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Original Article

miR-33b inhibits tumor EMT and migration in lung squamous cell carcinoma by targeting TWIST1

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Abstract: Lung squamous cell carcinomais the mayor cause of cancer death. Tumor metastasis isthe important reason for lung cancer-associated mortality. Current researches have shown that microRNAs (miRNAs) in metastatic cancer may offer new insights for therapeutic control. Based on our previous study using gene chips, we selected miR-33b as the subject of the current study. We found that the expression of miR-33b was significantly downregulated in primary lung squamous cell carcinoma. Furthermore, over-expression of miR-33b in lung squamous cell carcinoma cells remarkably inhibited cell growth in vitro. Mechanistically, TWIST1 is found to be a key transcription factor for the EMT and a direct target gene of miR-33b. Additionally, silencing TWIST1 maybe inhibit the proliferation, migration of lung squamous cell carcinoma, which parallels the function of miR-33b on lung squamous cell carcinoma cells. Importantly, miR-33b levels are negatively correlated with the expression of TWIST1 in human lung squamous cell carcinoma. Collectively, our data indicate that miR-33b is frequently decreased in lung squamous cell carcinoma and showed anti-metastatic activity by downregulating TWIST1, indicating a potential target in the treatment of human lung squamous cell carcinoma.

Keywords: microRNA-33b, EMT, lung squamous cell carcinoma, migration

Introduction

Lung cancer is the major cause of cancer-related mortality in the world wide, accounting for 29% and 26% of cancer deaths in male patients and female patients respectively [1]. In recent years, molecular mechanism assays have been used in the diagnosis of lung cancer. However the 5-year overall survival is only 16% despite the advances in diagnosis and treatment strategies in lung cancer [2, 3]. Non-small-cell lung cancer (NSCLC), the most common type of lung cancer, including two major histologic subtypes: adenocarcinoma and squamous cell carcinoma (SCC) [4]. Because different treatments or adverse effects depending on the SCC and adenocarcinoma, which has become an increasingly important issue in the management of lung cancer [5]. Altogether, it is imperative for us to develop new biomarkers that can improve early detection of NSCLC.

MiRNAs are endogenous, non-coding RNA molecules, which regulate gene expression by antisense inhibition and are complementary to target mRNAs [6]. The role of miRNAs in tumor development has become increasingly evident [7]. Some miRNAs have been shown to exert diverse functions in cancer cell proliferation and epithelial-to-mesenchymal transition (EMT) [8, 9]. In our previous work, we used TGF-β1 to induce the EMT of the A549 cell and perform a gene chip analysis. From the gene chip, we found that miR-33b was down-regulated, indicating that miR-33b may be involved in the EMT process of NSCLC [10]. Based on this finding, miR-33b was chosen for further investigation.

TWIST1, an important transcription factor of EMT, plays an essential role in mesoderm development [11, 12] and differentiation [13]. It may be act as a oncogene in many types of tumors, such as cancers from glioblastoma [14], breast cancer [15], lung cancer [16]. The role of TWIST1 in tumor growth has been attracting increasingly greater interest.

The function of miR-33b in lung SCC and its clinical pathologic significance has not been established. Here we found that miR-33b was

expressed at very low levels in lung SCC tissues compared with matched non-cancerous tissues, and there was a negative correlation between miR-33b and TWIST1 expression levels. Furthermore, bioinformatics analysis revealed that TWIST1 mRNA has a binding site for miR-33b in the 3'-untranslated region (3'UTR). miR-33b overexpression and TWIST1 inhibition produce similar changes, which inhibit lung SCC cell proliferation, migration in vitro. We found that miR-33b/TWIST1 axis maybe a new molecular mechanism for the growth of lung SCC.

Materials and methods

Cell lines and clinical specimens

The cell lines HBE, A549, SPC-a-1, PC-9, HTB-182 were purchased from American Type Culture Collection. These cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Invitrogen, USA), 100 IU/ml penicillin and 100 IU/ml streptomycin. These cell lines were grown at 37°C humidified atmosphere with 5% CO_2.

A total of 20 cases of lung SCC clinical samples were obtained from Cardiothoracic Surgery Department of Xingya hospital, Central South University. The study was approved by the Ethics Committee of Xiangya hospital and all patients were written informed consent.

Oligonucleotide construction and lentivirus production

The small interfering RNAs (siRNAs) targeting twist1, miR-33b mimic and miRNA mimic NC were synthesized by Shanghai GenePhama Co, Ltd. An unrelated sequence was used as a negative control (Shanghai GenePhama). The sequences were in Table S1. They were transfected into HTB182 cells during the logarithmic growth phase using Lipofectamine 2000 (Invitrogen USA) at a concentration of 100 nM according to the manufacturer's instruction.

MiR-33b was generated by lentiviral transduction (Table S2). The miR-33b sequence was amplified and cloned into the PGC-FU-SV40-EGFP-IRES-puromycin vector (GENECHEM) to generate pGC-FU-miR-33b. The negative control was pGC-FU-NC-LV. Theanti-miR-33b sequence was amplified and cloned into the pFU-GW-009 vector (GENECHEM) to generate the pFU-GW-miR-33b inhibition. The negative control was pFU-GW-RANI-NC. The lentiviral transduction was carried out according to instructions from GENECHEM. The sequences were in Table S2.

Quantitative RT-PCR

Total RNA was prepared with the Trizol reagent (Invitrogen) according to the manufacturer's instructions. For reverse transcription, cDNA was synthesized using the All-in-One™ First Strand cDNA Synthesis Kit (AORT-0050, Genecopoeia) and miRNA First Strand cDNA Synthesis Kit (AMRT-0050, Genecopoeia) according to the manufacturer's instructions. Quantitative RT-PCR was carried out using the All-in-One™qPCR mix (AOPR-1200, Genecopoeia) and All-in-One™miRNA qRT-PCR Detection Kit (AOMD-Q050, Genecopoeia) on an ABI 7300HT real-time PCR system (Applied Biosystems, USA). Relative quantification was achieved by normalization to the amount of GAPDH or U6. The primers used are shown in Supplementary information Table S3. The miR-33b (HmiRQP0432), U6 (HmiRQP9001) primer was purchased from Genecopoeia.

Western blotting

BCA Protein Assay Kit (Pierce) was used to determine the protein concentration. Whole cell proteins were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and blotted onto nitrocellulose membranes. The filters were hybridized with polyclonal anti-TWIST1 (ab175430, Abcam, 1:1000), anti-Vimentin (D21H3, Cell Signaling Technology, 1:1000), anti-E cadherin (ab76055, Abcam, 1:1000) at 4°C overnight, followed by incubation with secondary anti-rabbit or anti-mouse antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (14C10, Cell Signaling Technology, 1:1000) was used as a loading control, and goat anti-rabbit and goat anti-mouse (1:5000) immunoglobulin G (IgG; Invitrogen) as the secondary antibody.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as described [17]. The protocol according to the manufacturer's instructions (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). Negative controls were obtained by goat

serum. The sections were incubated with primary antibodies against Twist1 (1:200 dilution).

In vitro cell proliferation and migration assay

According to the manufacturer's protocol, the HTB182 cell transfected with a target vector was measured at 0, 24, 48, 72 and 96 h by using the MTT analysis. The absorbance at 490 nm was measured. Data are presented as the mean value for the triplicate experiments.

Cells (1×10^4) were plated on the top chamber of each insert (Corning Costar) for migration assays, and 1.0×10^4 cells were seeds on the top side of the polycarbonate Transwell filter coated with Matrigel (BD, New York, USA) for the invasion assays. The cells were seeded into the upper chambers with serum-free media, and normal growth medium was placed in the bottom chambers. After 24 hours of incubation at 37°C, cells which migrated or invaded were fixed and stained in a dye solution containing 20% methanol violet and 0.1% crystal and were counted under a microscope.

Immunofluorescence staining

Twenty-four hours after the transfection, cells were washed with PBS twice, 4% paraformaldehydeto fixed cells, 0.1% Triton X-100 used to permeabilized for 10 min. Then the transfected cells were blocked in 1% BSA for 1 hour at room temperature. Cells were incubated in appropriate primary antibodies TWIST1, vimentin, E-cadherin and β-catenin (a 1:100 dilution) overnight at 4°C. Cells were washed, incubated with specific secondary antibodies in PBS (a 1:100 dilution) for 1 hour. DAPI used to stained nuclei at room temperature for 10 min. Immunofluorescence was examined using a microscope (Leica Microsystems, Heidelberg, Germany).

Luciferase assays

RiboBio (Guangzhou, China) provided the wild type and mutant TWIST1 3'-UTR luciferase reporters. Briefly, the 3'-UTR region of the human TWIST1 mRNA and its mutant were cloned into the *Xho*I and *Not*I sites of the pmiR-RB-REPORT™vector (Promega), which was named pmiR-TWIST1-3'-UTR-wt and pmiR-TWIST1-3'-UTR-mut. The following primers were in Table S4. Then miR-33b mimic or a negative control and pmiR-TWIST1-3'-UTR-wt or pmiR-TWIST1-3'-UTR-mut were co-transfected into HEK-293 cell by Lipofectamine 2000. After 36 h of transfection, dual luciferase assay system (Promega) was used to measure the luciferase activity. The firefly luciferase activity of each sample was normalized to Renilla luciferase activity.

Animal studies

Four-week-old female BALB/c nude mice were purchased from the Animal Center of Central South University (Hunan, China). All mice experiments were carried out according to the regulations of the Animal Center of Central South University. To evaluate tumor growth in vivo, HTB182 cell was transfected miR-33b or empty vector controls were injected subcutaneously into the flanks of nude mice (1×10^7/200 L per mice, n=6). We measuring the length (a) and the width (b) of tumor to determine the volumes. The formula of tumor volume V (mm^3) $=a\times b^2/2$. After 21 days, mice that were injected subcutaneously were anesthetized and sacrificed, and the tumors were removed, photographed, weighed, and sectioned.

Statistical analysis

Comparative analysis between the groups for statistical significance was conducted with two-tailed paired Student's t-tests. Comparisons between the groups were performed using the χ^2 test for categorical variables. Pearson's test was used to calculate the correlation between miR-33b and ZEB1 expression. All error bars represent the mean ± SD derived from three independent experiments. $P < 0.05$ was considered statistically significant.

Results

miR-33b is frequently down-regulated in lungsquamous cell carcinoma cells and tissue specimens

To investigate the expression of miR-33b in lung SCC, we examed 20 pairs of primary lung cancer tissues and their adjacent non-tumor tissues by using qRT-PCR. Results showed that miR-33b expression in primary lung SCC tissues was significantly lower than that in pair-matched adjacent non-tumor tissues ($P < 0.05$; **Figure 1A**). Then we found miR-33b expression was reduced in several NSCLC cancer cell lines compared with the immortalized HBE cell (**Figure 1B**). Furthermore, on average, miR-33b

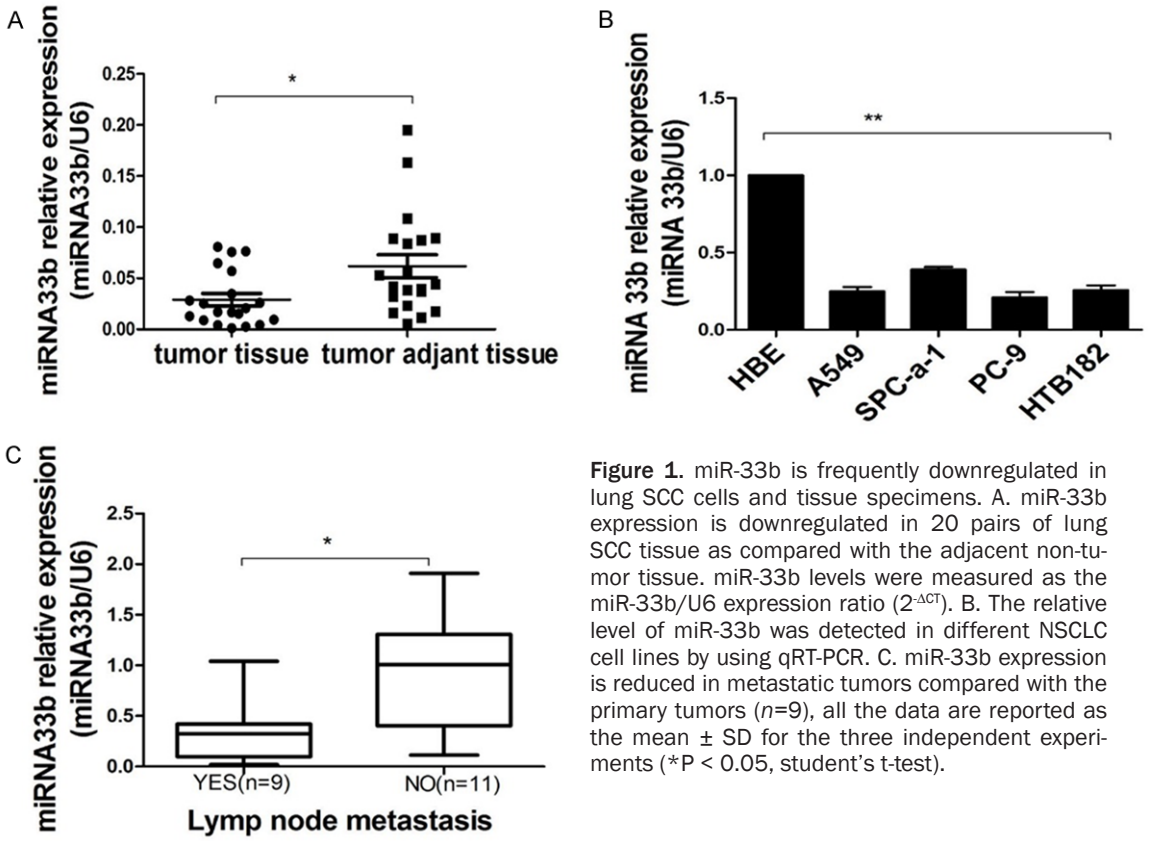


Figure 1. miR-33b is frequently downregulated in lung SCC cells and tissue specimens. A. miR-33b expression is downregulated in 20 pairs of lung SCC tissue as compared with the adjacent non-tumor tissue. miR-33b levels were measured as the miR-33b/U6 expression ratio ($2^{-\Delta CT}$). B. The relative level of miR-33b was detected in different NSCLC cell lines by using qRT-PCR. C. miR-33b expression is reduced in metastatic tumors compared with the primary tumors (n=9), all the data are reported as the mean ± SD for the three independent experiments (*P < 0.05, student's t-test).

Table 1. Association of microRNA-33b (miR-33b) expression with clinic pathological features of lung SCC tissues

Features	No of patients	miR-33b expression		χ^2	P
		Low	High		
Age					
< 60	8	5	3		
> 60	12	7	5	0.032	NS
Gender					
Male	13	6	7		
Female	7	5	2	0.755	NS
Clinical stage					
I-II	4	2	2		
III-IV	16	9	7	0.051	NS
Lymph node status					
Metastasis	9	8	1		
No metastasis	11	4	7	5.690	P < 0.01

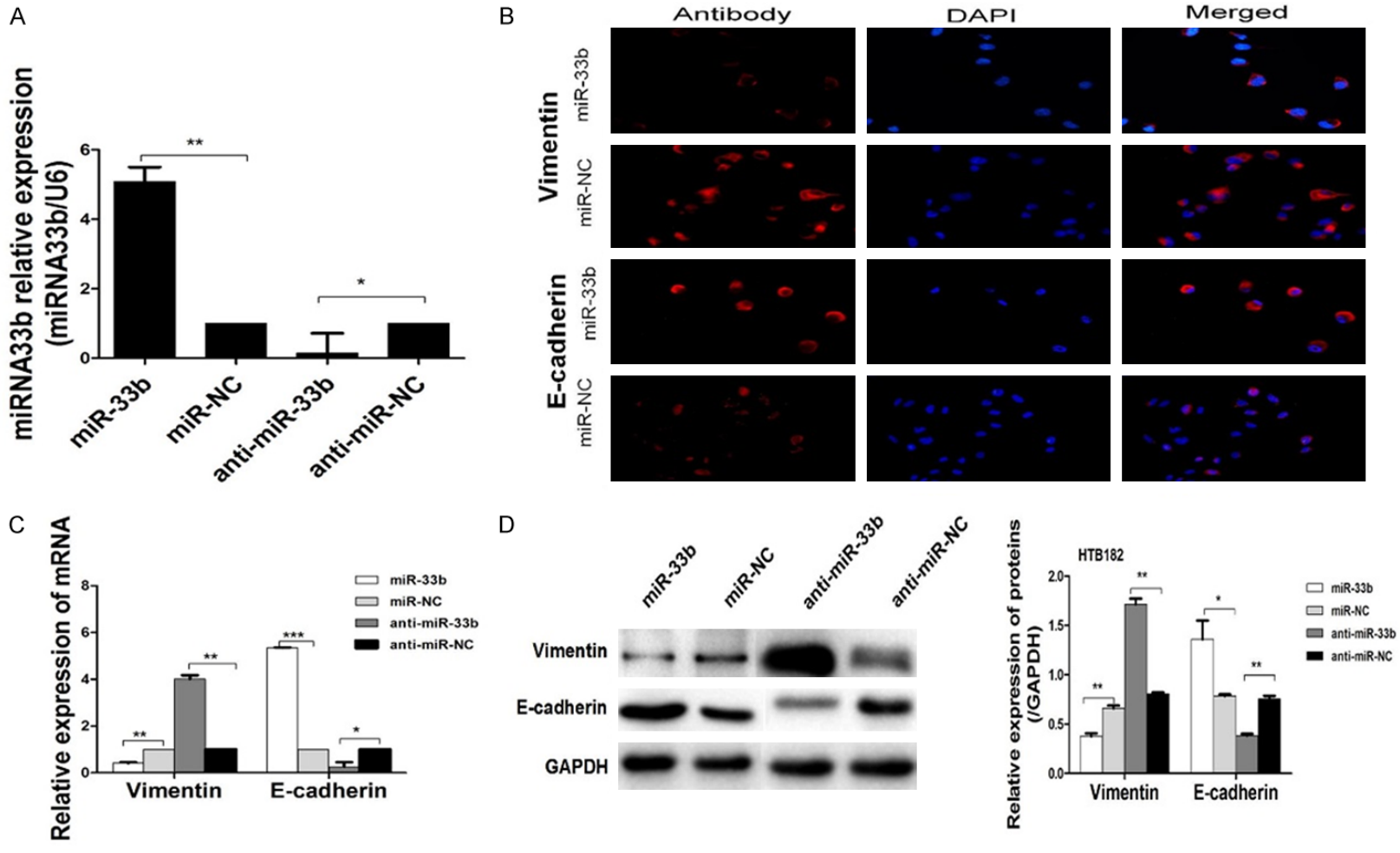
Note: 'NS' refers to the difference without statistical significance.

expression was reduced in metastatic tumors compared with primary tumors for 9 patients with metastatic lung SCC (P < 0.01; **Figure 1C**).

The clinical pathological features of patients with lung SCC was showed in **Table 1**. All the results indicated that miR-33b was significantly down-regulated in tumor tissues compared with adjacent normal tissues in lung SCC.

miR-33b suppresses EMT and lung SCC cell proliferation and migration in vitro

To determine the potential role of miR-33b in lung SCC proliferation and metastasis, cell migration assays were performed in HTB182 cells were transfected with miR-33b, miR-NC, anti-miR-33b and anti-miR-NC. The expression of mature miR-33b was confirmed by qRT-PCR (**Figure 2A**). Immunofluorescent staining, qPT-PCR and WB of HTB182 for E-cadherin revealed expression was increased in up regulation miR-33b, vice the expression was decreased in down regulation miR-33b, a typical marker of EMT, which located at cytomembrane. On the other hands, Vimentin expression was decreased in up regulation miR-33b, vice was increased in down regulation miR-33b, another hallmark of the mesenchymal phenotype (**Figure 2B-D**).


A
miRNA33b relative expression (miRNA33b/U6)
miR-33b
miR-NC
anti-miR-33b
anti-miR-NC
B
Antibody
DAPI
Merged
Vimentin
E-cadherin
miR-33b
miR-NC
C
Relative expression of mRNA
Vimentin
E-cadherin
D
Vimentin
E-cadherin
GAPDH
HTB182
Relative expression of proteins (/GAPDH)

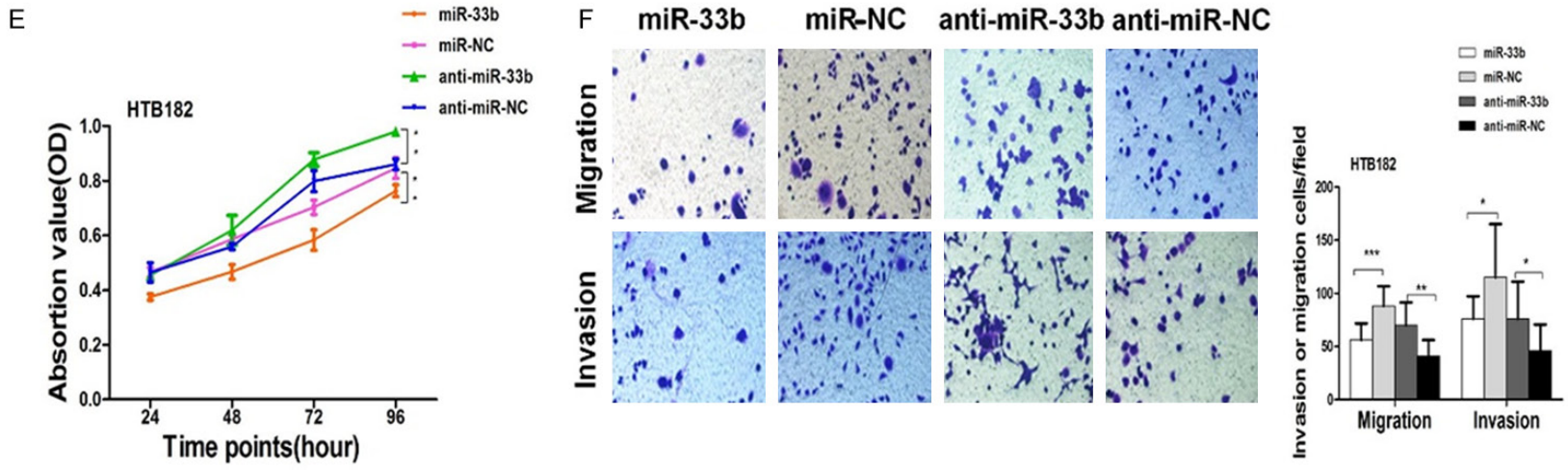


Figure 2. miR-33b suppresses EMT and lung SCC cell proliferation and migration in vitro. A. qRT-PCR validated miR-33b expression after transfected with miR-33b or miR-NC, anti-miR-33b or anti-miR-NC; B. The expression levels/patterns of E-cadherin, Vimentin by immunofluorescence between miR-NC and miR-33B (40×); C, D. qRT-PCR and WB analysis Vimentin, E-cadherin expression in HTB 182 cells transfected with miR-33b or miR-NC, anti-miR-33b or anti-miR-NC; E. MTT analysis growth curves of HTB182 cells transfected with miR-33b or miR-NC, anti-miR-33b or anti-miR-NC; F. HTB182 cells were transfected with the miR-33b or miR-NC, anti-miR-33b or anti-miR-NC and subjected to Transewell assays (40×). All data are reported as mean ± SD for three independent experiments. (*P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test).

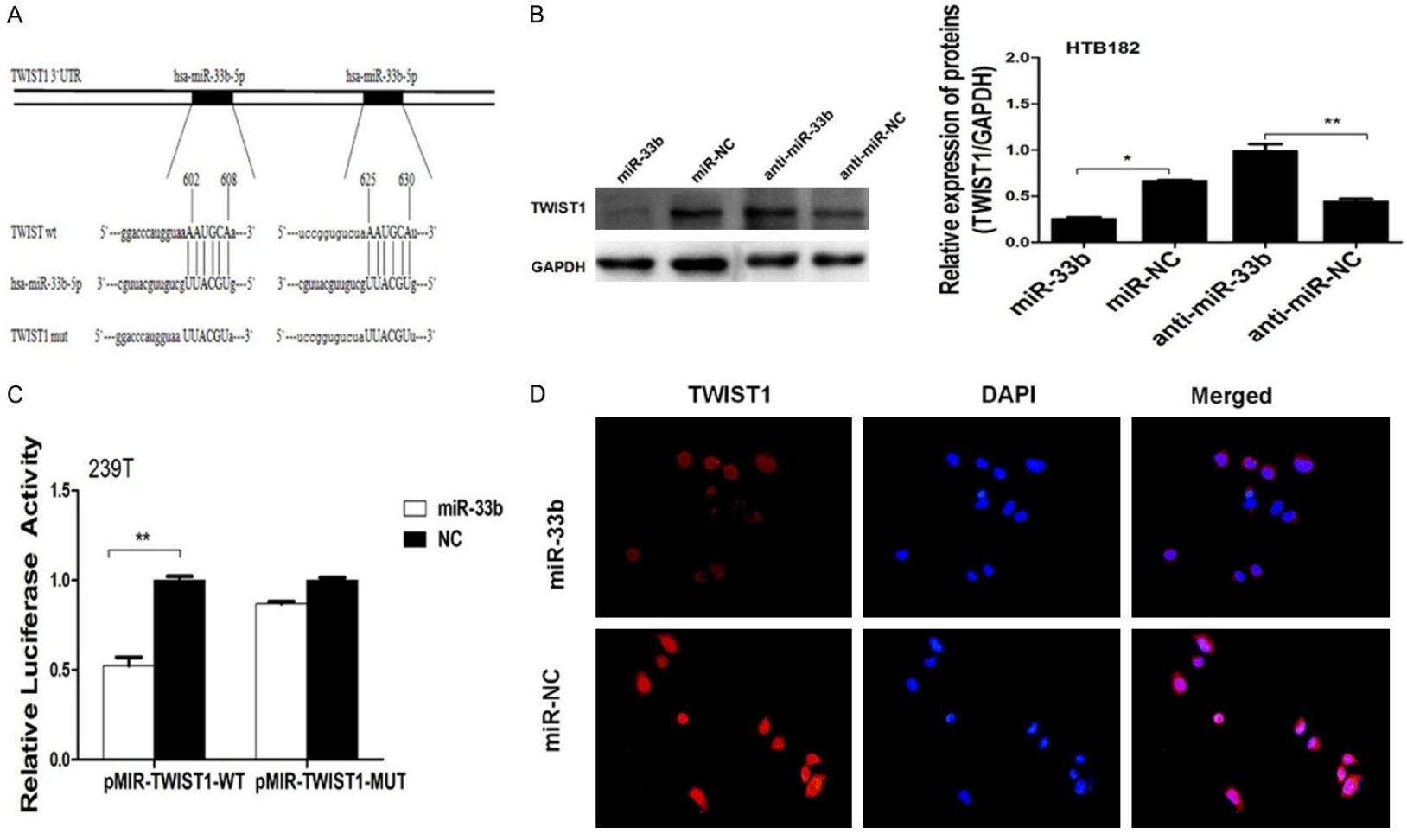
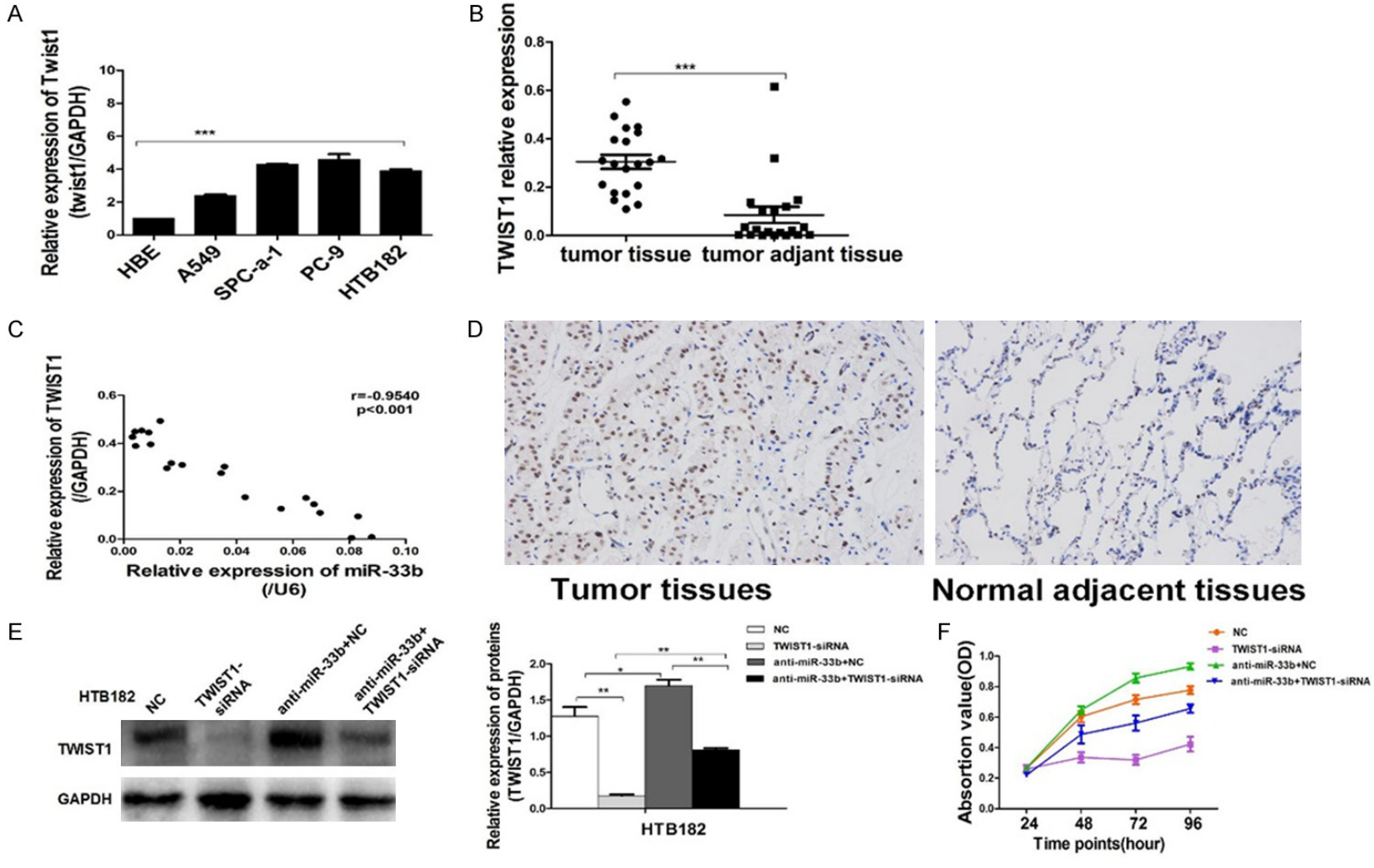


Figure 3. miR-33b directly targets TWIST1. A. miR-33b target sequences in 3'UTR of TWIST1 and mutant containing six mutated nucleotides in 3'UTR of TWIST1 (TWIST1-mut); B. WB analysis of TWIST1 expression in HTB182 transfected with miR-33b or miR-NC, anti-miR-33b or anti-miR-NC; C. Luciferase activity in the HEK239T cells upon transfection of miR-33b site mutant 3'UTR-driven reporter constructs; wt, wild-type (n=3); D. Immunofluorescence was used to compare the expression of TWIST1 in HTB182 transfected with miR-33b or miR-NC; error bars represent SEM for the three independent experiments. (*P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test).


A
Relative expression of Twist1 (twist1/GAPDH)
***
HBE
A549
SPC-a-1
PC-9
HTB182
B
TWIST1 relative expression
***
tumor tissue
tumor adjant tissue
C
Relative expression of TWIST1 (/GAPDH)
r=-0.9540
p<0.001
Relative expression of miR-33b (/U6)
D
Tumor tissues
Normal adjacent tissues
E
HTB182
NC
TWIST1-siRNA
anti-miR-33b+NC
anti-miR-33b+TWIST1-siRNA
TWIST1
GAPDH
Relative expression of proteins (TWIST1/GAPDH)
HTB182
F
Absortion value(OD)
Time points(hour)

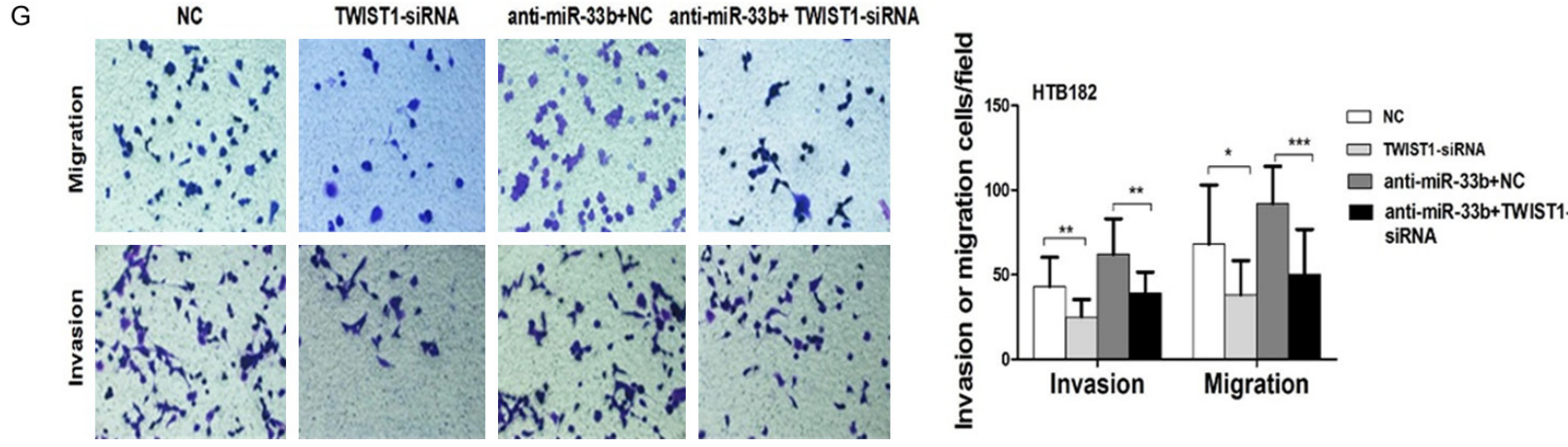


Figure 4. miR-33b regulates lung SCC cells development by down-regulating TWIST1 expression. A, B. qRT-PCR was used to examine the expression of TWIST1 in NSCLC cells and clinical lung SCC tissues. C. The inverse correlation between miR-33b and TWIST1 expression in lung SCC. Statistical analysis was performed using Pearson's correlation coefficient; D. The expression of TWIST1 was detected in lung SCC and in normal lung tissue utilizing immunohistochemistry (20×). E. The TWIST1 protein was measured by Western blot analysis; F. Growth curves of lung SCC cells with different treatments were analyzed by MTT. G. The migration or invasion ability of HTB182 with different treatments was detected using Transewell assay, respectively. All data are reported as the mean ± SD for the three independent experiments. (*P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test).

MTT was used to analysis the proliferation function of miR-33b. The rate of cell proliferation was decreased in the miR-33b group and increased in the miR-33b inhibition compared with the other control groups (**Figure 2E**). The inhibitory effect of miR-33b on cell migration and invasion was further confirmed by Transewell assays (**Figure 2F**). Consistently, miR-33b resulted in diminished proliferation and migration in lung SCC.

miR-33b directly targets TWIST1

To investigate the molecular mechanism that miR-33b used to suppress the metastasis potential of lung SCC cells, we used several methods to hunt for the potential targets of miR-33b in humans. We used the tool miRanda and Targetscan to identify the binding sites for miR-33b in the 3'UTR region of the TWIST1 gene (**Figure 3A**). To validate whether TWIST1 was a direct functional target of miR-33b, we conducted Western-blotting to compare TWIST1 expression in the HTB182 transfected with miR-33b, miR-NC, anti-miR-33b, and anti-miR-NC. TWIST1 expression significantly decreased when miR-33b was over-expressed. Additionally, TWIST1 expression was up-regulated after inhibiting miR-33b (**Figure 3B**). To obtain more direct evidence that miR-33b alters TWIST1 expression, we constructed a luciferase reporter plasmid containing the 3'UTR of TWIST1. As shown in **Figure 3C**, the luciferase activity in the Luc-TWIST1-UTR-transfected cells was significantly decreased compared with the luciferase activity in the miR-33b target site mutant TWIST1 3'UTR and negative control cells. Finally, immunofluorescence was used to compare the expression patterns of TWIST1, which located at nucleus (**Figure 3D**). All the results showed that miR-33b directly regulated TWIST1.

miR-33b regulates lung SCC cells development by down-regulating TWIST1 expression

We then examined the mRNA expression of TWIST1 in several NSCLC cells (**Figure 4A**). Additionally, the mRNA levels of TWIST1 were measured in primary lung cancer and paired adjacent normal tissues, suggesting that twist1 was up-regulated in primary lung cancer (**Figure 4B**: SCC vs. Normal 0.3047 ± 0.113 vs. 0.085 ± 0.150, $P < 0.001$). Of note, the expression of twist1 displayed a negative correlation with the miR-33b level in lung SCC tissues (**Figure 4C**, $r=-0.9540$ $P < 0.001$). To demonstrate TWIST1 in clinical samples, we further examined the TWIST1 expression in lung SCC by performing immunohistochemical staining in these clinical samples. We found the expression of TWIST1 was up-regulated in primary lung SCC compared with adjacent normal tissues (**Figure 4D**, $P < 0.01$).

To evaluate whether miR-33b suppresses cell proliferation and metastasis by downregulatingTWIST1, HTB182 cells were transfected with either siRNA-NC, twist1-siRNA and anti-miR-33b. The transfect efficiency was validated by a Western blotting analysis (**Figure 4E**). Silencing TWIST1 by siRNA in the HTB182 cell resulted in decreased proliferation, migration ability of the cell (**Figure 4F**, **4G**). Collectively, these results suggested that TWIST1 takes responses for the miR-33b-mediated regulation of the proliferation, migration of lung SCC cell.

miR-33b inhibits tumor growth through the TWIST1 in vivo

Next, we assessed the effects of miR-33b on tumor growth in vivo. Thus, the modified lung SCC cells with miR-33b overexpression were injected into the flanks of nude mice (1×10^7 per mouse, n=6) by subcutaneous injection. At the six days post-injection, the mean volumes of the xenograft tumors generated from the miR-33b-HT B182 cell were significantly smaller than the compared cell (**Figure 5A**, **5B**). Shown in **Figure 5C**, immunohistochemical staining for TWIST1 was shown as panels below (**Figure 5C**, $P < 0.01$). The results demonstrated that TWIST1 levels, was down-regulated after overexpression miR-33b.

Discussion

miRNAs can regulate different biological processes in tumorigenesis and invation [18, 19]. In this study, we found that miR-33b was downregulated in lung SCC tumor samples compared with adjacent normal lung tissues and downregulated in NSCLC cells. miR-33b expression was inversely correlated with lymph node metastatic status of lung SCC. Moreover, miR-33b could inhibit tumor proliferation, migration and invasion of HTB182 in vitro. Therefore, miR-33b may provide new functions in the metastasis of lung SCC.

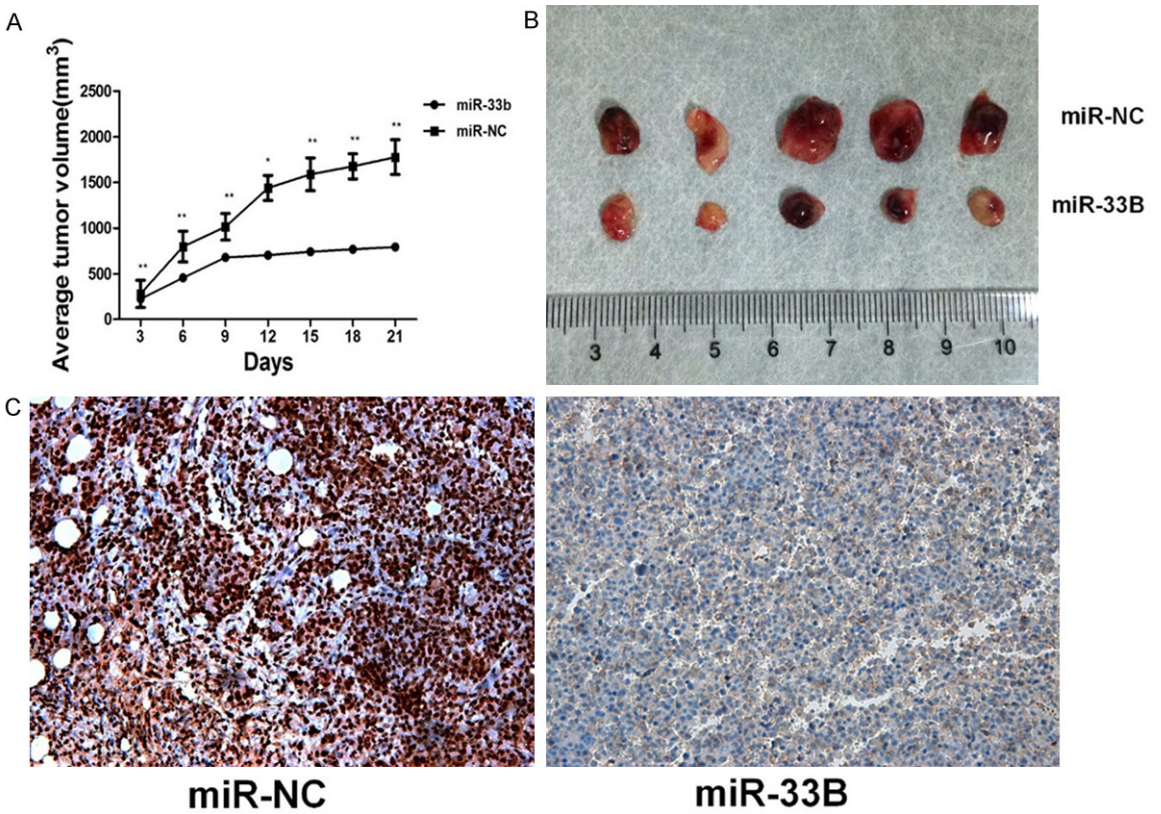


Figure 5. miR-33b inhibits tumor growth through the TWIST1 in vivo. A, B. Tumor growth curves in mice (n=6/group) inoculated with HTB182 cells at indicated days. At the experimental end point, tumors were dissected and photographed by subcutaneous injection. C. Immunohistochemically stained for TWIST1 was quantified according to staining intensity. All data are reported as the mean ± SD for the three independent experiments. (*P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test).

Tumor metastasis is one of the main reasons of death in lung SCC patients. MiRNA and EMT is the primary cause of metastasis. From our previous study, we select miR-33b as our research target [10]. Here, was showed that overexpression of miR-33b significantly suppressed proliferation features of lung SCC, and that it functions as an anti-metastatic miRNA. These results enrich our understanding of miR-33b in lung SCC molecular mechanisms metastasis. Differential expression of miR-33b has been reported in several different human cancers. It is obviously downregulated in newly diagnosed multiple myeloma patients and maybe a novel predictor associated with poor prognosis [20]. It is reported that miR-33b suppressed EMT and tumor invasion, metastasis in breast cancer, melamoma [15, 21, 22]. Our study is the first to identify miR-33b as a suppressor of lung SCC. Our data showed that miR-33b is frequently downregulated in lung SCC compared with adjacent normal lung tissues. Its low expression is associated with lymph node metastasis features in lung SCC. However, the role and mechanisms of miR-33b in lung SCC metastasis is still not known. In support of the tumor-inhibiting role of miR-33b in lung SCC, our current work provides evidence for the mechanical, as well as clinical significance of miR-33b in lung SCC.

As a critical metastasis promoter, epithelial-mesenchymal transition, TWIST1 was the important transcription factor of EMT and found to be directly regulated by miR-33b at the post-transcriptional level in the present study. It was recently reported that TWIST1 is directly regulated by some miRNA. miR-300 directly target twist1 to inhibit EMT and metastasis in human epithelial cancer [23]. miR-124 was found to be involved in cellular differentiation through the suppression of TWIST1 in glioma cells [24]. In the present study, we found that twist1 had over-expression in lung SCC cells and tumor tissue samples. Furthermore, the results showed that knock down of twist1 inhibited proliferation, invasion and migration of lung SCC cells. EMT was a key element in tumor cell metasta-

sis. Twist1 organize the entrance into a mesenchymal status by suppressing the expression of the epithelial marker E-cadherin and inducing the overexpression of the mesenchymal markers N-cadherin and vimentin [25]. We found that when miR-33b was upregulated, vimentin, affected by twist1, was down-regulated, while E-cadherin, also a downstream effector of twist1, was up-regulated, suggesting that miR-33b may inhibit the EMT of lung SCC cells.

In summary, our study provides significantly evidence that miR-33b inhibit cell proliferation and migration in lung SCC by directly targeting TWIST1. miR-33b is regarded as an important anti-metastatic miRNA that is frequently down-regulated in lung SCC. The present study implies the possibility of using this miR-33b as a promising therapeutic target for lung SCC.

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Disclosure of conflict of interest

None.

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Table S1. RNA oligo sequences

Oligo	Oligo sequences
Twist1-siRNA	Sense: 5'-GAUGGCAAGCUGCAGCUAUTT-3'
	Antisense: 5'-AUAGCUGCAGCUUGCCAUCTT-3'
siRNA-NC	Sense: 5'-UUCUCCGAACGUGUCACGUTT-3'
	Antisense: 5'-ACGUGACACGUUCGGAGAATT-3'
miR-33b mimic	Sense: 5'-GUGCAUUGCUGUUGCAUUGC-3'
	Antisense: 5'-AAUGCAACAGCAAUGCACUU-3'
miRNA mimic NC	Sense: 5'-UUCUCCGAACGUGUCAGCUTT-3'
	Antisense: 5'-ACGUGACACGUUCGGAGAATT-3'

Table S2. Lentiviral sequences

Lentiviral	Lentiviral sequences
Pri-miR-33b	Forward: 5'-GGATCCCTTTGGAGGCCCTGCATCAGGAGGGCTGGACAGCTGCTCCCGGGCCGGTG-GCGGGTGTGGGGGCCGAGAGAGGCGGGCGGCCCCGCGGTGCATTGCTGTTGCATTGCACGTGTGT-GAGGCGGGTGCAGTGCCTCGGCAGTGCAGCCCGGAGCCGGCCCCTGGCACCACGGGCCCCCATCCT-GCCCCTCCCAGAGCTGGAGCCCTGGTGACCCCTGCCCTGCCTGCCACCCCCAGGCCGTGCAGCT-GTTCCTGTGTGACCTGC-3'
	Reverse: 5'-GCAGGTCACACAGGAACAGCTGCACGGCCTGGGGGTGGCAGGCAGGGCAGGGGTCAC-CAGGGCTCCAGCTCTGGGAGGGGCAGGATGGGGGCCCGTGGTGCCAGGGGCCGGCTCCGGGCT-GCACTGCCGAGGCACTGCACCCGCCTCACACACGTGCAATGCAACAGCAATGCACCGCGGGGC-CGCCCGCCTCTCTCGGCCCCCACACCCGCCACCGGCCCGGGAGCAGCTGTCCAGCCCTCCTGATG-CAGGGCCTCCAAAGGGATCC-3'
Anti-miR-33b	Forward: 5'-GTGCATTGCTGTTGCATTGC-3'
	Reverse: 5'-GCAATGCAACAGCAATGCAC-3'
Anti-miR-NC	Forward: 5'-TTCTCCGAACGTGTCACGT-3'
	Reverse: 5'-ACGTGACACGTTCGGAGAA-3'

Table S3. Primers in the present study

Genes	Primers 5-3'
Human GAPDH	F: 5'-AGGGCTGCTTTTAACTCTGGT-3'
	R: 5'-CCCCACTTGATTTTGGAGGGA-3'
Human Twist1	F: 5'-GAGCAAGATTCAGACCCTCAAG-3'
	R: 5'-CCATCCTCCAGACCGAGAAG-3'
Human Vimentin	F: 5'-GTACCGGAGACAGGTGCAGT-3'
	R: 5'-AACGGCAAAGTTCTCTTCCA-3'
Human E-cadherin	F: 5'-GGGTTATTCCTCCCATCAGC-3'
	R: 5'-GTCACCTTCAGCCATCCTGT-3'

Table S4. mRNA oligo sequences

Genes	Sequences
TWIST1-3'-UTR wild type	F: 5'-GTTAATTCTTTTTTTCATCCTTCCTCTGAGGGGAAAAACAAAAAAAAAC TTAAAATACAAAAAACAACATTCTATTTATTTATTGAGGACCCATGGTAAAAT GCAAATAGATCCGGTGTCTAAATGCATTCATATTTTTATGATTGTTTTGTAAAT ATCTTTGTATATTTTTCTGCAATAAATAAATATAAAAAATTTAGAGAA-3'
	R: 5'-TTCTCTAAATTTTTTATATTTATTTATTGCAGAAAAATATACAAAGATATTT ACAAAACAATCATAAAAATATGAATGCATTTAGACACCGGATCTATTTGCATTT TACCATGGGTCCTCAATAAATAAATAGAATGTTGTTTTTTGTATTTTAAGTTTTT TTTTGTTTTTCCCCTCAGAGGAAGGATGAAAAAAAGAATTAAC-3'
TWIST1-3'-UTR mutant	F: 5'-GTTAATTCTTTTTTTCATCCTTCCTCTGAGGGGAAAAACAAAAAAAAACT TAAAATACAAAAAACAACATTCTATTTATTTATTGAGGACCCATGGTAACTATAG AATAGATCCGGTGTCTACTATAGTTCATATTTTTATGATTGTTTTGTAAATATCTTT GTATATTTTTCTGCAATAAATAAATATAAAAAATTTAGAGAA-3'
	R: 5'-TTCTCTAAATTTTTTATATTTATTTATTGCAGAAAAATATACAAAGATATTTA CAAAACAATCATAAAAATATGAACTATAGTAGACACCGGATCTATTCTATAGTTA CCATGGGTCCTCAATAAATAAATAGAATGTTGTTTTTTGTATTTTAAGTTTTTTTT TGTTTTTCCCCTCAGAGGAAGGATGAAAAAAAGAATTAAC-3'