF1

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Abstract: The non-small cell lung cancer (NSCLC) is among the lethal malignancies with high metastasis and recurrence. The microRNAs are classified into noncoding RNAs and dictate various biological processes. However, few reports have focused on miR-520f in NSCLC. In current work, we showed that miR-520f behaved like a tumor suppressor and effectively inhibited NSCLC development. We found decreased miR-520f expression in NSCLC specimens and cell lines. Meanwhile, overexpression of miR-520f could inhibit NSCLC proliferation, migration and invasion. Bioinformatics screening identified transmembrane4-L-six-family-1 (TM4SF1) as a putative target of miR-520f and transfection with miR-520f can significantly downregulate TM4SF1 expression at both mRNA and protein levels. The miR-520f can further decrease VEGF expression via TM4SF1 and fulfill its role in epithelial-mesenchymal transition. We also found that TM4SF1 was upregulated in NSCLC samples. A significantly negative correlation between miR-520f and TM4SF1 has also been identified. Immunohistochemistry also showed enhanced TM4SF1 and VEGF staining in NSCLC tissues compared with normal specimens. These data suggest a tumor suppressive role of miR-520f and may provide novel insight into lung cancer oncogenesis.

Keywords: miR-520f, TM4SF1, VEGF, NSCLC

Introduction

The lung cancer is one of the leading deadly cancers world-wide [1]. Among all the lung cancer patients, over 80% patients have non-small cell lung cancer phenotypes (NSCLC) along with terribly poor prognosis [1]. In China, especially, more than half million patients died from lung cancer each year [2]. The onset of lung cancer can be due to multiple origins [3]. Owing to the serious threat to life, unravelling novel biomarkers such as non-coding RNAs may provide critical insight.

The microRNAs denote a class of noncoding RNAs about 18~22 nt in length which can regulate gene expression by binding the 3'-untranslated regions (3'-UTR) of targets [4, 5]. In NSCLC, microRNAs have been reported to be important biomarkers and may regulate tumorigenesis in different manners. For example, miR-126 can behave like a tumor suppressor and inhibit NSCLC development via targeting PIK3R2 [6]. A novel microRNA named miR-1976 can be used as a prognostic marker in NSCLC as demonstrated by Chen and coworkers [7]. The miR-361-3p also displays tumor suppressive roles in NSCLC while the effect is largely ascribed to SH2B1 targeting [8]. Zhang et al. also confirmed that downregulated is associated with NSCLC and further identifying that miR-663a can suppress NSCLC metastasis [9]. Instead, the miR-410 has shown an oncogenic role in NSCLC by regulating bromodomain-containing protein 7 (BRD7) [10]. Furthermore, miR-224 can also target apoptotic pathways such as caspase-3 and caspase-7 to promote occurrence of NSCLC [11]. Therefore, different microRNAs may play various roles in tumorigenesis of NSCLC and therefore identifying the intricate relations between microRNAs and carcinogenesis represents an important facet in tumor biology. Although numerous reports have focused on the role of microRNAs in

NSCLC, the specific mechanisms about how miR-520f affects NSCLC progression remains poorly understood.

The transmembrane4-L-six-family-1 (TM4SF1) is a member of the TM4SF family proteins which was first identified as an antigen for immunotherapy in lung cancer [12]. The expression of TM4SF1 is raised in various human epithelial carcinomas [13]. TM4SF1 is highly expressed in vascular endothelium of tumor tissues and significantly correlated with tumor incidence [14]. Previous evidence has also shown a critical role of TM4SF1 in regulating motility and proliferation in colon cancer, ovarian cancer, and hepatocellular carcinoma [15, 16]. However, the role of miR-520f and TM4-SF1 in NSCLC remains elusive.

In this work, we identified significantly decreased expression of miR-520f in NSCLC tissues as well as cell lines. Expression of miR-520f can inhibit NSCLC proliferation, migration and invasion. Furthermore, we identified TM4SF1 as a direct target of miR-520f via bioinformatics strategies. We also found that TM4SF1 mRNA expression was up-regulated in NSCLC specimens compared with normal adjacent tissues. TM4SF1 can positively regulate vascular endothelial growth factor (VEGF) expression while miR-520f can inhibit it. We also detected a significantly inverse correlation between TM4SF1 expression and miR-520f levels. Our results demonstrate a critical rule of miR-520f in NSCLC tumorigenesis and may provide a novel therapeutic target for future intervention.

Materials and methods

Cell culture and human specimens

Five NSCLC cell lines (H1299, H1703, H522, H838 and H920) and WI-38 normal lung cell line all were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RMPI-1640 medium with 8% fetal bovine serum (FBS, Sigma, Shanghai, China) plus streptomycin (50 μ g/ml. Sigma, Shanghai, China). The 293T cell line was also obtained from the Shanghai Institute of Cell Biology (Shanghai, China). The surgically resected NSCLC specimens were acquired from the Affiliated Hospital of Jining Medical University from June 2013 to May 2015. All patients have

signed formal consent forms. The research for human samples was approved by Ethics Committee of Affiliated Hospital of Jining Medical University (NO. 2013L008).

MiR-520f mimics and siRNA

The hsa-miR-520f mimics (miR-520f) and negative controls were chemically synthesized by TIANGEN (Shanghai, China). The si-TM4SF1 and TM4SF1 plasmid DNA were commercially synthesized and obtained from TIANGEN (Shanghai, China). Totally 1×10^6 cells per well were seeded into 12-well plates before transfection. The transfection with miR-520f or si-TM4SF1 was performed using Lipofectamine 2000 (TIA-NGEN, Shanghai, China) according to the manufacturer's protocols. The medium was refreshed after 6 h's transfection, and total RNAs and proteins were extracted after 2 days.

RNA extraction and quantitative real-time PCR

Total RNAs were isolated from both NSCLC cell lines (H1299 and H522) and human samples with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The expression of TM4SF1 and VEGF was measured by gRT-PCR using SYBR-Green assays (Applied Biosystems, Foster City, CA, USA) [17]. The cDNA was synthesized from 2 ng of total RNAs with a reverse reaction kit (Promega, Madison, WI, USA). The mixture was maintained in 70°C for 5 min and then the mix containing 5 × RT buffer, 200 U/µl reverse transcriptase, 20 U/µl RNase inhibitor was added (TIANGEN, Shanghai, China). Standard TaqMan protocol was used as previously described [18]. We used GAPDH as the control. Reactions were performed by the ABI PRISM[®] 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. The relative expression was calculated using the 2-ADCt method. Primers used were listed below: 5'-TCGCTAACGCCTATTGC-TT-3' (forward), 5'-TAATGATCCATAGGT-3' (reverse) for TM4SF1; 5'-ACCCATGGATTGGGACCA-A-3' (forward), 5'-ATTAGCATCCTAGTTTTTGC-3' (reverse) for VEGF.

Migration and invasion assays

A 12-well transwell plate (TIANGEN, Shanghai, China) with 8-µm-pore membranes was used to measure the migration and invasion. For migra-



Figure 1. The expression of miR-520f in NSCLC. A. qRT-PCR quantification of miR-520f in 90 NSCLC samples and paired adjacent normal tissues (NT). B. The expression of miR-520f in five NSCLC cell lines (H1299, H1703, H522, H838 and H920) and WI-38 normal lung cell line. **: P < 0.01.

Characteristics	No.	miR-520f level		
		Low (n, %)	High (n, %)	Р
Age				
< 60	47	21 (44.7%)	26 (55.3%)	0.226
≥ 60	43	23 (53.5%)	20 (46.5%)	
Gender				
Male	41	18 (43.9%)	23 (56.1%)	0.257
Female	49	26 (53.1%)	23 (46.9%)	
Tumor size				
≤ 3	38	12 (31.6%)	26 (68.4%)	0.006**
> 3	52	32 (61.5%)	20 (38.5%)	
TNM stage				
0/1	50	17 (34.0%)	33 (66.0%)	0.001**
II-IV	40	27 (67.5%)	13 (32.5%)	
Histological type				
Squamous cell carcinoma	33	13 (39.4%)	20 (60.6%)	0.124
Adenocarcinoma	57	31 (54.4%)	26 (45.6%)	
Metastasis				
Absent	42	15 (35.7%)	27 (64.3%)	0.016*
Present	48	29 (60.4%)	19 (39.6%)	

 Table 1. Correlation between the miR-520f and clinicopathological features

*: *P* < 0.05; **: *P* < 0.01.

tion assay, 1×10^5 transfected cells were suspended in serum-free medium and then plated into upper chambers. For invasion assay, the upper chamber was coated with Matrigel (Invitrogen, Shanghai, China) overnight. The lower chamber was covered with RPMI-1640 medium and 5% FBS as chemo-attractants. After 24 h, the cells on the top were removed by cotton swabs. Migrating cells into the lower chamber were fixed with 3% PFA and stained by 0.2% crystal violet. Leica microscope fluorescent microscope (DM-IRB, Leica, Germany) was used to visualize the images.

Luciferase reporter assay

The TM4SF1 3'-UTR-WT was cloned into the pMIR-GLO luciferase vector (TIANGEN, Shanghai, China). The TM4SF1 3'-UTR MUT was generated using a site-directed mutagenesis kit (TIANGEN, Shanghai, China). The recombinant plasmids were transfected into the 293T cells by lipofectamine 2000 system (Invitrogen, Carlsbad, CA, USA). Luciferase activities were quantified using Dual Luciferase Assay (Promega, Shanghai, China) following the manufacturer's instructions.

Western blot

Cells were lysed with RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS). 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins. The polyvinylidene difluoride (PVDF, TIANGEN, Shanghai, China) membranes were used. Blots were probed with antibodies against TM4SF1, VEGF or GAPDH (Sigma, Shanghai, China). After being washed with TBST, the blots were incubated with

horseradish peroxidase-conjugated secondary antibodies (TIANGEN, Shanghai, China). Chemiluminescence was used to show the results (GE, Fairfield, CT, USA).

Immunohistochemistry

We used the immunohistochemistry technique with the Expose HRP/DAB detection system kit (Abcam, Shanghai, China) according to the manufacturer's instructions. The samples were



Figure 2. Effect of miR-520f on NSCLC cell proliferation, migration and invasion. A. H1299; B. H522 cells were either untreated or transfected with miR-520f mimics. A five-day proliferation assay was performed. C. Migration assays H1299 and H522 cells. The quantification was displayed on the right panel. D. Transwell invasion assays for H1299 and H522 cells. **: P < 0.01.

treated with anti-TM4SF1 or anti-VEGF primary antibodies (Sigma, Shanghai, China) washed with PBS, and incubated with secondary antibody for one hour. Then, the samples were stained with hematoxylin and diaminobenzidine (DAB) for microscopic visualization.

Proliferation assay

We used the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) to quantify proliferation. After treatment for 24 h, H1299 and H522 cells were suspended and seeded into a 12-well plate (10^5 cells/well) for 5 days. A total 10 µL MTT solutions were added into the culture with a final concentration of 10 mg/ml. The crystalline formazan was resolved in 150 µL sodium dodecyl sulfate (SDS, 8%) solution for 24 h and the optical density (O.D.) at 490 nm was monitored with the Spectramax M5 microplate monitor (Molecular Devices, USA).

Statistical analysis

Data were represented as the mean ± standard deviation (SD). Statistical significance was calculated by Student's t-test (SPSS 15.0, Inc., Chicago, IL, USA). The significance was evident if P < 0.05. Pearson correlation was used to measure the association between miR-520f and TM-4SF1 expression. Fisher exact test was used to evaluate the correlation between miR-520f and clinicopathological features. All experiments were performed with at least three replicates.

Results

miR-520f is decreased in NSCLC tissues and cell lines

To identify the role of miR-520f in NSCLC, we measured the expression of miR-520f in 90 NSCLC specimens as well as corresponding normal adjacent tissues. We found that the miR-520f expression was significantly downregulated in NSCLC samples compared with normal adjacent tissues (**Figure 1A**). We

also confirmed that the expression of miR-520f was also decreased in at least five NS-CLC cell lines (**Figure 1B**). We also found that the miR-520f is significantly correlated with tumor size (P = 0.003), TNM stages (P = 0.001) and metastasis (P = 0.016) (**Table 1**). Meanwhile, miR-520f was not associated with age, gender and Histological type (**Table 1**). These results suggested that miR-520f is downregulated in NSCLC tissues and cell lines, suggesting that miR-520f may function as a tumor suppressor. Since H1299 and H522 cells were most significantly downregulated in miR-520f expression, we chose these two cell lines for further analysis.

miR-520f may inhibit proliferation, migration and invasion in NSCLC cells

We the quantified cell proliferation using the Cell Counting Kit-8 kit in H1299 and H522 cells



Figure 3. The miR-520f targets TM4SF1. A. The base pairing between miR-520f and TM4SF1 3'-UTR. The mutant construct (MUT) was also shown. B. Relative luciferase activities of 293T cells transfected with either WT or mutant TM4SF1 plasmids. The luciferase activity data were normalized to those transfected with miR-negative control (miR-NC). C. The mRNA level of TM4SF1 in H1299 and H522 cells with or without miR-520f mimics transfection. D. The protein levels of TM4SF1 in H1299 and H522 cells with or without miR-520f mimics transfection using western blot.

with or without miR-520f transfection. We found that miR-520f transfection can significantly decrease lung cancer cell proliferation in H1299 cells (Figure 2A). Qualitatively similar results can be obtained in H522 cells (Figure 2B). To further confirm the tumor suppressive role of miR-520f, we carried out migration assays for H1299 and H522 cells. We found that miR-520f transfection can significantly decrease the migration of H1299 and H522 cells (Figure 2C). The reduction in migration can even decline by 2~3 fold (Figure 2C, right). We also performed transwell invasion assays and the results suggested that miR-520f also lead to decreased invasion of H12-99 and H522 cells (Figure 2D). These data argued that miR-520f can suppress tumorigenesis of NSCLC cells by modulating proliferation, migration and invasion.

miR-520f can target TM4SF1 in NSCLC cells

We used the online database DIANA-MICR-OT (http://diana.cslab.ece.ntua.gr), MICROR-NA.ORG (http://www.microrna.org) and TARGE-TSCAN-VERT (http://www.targetscan.org) were used for targets prediction. We found that TM4SF1 might be putative target for miR-520f. The theoretical base pairing between miR-520f and TM4SF1 was shown in **Figure 3A**. To verify the prediction in experiments, we performed luciferase reporter assay and confirmed that the luciferase intensities were significantly downregulated with wild type TM4SF1 transfection (Figure 3B). The mutant TM4SF1 failed to result in decreased luciferase activities possibly due to incapability of basepairing (Figure 3A and 3B). Overexpression of miR-520f in H1299 and H522 cells again confirmed that TM4SF1 mRNA expression was decreased (Figure 3C). The protein level of TM4SF1 with miR-520f transfection was consistently reduced (Figure 3D). These results suggested that miR-520f can directly target TM4SF1 and decrease its expression at both mRNA and protein levels.

TM4SF1 transfection can promote VEGF expression and restore the oncogenic potential of NSCLC cells

Since EMT play essential roles in the tumorigenesis of lung cancer [19], we further evaluated whether miR-520f can affect the EMT marker expression. We observed significant differences in VEGF expression in H1299 and H522 cells (Figure 4A). However, selected other EMT markers such as β -catenin, Slug, α -SMA, and Twist were not substantially altered (data not shown). We then investigated whether TM-4SF1 knockdown can influence the expression of VEGF. The si-TM4SF1 efficiency was confirmed (Figure 4B). Furthermore, we found that TM4SF1 knockdown downregulate VEGF expression at transcript levels (Figure 4C). The protein level of VEGF was consistently decreased with si-TM4SF1 (Figure 4D). Next, we performed a rescue experiment to confirm that miR-520f can inhibit the malignant phenotypes of NSCLC cells by directly reducing TM4-SF1. Transfection of TM4SF1 plasmids can efficiently restore VEGF expression in miR-520f transfected H1299 and H522 cells (Figure 4E). By restoring VEGF expression, the invasive capacities of H522 cells were also recovered (Figure 4F). These results miR-520f can regu-



Figure 4. miR-520f can modulate VEGF via TM4SF1. A. Western blot analysis of epithelial-mesenchymal transition (EMT) marker VEGF in H1299 and H522 cells with or without miR-520f transfection. B. Relative TM4SF1 and VEGF mRNA expression after transfection with si-TM4SF1 in H1299 and H522 cells. C. VEGF mRNA levels were measured under si-TM4SF1 treated conditions. D. Quantification of VEGF proteins levels under si-TM4SF1 treated ment. E. VEGF expression in H1299 and H522 cells left untreated, transfected with miR-520f mimics or co-transfected with miR-520f and TM4SF1 plasmids. F. Invasion assay for H522 cells with TM4SF1 restoration. **: P < 0.01.

late EMT possibly through directly regulating TM4SF1 and further modulating VEGF expression.

TM4SF1 level is dramatically increased in NSCLC specimens

To further identify the role of TM4SF1, we analyzed the TM4SF1 expression in 90 NSCLC samples as well as normal tissues. We found that TM4SF1 transcripts were significantly upregulated in NSCLC tissues compared with paired normal ones (**Figure 5A**). Furthermore, we performed correlation analysis and found that TM4SF1 and miR-520f showed significantly negative correlation (R = -0.5356, P < 0.0001, **Figure 5B**). We also found positive staining in TM4SF1 and VEGF expression in NSCLC specimens compared with normal tis-

sues (**Figure 5C**). Collectively, these data suggested that TM4SF1 might be positively associated with metastasis of NSCLC.

Discussion

In current study, we revealed that miR-520f may exhibit tumor suppressive role in NSC-LC. We identified decreased expression of miR-520f in human NSCLC samples as well as cell lines. Furthermore, we noticed that TM4SF1 might be a direct target of miR-520f. Luciferase reporter assays confirmed this prediction. Meanwhile, miR-520f can indirectly downregulate VEGF expression via TM4SF1 and fulfill its role in EMT. In NSCLC specimens, we also identified a significantly inverse correlation between miR-520f and TM4SF1 expression. These results collectively argued that miR-520f may function as a tumor suppressor in NSCLC and might be used as a putative biomarker.

The miR-520 family member miR-520h can downregulate ABCG2 and inhibit pancreatic

cancer cell invasion [20]. Instead, miR-520c may reactive MAPK signaling and promote fibrosarcoma occurrence [21]. To date, little information is available for the role of miR-520f especially in cancer. Therefore, our results may provide first evidence that miR-520f can effectively participate in lung cancer progression via TM4SF1/VEGF signaling.

TM4SF1 denotes a small plasma membrane glycoprotein which modulates motility and growth in several cancers [16, 22]. TM4SF1 is ubiquitously expressed on plasma membrane as well as intracellular vesicles [16]. TM4SF1 is critically involved in both metastasis and angiogenesis [23]. Many recent findings further argued that TM4SF1 might serve as a prognostic marker in various tumors. For example, TM4SF1 may represent a prognostic marker in



Figure 5. Analysis of TM4SF1 expression in specimens. A. Measurement of TM4SF1 levels in 90 NSCLC samples and paired normal adjacent tissues. B. Pearson correlation between TM4SF1 and miR-520f expression in 90 human samples. R = -0.5356, P < 0.0001. C. The expression of TM4SF1 and VEGF in NSCLC specimens compared with normal tissues by immunohistochemistry. **: P < 0.01. ***: P < 0.001.

pancreatic ductal adenocarcinoma where TM-4SF1 is frequently upregulated in tumor tissues [24]. In addition, TM4SF1 can also promote pancreatic cancer progression via regulating MMP-2 and MMP-9 levels [23]. TM4-SF1 also play essential roles in hepatocellular carcinoma where increased expression of TM4SF1 is identified [25]. In present work, we found that miR-520f targets TM4SF1 via its 3'-UTR region can suppress TM4SF1 expression. Our novel finding may shed light on the complex regulatory patterns converging on TM4SF1.

It is well documented that the epithelial-mesenchymal transition (EMT) is significantly correlated with NSCLC carcinogenesis and metastasis [26, 27]. EMT is a highly coordinated, during which the polarity of epithelial cells might be lost and failure in cell to cell adhesion may occur [27]. Multiple signaling pathways may participate in this process such as bone morphogenetic protein (BMP), Wnt- β -catenin, Notch and transforming growth factor β (TGF- β) pathways [28]. During the development of EMT, the dissemination of epithelial cells may greatly depend on remodeling of cytoskeleton and possibly result in invasive and migratory phenotypes [29]. The VEGF can promote angiogenesis and elicit an angiogenic switch [30]. One switched on, the angiogenic switch can lead to increased expression of angiogenic factors and allow metastasis [30]. Overexpression of VEGF is evident during the progression from normal tissues to carcinomas with acquisition of invasive properties [30]. Other reports also suggest that VEGF can induce a mesenchymal phenotype with altered TGF-B1 signaling with augmented feedback loops [31-33]. Therefore, VEGF plays important roles in the development of metastatic cancer phenotypes in numerous tumors. More studies have demonstrated that many microRNA families such

as miR-200, miR-101 and some long non-coding RNAs can function in EMT [34]. For example, miR-15 and miR-16 may inhibit fibroblast growth factor (FGF) and consequently decrease EMT [34]. Instead, miR-200 family members may target ZEB1 and ZEB2 to suppress EMT progression [34]. The miR-34 and miR-203, however, can mediate EMT suppression via targeting SNAI1 and SNAI2 [34]. However, no roles for miR-520f have been implicated especially in EMT. In current study, we found that miR-520f can mediate suppression of EMT by downregulating VEGF expression. This indirect effect is largely ascribed to TM4SF1. Therefore, we have identified a novel role of miR-520f which can regulate EMT through TM4SF1/VEGF pathway and suppress tumor development.

Taken together, the current study indicates that miR-520f might be a potential tumor suppressor at least in NSCLC. As VEGF is critically involved in EMT and metastasis, targeting the intricate interplay between miR-520f and TM4-SF1/VEGF might be an effective rationale for diagnosis.

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Disclosure of conflict of interest

None.

Authors' contribution

YL and HMZ conceived the study. YL, SMZ performed the experiments. YL, SMZ and MS analyzed the data. YL and HMZ wrote the paper. All authors have read and approved the final version of the paper.

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