Original Article MicroRNA let-7f suppresses the proliferation, migration and invasion of non-small cell lung cancer by targeting TGFBR1

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Abstract: MicroRNAs that negatively regulate gene expression have emerged as novel therapeutic tools for cancer treatment. In the present study, we investigated the role of let-7f in non-small cell lung cancer (NSCLC) tumorigenesis and progression. Our results showed that let-7f was down-regulated in NSCLC tissues. Overexpression of let-7f inhibited NSCLC cell proliferation, migration and invasion. Further data demonstrated that TGFBR1 was a direct target of let-7f, and played a crucial role in let-7f mediated suppressive effect on NSCLC cell migration and invasion abilities. In summary, these findings indicated that let-7f acts as a tumor suppressor in NSCLC, and it reduces the cancer cell motility and invasive capacity by targeting TGFBR1, providing a potential anti-cancer drug candidate for NSCLC.

Keywords: Let-7f, non-small cell lung cancer, proliferation, migration, invasion, TGFBR1

Introduction

Lung cancer is the leading cause of cancer mortality worldwide, among which non-small cell lung cancer (NSCLC) takes a major part of over 70% [1]. Despite the continuous advancement in diagnosis and therapies for NSCLC, five-year overall survival rate remains under 15% [2]. Malignant proliferation, adjacent invasion and distant metastasis of cancer cells account for the poor outcomes [3]. Therefore, it is of great significance to discover novel targets for NSCLC, and explore the correlated signal pathways, which could be beneficial for developing new therapeutics for NSCLC.

MicroRNAs (miRNAs) are a class of small noncoding RNAs including 19 to 25 nucleotides. The miRNAs interact with 3'-untranslated region (UTR) of the target mRNA, resulting in mRNA degradation [4]. Numerous studies have shown that miRNAs play a vital role in the development and progression of cancers. The aberrant miRNAs may be potential diagnostic or prognostic biomarkers, and even serve as promising therapeutic tools for cancer treatment.

Let-7 was the first known human miRNA. The let-7 family contains a dozen of most actively studied tumor suppressors [5]. Let-7a, let-7c, and let-7g have been found to inhibit NSCLC cell proliferation, migration and invasion [6-9]. Let-7f was reported to be down-regulated in a variety of cancers, such as gastric cancer, glioma, and breast cancer [10-12]. Meanwhile, lower level of let-7f in serum and tumor tissues was also demonstrated in NSCLC patients [13, 14]. Takamizawa et al [15] found that overexpression of let-7f inhibited A549 cell proliferation. However, there are no further studies about the effect of let-7f in migration and invasion of NSCLC, or the underlying mechanisms.

In this study, we investigated the role of let-7f in NSCLC and identify the potential target of let-7f that might mediate the biological function in NSCLC cells. Let-7f was down-regulated in NSCLC tissues. Over-expression of let-7f inhibited NSCLC proliferation, migration and inva-

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Target genes	Forward primers	Reverse primers
COL1A1	5'-CAAGGTGTTGTGCGATGACG-3'	5'-TTGGTCGGTGGGTGACTCTG-3'
COL1A2	5'-GGAAAAGGAGTTGGACTTGG-3'	5'-GGTCCTTGGAAACCTTGAGG-3'
TGFBR1	5'-ACAACCGCACTGTCATTCACC-3'	5'-GGTAAACCTGAGCCAGAACCT-3
TGFBR3	5'-GCAAACTTCTCCTTGACAGC-3'	5'-TCTTGCTATCTTGAGTTCGG-3'
CCR7	5'-ATTCTGTCATCTGCTTCGTG-3'	5'-AGGAGGAAAAGGATGTCTGC-3'
AKT2	5'-ATTGCCAAGGATGAAGTCGC-3'	5'-CGGGACAGGTGGAAGAACAG-3
BCL2L1	5'-AGGGACTGAATCGGAGATGG-3'	5'-GCATCCAAACTGCTGCTGTG-3'
E2F2	5'-TCCCCTTGGAGGCTACTGAC-3'	5'-AAGCCCCACAGGTAGTCGTC-3'
IGF1R	5'-GACCACCGCAACCTGACCAT-3'	5'-AGGCTCTCTCCCCGTTGTTC-3'
MAP3K1	5'-TGGAGTGTTGGCTGTGCTAT-3'	5'-GCCACATCTCGTAAACCAGG-3'
MAP4K4	5'-CCAGAATGTGTTGCTGACTG-3'	5'-GGCATCTGGGTTCTCATCAC-3'

Table 1. The sequences of forward and reverse primers

sion. TGFBR1 was the direct target of let-7f, and contributed to the suppressive effect on NSCLC cell migration and invasion. These findings may help in a better understanding of the mechanisms of NSCLC development, and indicate that let-7f is possibly a new target for NSCLC treatment.

Materials and methods

Patient specimens and cell cultures

The resected cancer samples from 20 NSCLC patients and the matched adjacent normal tissues were provided by Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. None of patients received radiotherapy or chemotherapy before operation. All specimens were immediately frozen in liquid nitrogen after surgery and stored at -80°C until RNA extraction. This study was approved by the Tongji Hospital, Tongji Medical College Ethics Committee, and written informed consent was obtained from all patients.

The NSCLC cell lines A549 and SPC-A-1 were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured with 5% CO_2 in RPMI 1640, supplemented with 10% fetal bovine serum (Every Green, Hangzhou, China).

RNA extraction and quantitative real-time PCR

Total RNA of cells and tissue specimens were extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA using TransScript First-Strand cDNA

Synthesis SuperMix (Transgen Biotech, Beijing, China) in accordance with the manufacturer's instructions. Quantitative real-time PCR (gRT-PCR) was conducted to detect with SYBR green mRNA assay by using ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA. USA). The parameters of PCR were as follows: 95°C for 20 s, 40 cycles of 95°C for 3 s.

60°C for 30 s. The calculating method of relative expression level was the 2^{-ΔΔCt} method. U6 and β-actin were used as internal controls. Reverse-transcribed and real-time primers for let-7f and U6 were synthesized by Ribobio (Guangzhou, China). The sequences of forward and reverse primers of the potential target genes (COL1A1, COL1A2, TGFBR1, TGFBR3, CCR7, AKT2, BCL2L1, E2F2, IGF1R, MAP3K1, and MAP4K4) mRNAs, and β-actin (AuGCT, Beijing, China) are listed in **Table 1**.

Cell transfection

Let-7f mimics and inhibitor with respective negative controls (NC) were transfected into cells with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Let-7f mimics and NC were transfected at 50 nM, while let-7f inhibitor and inhibitor NC were transfected at 100 nM. TGFBR1 siRNA and si-NC were transfected at 100 nM.

Cell proliferation assay

The cell proliferation effects of let-7f on cells were determined by Cell counting kit-8 assay. Logarithmic growing A549 and SPC-A-1 cells were seeded into each well of 96-well plates at a density of 5.0×10^3 cells/well and transfected with let-7f mimics, let-7f inhibitor, TGFBR1 siRNA and respective NC at an appropriate concentration. The optical density (OD) on cell proliferation was measured at different time points (24, 48, 72, 96 h) by a microplate reader (Beckman Coulter, Fullerton, CA, USA) at 492 nm. Each well was carried out in three-replicate wells and triplicate independent experiments were performed.

	NO	N1-3	P-value
Number of patients	8	12	
Gender			1
Male	6	9	
Female	2	3	
Age			0.85
≥60	3	5	
<60	5	7	
Smoking condition			0.26
Smoker	6	6	
Non-smoker	2	6	
TNM stage			-
I-II	8	-	
III-IV	-	8	
Histological type			0.25
Squamous cell carcinoma	4	3	
Adenocarcinoma	4	9	
Tumor differentiation			0.71
Well-moderate	4	5	
Poor	4	7	

 Table 2. Clinicopathological features of patients included for detection of let-7f expression



Figure 1. Let-7f is down-regulated in NSCLC tissues. The let-7f expression was detected in 20 pairs of human NSCLC tissues and corresponding adjacent normal tissues by using qRT-PCR. **P<0.01.

Transwell assay

Transwell assay was used to measure cell migration and invasion. The transfected A549 and SPC-A-1 cells were seeded into Transwell chambers (Costar, Cambridge, MA, USA) at densities of 3.0×10^4 cells/well in serum-free

RPMI 1640 medium to measure cell migration. The lower chambers in 24-well plate were contained with 10% FBS RPMI-1640 medium. After 24 hours of incubation, cells that migrated onto the lower surface of the membrane were fixed with 100% methanol and stained with 0.1% crystal violet. Then the non-migrating cells on the upper membrane surface were removed with cotton swabs. Cells attaching to the lower chambers were photographed and manually counted at 200 × magnification from 8 different fields of each filter to quantify cell migration ability. The invasion ability of cells was measured in the almost equal condition with Matrigel-coated chambers, except that the quantity of seeded A549 and SPC-A-1 was 5.0 \times 10⁴ cells/well. Data are presented as the mean value for triplicate experiments.

Western blot

After transfection for three days, equal amounts of protein were separated on 10% SDS-PAGE gels and transferred to PVDF membrane. Anti-Smad2/3, p-Smad2, p-Smad3, and GAPDH antibodies were purchased form Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-TGFBR1 antibody was from Proteintech (Chicago, CA, USA). Triplicate independent experiments were performed.

Dual-luciferase reporter assay

The wide type or mutant type TGFBR1 3'-UTR containing let-7f binding sites were subcloned into pGL3/luciferase vector (Promega, Madison, WI, USA). The constructed recombinant vectors were co-transfected with let-7f mimics or NC respectively into HEK293 cells by using lipofectamine 2000 reagent. After 48 hours of transfection, the Dual-Luciferase Reporter Assay System (Promega) was used to measure luciferase activities according to the manufacturer's instruction. Triplicate independent experiments were performed.

Statistical analysis

Differences between two groups were estimated using Student's t test. The relationship between let-7f expression and TGFBR1 mRNA was analyzed using the Pearson's test. *P* value <0.05 was considered statistically significant. All of the statistical analyses were performed using SPSS version 18.0.



Figure 2. Up-regulation of let-7f inhibits NSCLC cell proliferation, migration and invasion. (A) The transfection efficiency of let-7f mimics in A549 and SPC-A-1 was confirmed by qRT-PCR analysis. (B) The proliferative ability of A549 and SPC-A-1 was detected by CCK-8 assay. The migration (C) and invasion (D) abilities of NSCLC cells were tested in Transwell chambers without or with Matrigel. Cells that had migrated or invaded through the membrane were manually counted at 200x magnification from 8 different fields of each filter. Data are presented as the mean value for triplicate experiments. *P<0.05, **P<0.01.

Results

Let-7f is down-regulated in NSCLC tissues

Firstly, we included 20 NSCLC patients who were equally divided into with or without lymph node metastasis groups, comparable according to gender, age, smoking status, histological type, and tumor differentiation (**Table 2**). Subsequently, we investigated let-7f level in the 20 pairs of human NSCLC tissues and corresponding adjacent normal tissues. Significant lower expression of let-7f was found in NSCLC

samples compared with adjacent areas (**Figure 1**). These data suggested that let-7f might act as a tumor suppressor in NSCLC.

Overexpression of let-7f inhibits NSCLC cell proliferation, migration and invasion

To investigate the role of let-7f in the growth of NSCLC, we transfected A549 and SPC-A-1 with let-7f mimics and NC. The transfection efficiency was validated using gRT-PCR (Figure 2A). CCK8 assay was performed to detect the effect of let-7f on NSCLC cell growth. The results showed that let-7f significantly suppressed the cell proliferation in both cell lines (Figure 2B). We further explore the role of let-7f in NSCLC cell migration and invasion. It was obvious that ectopic expression of let-7f inhibited the migration (Figure 2C) and invasion (Figure 2D) abilities of NSCLC cells. All the above data demonstrated the suppressive effect of let-7f on NSCLC cell proliferation, migration and invasion.

Down-regulation of let-7f promotes NSCLC cell proliferation, migration and invasion

To ascertain the role of let-7f in NSCLC development and progression, we transfected A549 and SPC-A-1 with let-7f

inhibitor and inhibitor NC. The transfection efficiency was validated using qRT-PCR (**Figure 3A**). We detected that down-regulation of let-7f increased A549 and SPC-A-1 cell proliferation using CCK-8 assay (**Figure 3B**). Moreover, A549 and SPC-A-1 cells that migrated through membrane were more in let-7f inhibitor group compared to inhibitor NC group in both Transwell migration and invasion assays (**Figure 3C** and **3D**). These results suggested a promoted effect of let-7f inhibition on NSCLC cell proliferation, migration and invasion, which confirmed the



Figure 3. Down-regulation of let-7f promotes NSCLC cell proliferation, migration and invasion. (A) The transfection efficiency of let-7f inhibitor in A549 and SPC-A-1 was confirmed by qRT-PCR analysis. (B) The proliferative ability of A549 and SPC-A-1 was detected by CCK-8 assay. The migration (C) and invasion (D) abilities of NSCLC cells were tested in Transwell chambers without or with Matrigel. *P<0.05, **P<0.01.

suppressive effect of let-7f on NSCLC develo-p ment.

TGFBR1 is a direct target of let-7f via Smad2/3 signaling

To explore the target genes of let-7f which may participate in regulating NSCLC tumorigenesis, we searched for putative targets in miRNA target prediction software, including TargetScan, miRanda and Pictar. We chose eleven potential target genes (COL1A1, COL1A2, TGFBR1, TGFBR3, CCR7, AKT2, BCL2L1, E2F2, IGF1R, MAP3K1, and MAP4K4) related to cancer cell proliferation, migration or invasion, and identified their relation with let-7f using qRT-PCR. Let-7f mimics were transfected into A549, then CO-L1A1 and TGFBR1 were found to be down-regulated compared with NC group (Figure 4A). A similar result of TGFBR1 was shown in SPC-A-1, while difference of COL1A1 no mRNA level between the two groups was observed (Figure 4B). Next, we transfected A549 and SPC-A-1 with let-7f inhibitor or inhibitor NC. TGFBR1 mRNA expression was significantly reversed by let-7f inhibitor. However, there was no statistical difference of COL1A1 expression between the two groups in either NSCLC cell lines (Figure 4C). Furthermore, Western blot analysis showed that protein level of TGFBR1 was downregulated by let-7f mimics transfection (Figure 4D). In human NSCLC tissues, TGF-BR1 mRNA expression was markedly higher compared with adjacent normal tissues (Figure 4E). Additionally, TGF-BR1 mRNA level was inversely correlated with let-7f expression in tumor tissues (Figure 4F). To confirm the relationship between let-7f and TGF-BR1, we analyzed the 3'-UTR of TGFBR1 and identified one binding site for let-7f at the

position 75-82nt (**Figure 4G**). We constructed the wide type and mutant type of TGFBR1 3'-UTR fragment into luciferase vector. Treatment of HEK293 cells with let-7f mimics significantly reduced relative luciferase activity, while the mutative binding site of let-7f reversed the suppressive response (**Figure 4H**), which indicated that TGFBR1 is a direct target gene of let-7f. Heteromeric receptor complex formed by TGFBR1 and TGFBR2, which allowing the activation of Smad2 and Smad3, plays an important role in TGF- β signaling. In our study, there was no significant change of total Smad2 and Smad3 expression in A549 and SPC-A-1 trans-



Figure 4. TGFBR1 is a direct target regulated by let-7f via Smad2/3 signaling. (A) A549 was transfected with let-7f mimics, and the mRNA level of eleven potential target genes (COL1A1, COL1A2, TGFBR1, TGFBR3, CCR7, AKT2, BCL2L1, E2F2, IGF1R, MAP3K1, and MAP4K4) was identified by using qRT-PCR. TGFBR1 and COL1A1 were found to be significantly down-regulated by overexpression of let-7f. The relation of TGFBR1 and COL1A1 with let-7f was further examined in SPC-A-1 transfected with let-7f mimics and NC (B), as well as in both cell lines transfected with let-7f inhibitor and inhibitor NC (C). TGFBR1 was finally determined as the potential target of let-7f, and comfirmed by Western blot (D). (E) The mRNA level of TGFBR1 in human NSCLC tissues and corresponding normal tissues was analyzed by qRT-PCR. (F) An inverse correlation between TGFBR1 mRNA and Let-7f expression was found in clinical specimens (Pearson's correlation r = -0.4728, P = 0.0353). (I) Activation of Smad2/3 was involved in let-7f mediated TGFBR1 signaling, and comfirmed by Western blot. (G) Let-7f and its putative binding sequences in 3' UTR of TGFBR1. Mutations were generated in the complementary site that binds to the seed region of let-7f. (H) Luciferase report assay was performed to confirm the let-7f binding target. The luciferase activity was detected in HEK293 cells after co-transfection of 3'UTR-TGFBR1 WT vector or 3'UTR-TGFBR1 MUT vector, let-7f mimics and NC. *P<0.05, **P<0.01.

fected with let-7f mimics, but p-Smad2 and p-Smad3 expression was repressed to some degrees (**Figure 4I**). The above results implicated that activation of Smad2 and Smad3 participates in let-7f mediated TGFBR1 signaling.

Knockdown of TGFBR1 inhibits NSCLC cell migration and invasion

Previous evidence had demonstrated that let-7f inhibited NSCLC cell proliferation, migration,

and invasion. Besides, let-7f directly targeted TGFBR1. Then we wondered whether TGFBR1 was involved in let-7f mediated suppressive effect on NSCLC growth and progression. Hence, we silenced TGFBR1 in A549 using si-TGFBR1. Both qRT-PCR and Western blot were performed, and cells transfected with si-TGF-BR1 #1 showed a lowest expression in TGFBR1 mRNA and protein levels (**Figure 5A** and **5B**). Therefore, si-TGFBR1 #1 was chosen to knock-



Figure 5. Knockdown of TGFBR1 inhibits cell migration and invasion. The mRNA (A) and protein (B) levels of TGFBR1 were down-regulated by TGFBR1 si-RNAs to different degrees in A549. Eventually, si-TGFBR1 #1 was chosen to knockdown TGFBR1. After transfection of si-TGFBR1 #1, (C) the proliferative ability of A549 and SPC-A-1 was detected by CCK-8 assay. The migration (D) and invasion (E) abilities of NSCLC cells were tested in Transwell chambers without or with Matrigel. **P<0.01.

down TGFBR1 in the following experiments. CCK-8 assay didn't demonstrate that si-TGF-BR1 significantly inhibited NSCLC cell proliferation in either A549 or SPC-A-1 (Figure 5C). However, down-regulation of TGFBR1 was found to depress NSCLC cell migration and invasion abilities (Figure 5D and 5E). The above data suggested that TGFBR1 might not play a role in regulation of NSCLC cell proliferation, but it participated in inhibiting NSCLC cell migration and invasion.

Discussion

Let-7 family in human is a dozen of actively studied highly conservative miRNAs, including

let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, and miR-98 [5]. Let-7a, let-7c, and let-7g have been proved to be involved in NSCLC oncogenesis and development [6-9]. Let-7f was reported to inhibit A549 cell proliferation, while no further experiments for confirmation or investigation of its effects on NSCLC cell migration and invasion, as well as the regulating mechanisms. In the present study, the data demonstrated that expression of let-7f is downregulated in human NSCLC tissues, and it serves as a tumor suppressor. Moreover, let-7f directly targets TGFBR1, which may play an important role in let-7f mediated regulation of NSCLC cell migration and invasion.

The expression of let-7f is conflicting in various malignancies. Let-7f is down-regulated in head and neck cancer, hepatocellular carcinoma, and ovarian carcinoma, while upregulated in primary breast cancer [16-18]. Those findings suggested that let-7f might show contrary effects on different types and stages of cancers. A few studies reported low expression of let-7f in both human NSCLC tissues and serum [13, 14]. In our

study, down-regulation of let-7f was found in cancer samples, which are in consistent with previous evidence. This result indicated that let-7f might serve as a tumor suppressor in NSCLC.

Let-7f is involved in a variety of physiological and pathological processes, including angiogenesis, growth arrest, immune tolerance, mesenchymal stem cell differentiation, and pulmonary arterial hypertension [18-22]. In Takamizawa et al's research, over-expression of let-7f demonstrated a dramatic growth-inhibitory effect in A549 cell line using colony formation assay [15]. Here, we confirmed the suppressive effect of let-7f on NSCLC cell proliferation by performing CCK-8 assay in A549 and SPC-A-1 cell lines. Furthermore, let-7f was reported to reduce vasculogenic mimicry by disturbing periostin-induced migration of glioma cells [23]. In gastric cancer, let-7f inhibited tumor invasion and metastasis by targeting MYH9 [10]. Our results showed that ectopic expression of let-7f significantly repressed NSCLC cell migration and invasion. Taking together, these data suggested that let-7f might be critical for the regulation of NSCLC tumorigenesis and development.

To date, few targets of let-7f have been reported, and the underlying molecular mechanism of the effects of let-7f on NSCLC remains unclear. We attempted to determine the putative targets of let-7f in NSCLC by bioinformatics analysis. Finally, TGFBR1 was identified as the direct target gene of let-7f. TGFBR1, a transmembrane serine/threonine kinase, belongs to TGF-β signaling pathway. The canonical axis of TGF-β signaling pathway is TGFBR1/Smad2/3 axis. The complex formed by TGFBR1 and TGFBR2 subsequently phosphorylates Smad2 and Smad3. Activated Smad2 and Smad3 form hetero-oligomeric complexes with Smad4, and migrate to nucleus, regulating transcription of target genes [24]. The TGF-β signaling pathway participates in regulation of tissue homeostasis, including inflammation, angiogenesis, apoptosis, and endothelial mesenchymal transition (EMT) [25, 26].

There is discrepancy about the effect of TGFB1/ Smad2/3 axis signaling in cell proliferation. A recent study demonstrated that let-7g downregulated TGFBR1 and promoted cell apoptosis in follicular granulosa cells [27]. TGFBR1 overexpression was also involved in fibroproliferative diseases [28]. In addition, it should be noted that ERK1/2 is another downstream molecular signaling pathway of TGFBR1, which results in lung endothelial cell survival and promoted proliferation [29]. On the other hand, TGFBR1 was reported to facilitate in encoding cell cycle inhibitor p21, and found lowerexpressed in epithelial ovarian cancer tissues [30]. In the present study, we observed a significant higher level of TGFBR1 in NSCLC tissues. while the block of TGFBR1 didn't show inhibition effect on cell proliferation, which is inconsistent with the result of a previous similar research in A549 [31]. Hence, the regulation of TGFBR1 in NSCLC cell proliferation is still under question and requires more researches for confirmation. Since cancer cell proliferation is regulated by complicated signaling pathway network, TGFBR1 might not play a critical role in let-7f mediated proliferation suppressive effect.

Plenty of evidence has elucidated that TGF-B signaling positively correlates with EMT through Smad and non-Smad signaling pathways. TGF-B/Smad signaling induces multiple transcription factors, including δEF1/ZEB1, SIP1/ ZEB2, and Snail/SNAI1, which activate cell motility and invasive capacity [32]. Downregulation of TGFBR1 has been proved to inhibit cell migration and invasion abilities in various cancers, such as hepatocellular carcinoma, glioblastoma, gastric cancer, and thyroid carcinoma [33-36]. In NSCLC, knockdown of TGFBR1 was reported to demonstrate the suppression of cell invasion [31]. Here, our study showed similar results, revealing that TGFBR1 is a key part of let-7f mediated inhibition effect on NSCLC cell migration and invasion.

Conclusion

Taking together, our study showed that let-7f suppresses NSCLC cell proliferation, migration and invasion. Additionally, the cancer cell motility and invasive capacity was depressed by regulating TGFBR1 expression in let-7f mediated signaling. These findings may not only increase our knowledge about the effect of let-7 family on NSCLC carcinogenesis and progression, but also help with therapeutic strategies for NSCLC.

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Disclosure of conflict of interest

None.

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