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MicroRNA-132 inhibits migration, invasion and epithelial-mesenchymal transition by regulating TGFβ1/Smad2 in human non-small cell lung cancer

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Abstract. – OBJECTIVES: Increasing evidence shows that microRNA involves in the development of several types of cancers, however, the role of microRNA-132 (miR-132) in non-small cell lung cancer (NSCLC) metastasis remains largely unknown. In this study, we aimed to investigate the effect of miR-132 on the epithelial-mesenchymal transition (EMT) and the potential mechanisms in NSCLC.

PATIENTS AND METHODS: The Quantitative real-time PCR (QRT-PCR) was used to detect the miR-132 levels in 15 NSCLC tissues and cell lines. Transwell and wound healing assays were used to evaluate the function of miR-132 in NSCLC cell metastasis. EMT-related markers were determined by using qRT-PCR. EMT-related TGFβ1/Smad2 signaling pathway was explored using Western blot.

RESULTS: MiR-132 expression level was lower in NSCLC tissues compared with the matched adjacent normal tissues. It was also downregulated in A549 cell lines compared to normal lung epithelial cell BEAS-2B. MiR-132 overexpression obviously inhibited migration and invasion capacities in A549 cells while miR-132 down-regulation would enhance such capacities. Expression of EMT-related markers and TGFβ1/Smad2 was higher in A549 cells transfected with miR-132 inhibitor compared with those transfected with miR-132 mimic. Moreover, expression of EMT-related markers and Smad2 was increased in NSCLC tissues compared to in the adjacent normal tissues and the reverse expression of miR-132 and Smad2 was observed.

CONCLUSIONS: These results indicate that miR-132 may play a suppressive role in the metastasis of NSCLC cells by promoting EMT via TGFβ1/Smad2 signaling pathway.

Key Words:
Non-small cell lung cancer (NSCLC), microRNA-132, Metastasis, Epithelial-mesenchymal transition (EMT).

Introduction

Non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancer, and the metastases are detected in about 75% of the patients those clinically diagnosed[1]. The lymph node, brain, bone and liver are the most common metastatic sites in NSCLC and the uncontrolled metastasis of NSCLC is the main causes of death in Asian and Western populations[2]. Numerous investigations revealed that tumor-associated molecules would be the therapeutic targets for cancer metastasis. However, the molecular mechanisms of metastatic NSCLC are not well understood, especially the role of microRNAs, which are the most topical researches in recent years in the progression of cancer. Therefore, identifying new microRNAs and explore their functions in NSCLC are necessary for the disease individualized diagnosis and treatment in the future.

MicroRNAs are small double-stranded, non-protein coding RNAs with 21-25 nucleotides and can mediate sequence-specific, posttranscriptional regulatory to target RNA by binding to the homologous 3'UTR region as an imperfect match[3,4]. Accumulating studies demonstrate that microRNAs involves in cancer cell proliferation, differentiation, apoptosis and other cellular activities, including tumor initiation and progression[5-7]. Investigations in recent years reported that multiple microRNAs play a key role in the development and progression of NSCLC[8-10].

In humans, microRNA-132 (miR-132) is reported to regulate cancer cell progression: in ovarian cancer cells, miR-132 can suppress the cell proliferation, invasion and migration by targeting E2F5[11]; in glioma cells, miR-132 can result in caspase-dependent apoptotic death[12]; in

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colorectal cancer, down-regulation of miR-132 is associated with poor prognosis[13]. In NSCLC, reduced miR−132 expression was significantly associated with lymph node metastasis cells in patients and increased miR-132 was shown can reduce NSCLC cell proliferation, invasion and migration, and promoted cell apoptosis[14]. However, the potential molecular mechanism of miR-132 in NSCLC progression remains largely unclear.

In this study, we focus on the role of miR-132 in NSCLC metastasis. The expression of miR-132 in NSCLC tissues that occurred lymph node metastasis and A549 cells were measured. Based on the result that the mRNA levels of miR-132 in cancer tissue and cancer cells were down-regulated, we analyzed the role of miR-132 in cancer cells metastasis and explored the potential molecular mechanism by evaluating the expression of EMT-related markers and EMT-associated signaling pathway.

Patients and Methods

Patients and Tissue Samples

NSCLC and the matched adjacent normal tissue specimens were obtained from 15 NSCLC patients confirmed have occurred lymph node metastasis by the clinical and pathological diagnosis at the hospital. None of the patients received chemotherapy or radiation therapy before the surgery in the current study. The tissue samples were stored in liquid nitrogen after resection. This study was approved by the Ethics Committee of the hospital and written informed consents were obtained from all patients.

Cell Culture

Normal lung epithelial cell BEAS-2B and human NSCLC cell lines A549 were from the Institute of Basic Medical Science, Chinese Academy of Medical Science and Peking Union Medical College (Beijing, China). The cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) medium with 10% fetal bovine serum(FBS) (Gibco) in the condition containing 5% CO_2 at 3795°C.

Cell Transfection

The hsa-miR-132 mimics, hsa-miR-132 inhibitor and control mimics (NC) were synthesized by Genepharma (Shanghai, China). The sequences were as follows: miR-132 mimics, 5′-UAACAGUCUACAGCCAUGGUCG-3′; miR-132 inhibitor, 5′-CGACCAUGGCUGUAGACUGUUA-3′. Transfection was performed using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY, USA) at a final concentration of 50 nM according to the manufacturer's instructions when cells reached 50-70% confluence. The transfection efficiency was determined by quantitative real-time PCR (QRT-PCR).

Quantitative Real-time PCR

Total RNAs from cancer tissues and cells were isolated by using TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. Reverse transcription and QRT-PCR for EMT-related markers and Smad2 were performed using PrimeScript™ RT Master Mix and the SYBR® Premix Ex Taq™ II kit (TaKaRa Bio, Otsu, Shiga, Japan). Reverse transcription and qRT-PCR for detecting miR-132 was performed by using hairpin-it miRNAs qRT-PCR kits (Genepharma, Shanghai, China). The samples were run on an ABI7500 Real-time PCR Detection System (Applied Biosystems, Foster City, CA, USA) with the conditions of 95°C for 5 min, followed by 40 repetitions of 95°C for 5 s and 60C for 20 s. U6 and GAPDH were used as the internal controls for miRNA and genes, respectively, using the 2^{-Ct} method. The primers of miRNA were used as described previously[14]. Other primers used were listed in Table I.

Migration and Invasion Assays

24-well transwell chambers (8 μm; Corning, Inc.) were used for the invasion assay. About 10 μg/ml Matrigel (BD Biosciences, San Jose, CA, USA) was used to cover the reverse side of upper chambers and the facade was coated with 1 mg/ml Matrigel 70 μl for cell invasion assays. 2×10^5 cells suspended in 200 μl serum-free medium with 1% bovine serum albumin (BSA) were seeded into the upper chamber and 700 μl of complete medium was added to the bottom well. After 24 h incubation, cells that not migrated were removed using cotton swabs from the upper surfaces. The cells that had migrated or invaded to the lower surfaces of the filters were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 10 min. Five random fields were captured from each membrane and the number of migration cells was counted to analyze.

Table I. Primer sequences of EMT-related markers and SMAD2 for QRT-PCR analyses.

Gene	Primer sequences	
N-Cadherin	Forward:	5′- TTCCATCCTGCGCGTGAAG -3′
	Reverse:	5′- CGGCGTTTCATCCATACCACA -3′
ZEB1	Forward:	5′- GCAGATGAAGCAGGATGTAC -3′
	Reverse:	5′- TCCATTTTCATCATGACCACT -3′
SNAIL	Forward:	5′- GAGTGGTTCTTCTGCGCTAC -3′
	Reverse:	5′- TCCAGAGTTTACCTTCCAGCAG -3′
VIM	Forward:	5′- ATTGCAGGAGGAGATGCTTCA -3′
	Reverse:	5′- GGATTTCCTCTTCGTGGAGTT -3′
SMAD2	Forward:	5′- GACACACCGAGATCCTAACA -3′
	Reverse:	5′- GAGAGCCTGTGTCCATACTTT -3′
GAPDH	Forward:	5′- AATGAATGGGCAGCCGTTAGGA -3′
	Reverse:	5′- TCTGATTTGGTCGTATTGGGCG -3′

ZEB1, zinc finger E-box binding homeobox 1; SNAIL, snail family transcriptional repressor 1; VIM, vimentin; SMAD2, SMAD family member 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Western Blot

Cell extracts using RIPA buffer were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk for 1 hour and incubated with primary antibody overnight. The primary antibodies used in this study were as follows: anti-TGFβ1 antibody 1:1000, anti-p-Smad2 antibody 1:1000, anti-Smad2 antibody 1:1000, (Abcam, Cambridge, UK) and anti-β-actin antibody (1:5000, Sigma, St. Louis, MO, USA). Then the membranes were incubated with anti-rabbit, anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical Analysis

The data were presented as mean ± standard deviations (SD). Student's *t*-test was used to determine the statistical significance between the groups. Two-tailed Pearson's correlation was used to assess the relationship between miR-132 and SMAD2 expressions. Statistical analysis was performed using SPSS16.0 software (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered significant.

Results

MiR-132 Expression was Down-regulated in NSCLC Tissues and Cells

QRT-PCR assay showed the expression of miR-132 was significantly lower in NSCLC tissues with lymph node metastasis than that in the matched adjacent normal tissues (Figure 1 A). As well as, the mRNA level of miR-132 was showed remarkably higher in normal lung epithelial cell BEAS-2B compared with that in NSCLC A549 cells (Figure 1 B). These data suggest that down-regulation of miR-138 may be related to NSCLC or NSCLC metastasis.

MiR-132 Inhibited the Migration and Invasion of NSCLC Cells

The miR-132 level of A549 transfected with miR-132 mimic, mimic control or inhibitor was determined by QRT-PCR. Figure 1C and 1D showed miR-132 expression in cells transfected with miR-132 inhibitor was significant decreased while transfection of miR-132 mimic remarkably increased the miR-132 level, compared to the mimic control cells (NC). Next, we investigated the effect of miR-132 on metastasis of NSCLC cells by transwell assays. The results showed that miR-132 overexpression significantly inhibited cell migration and invasion compared to the control group ($p < 0.05$), while miR-132 expression inhibition would occur the opposite effect (Figure 2). The results demonstrate that miR-132 can suppress the migration and invasion of NSCLC cells in vitro.

MiR-132 Decreased the Expression of EMT-related Markers

To explore whether miR-132 overexpression changes EMT, we examined the expression of EMT-related markers in NSCLC cell lines. The data revealed that miR-132 inhibition dramatical-

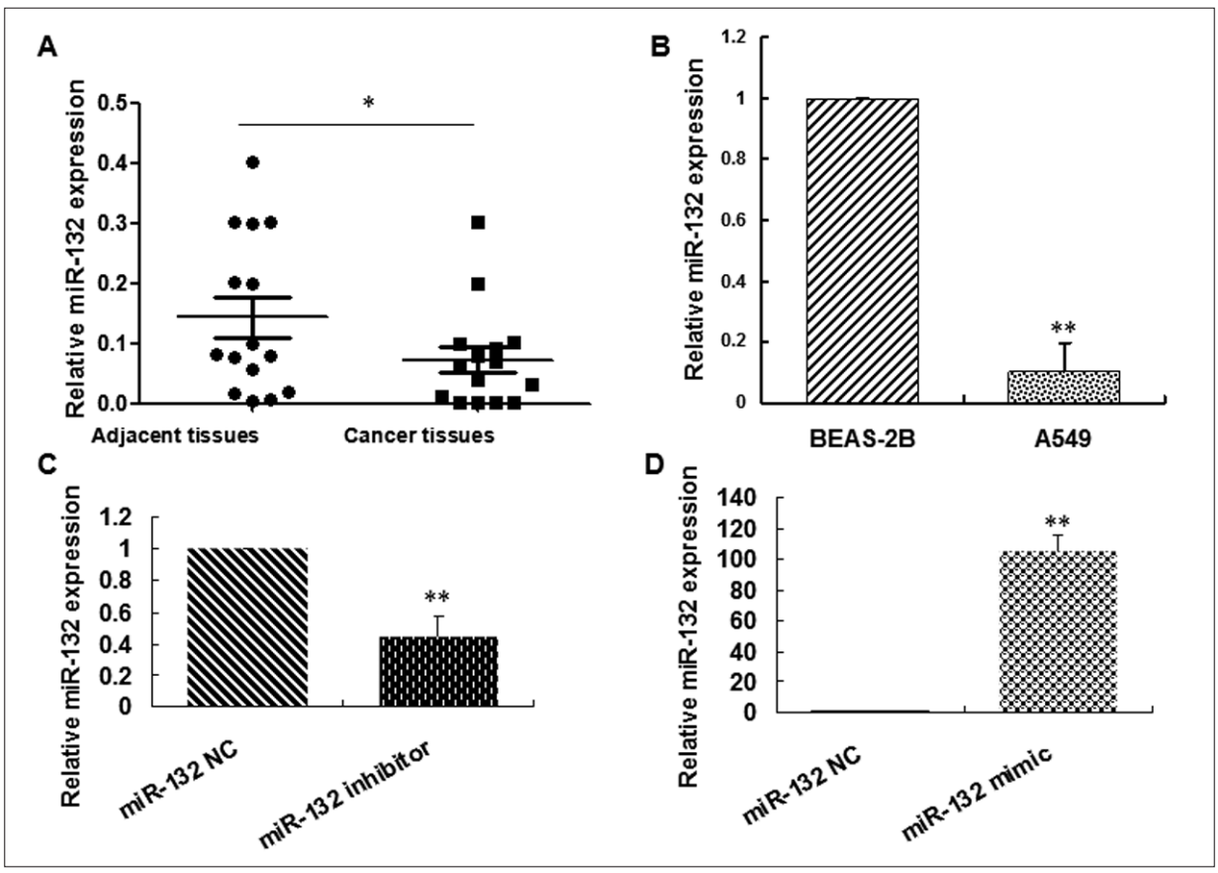

Figure 1. MiR-132 expression in NSCLC tissues and cells. QRT-PCR analysis of miR-132 expression level in NSCLC tissues is lower than that in the matched normal tissues ***(A)***. QRT-PCR analysis of miR-132 expression in normal lung epithelial cell BEAS-2B is higher than that in NSCLC A549 cells ***(B)***. QRT-PCR analysis of miR-132 expression in A549 cells transfected with miR-132 inhibitor was decreased compared to the mimic control cells ***(C)***. MiR-132 expression in cells transfected with miR-132 mimic is increased compared to the mimic control cells ***(D)***. *$p < 0.05$; **$p < 0.01$.

ly promoted the expression of mesenchymal markers N-Cadherin, Zeb1, Snail and Vimentin. Overexpression of miR-132 can significantly inhibit the expression of mesenchymal markers ($p < 0.05$) (Figure 3). These results suggest that miR-132 expression involves in EMT in NSCLC cells.

TGFβ1/Smad2 was Increased in miR-132 Down-regulated NSCLC Cells

To explore the potential mechanism of the EMT inhibition by miR-132 in NSCLC cells, TGFβ1/Smad2 expression was examined. We observed the expression of TGFβ1, Smad2 and p-Smad2 was increased in A549 cells transfected with miR-132 inhibitor compared to miR-132 NC cells. Consistently, TGFβ1/Smad2 expression was lower in A549 cells transfected with miR-132 mimic than that in NC cells (Figure 4). The results indicate that TGFβ1/Smad2 signaling pathway maybe promote the EMT in NSCLC metastasis induced by miR-132.

The Correlation of the Expression of SMAD2 and miR-132 in NSCLC Tissues

As a type of conservative miR-132, miR-132-3p is predicted can combine to the 3′UTR of Smad2 on www.targetscan.org (Figure 5A). To further determine the association between Smad2 and miR-132, the expression of SMAD2 in NSCLC tissues was tested by QRT-PCR. Semi-quantitative results showed the expression of SMAD2 was increased in the tumor tissue compared to in the matched normal tissues in some NLCLC patients (Figure 5B). The QRT-PCR results revealed that expression of SMAD2 was significantly increased in NLCLC tissues compared to in normal tissues ($p < 0.01$) (Figure 5C).

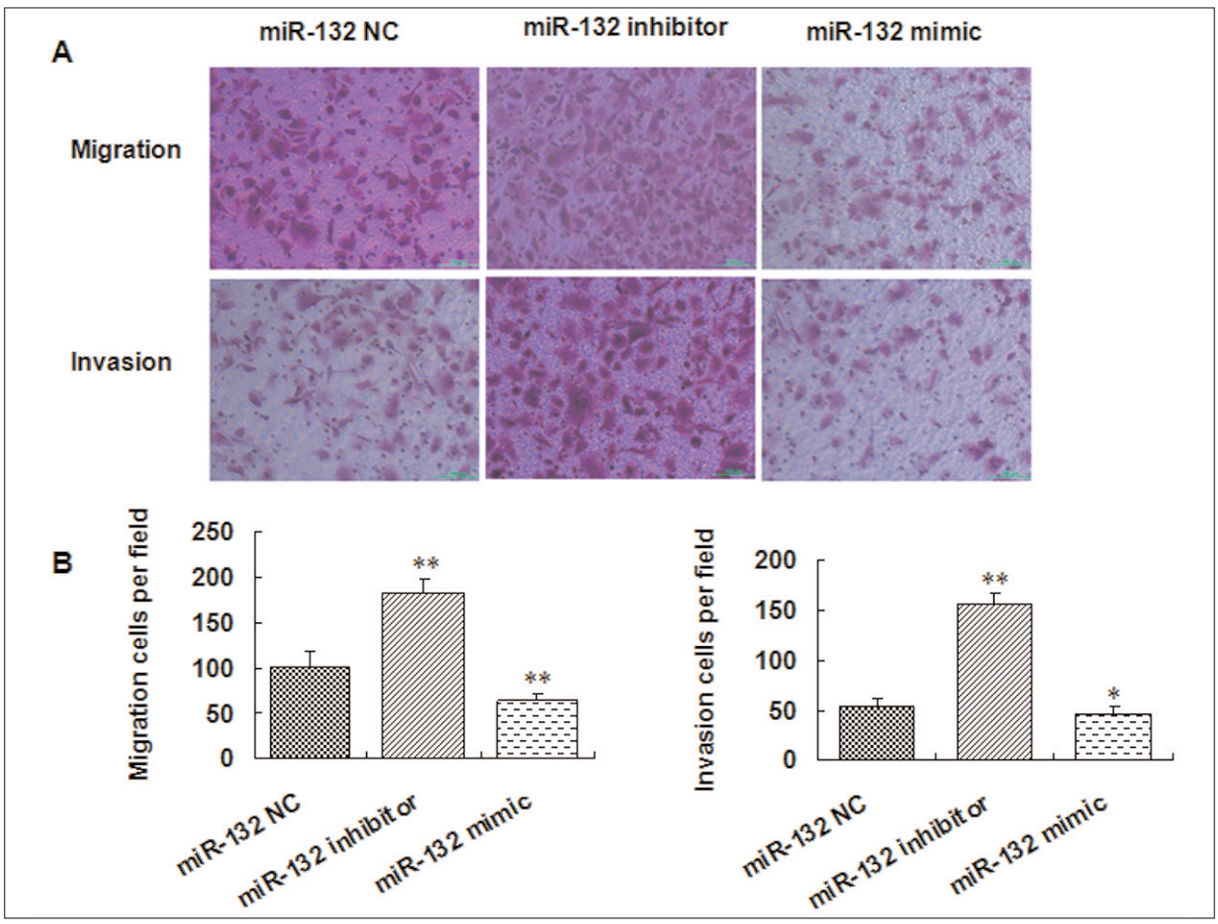

Figure 2. MiR-132 inhibited the migration and invasion of NSCLC cells. Transwell assays for A549 cells transfected with miR-132 mimic, miR-132 inhibitor or miR-132 NC ***(A)***. Migrated and invaded cells were stained and counted in at least 5 microscopic fields. The migrated or invaded cells were increased in cells transfected with miR-132 inhibitor while were decreased in cells transfected with miR-132 mimic, compared to cells transfected with miR-132 NC, respectively ***(B)***. (Magnification x100). *$p < 0.05$; **$p < 0.01$.

Pearson's correlation analysis displayed the negative correlation between SMAD2 expression and miR-132 expression (r = -0.56, $p < 0.05$) (Figure 5D). The results imply that SMAD2 maybe one of the target proteins of miR-132 in NLCLC metastasis.

Discussion

Dysregulation of miRNAs was reported involves in many human tumor types, including NSCLC. MiR-132 has been found to be a tumor suppressor or an oncogene in several cancers[11-13]. Liu et al[14] reported that miR-132 was downregulated in NSCLC tissues and cell lines, and overexpression of miR-132 can reduce cell invasion and migration. However, the potential mechanism of miR-132 in NSCLC metastasis is not well understood. In NSCLC tissues with lymph node metastasis and A549 cells, we detected miR-132 expression and demonstrated the miR-132 role in cells metastasis. Then we detected the EMT-related markers expression in miR-132 overexpression and downregulation NSCLC cells. Further, TGFβ1/Smad2 changes were explored in NSCLC cells and correlation of SMAD2 and miR-132 was evaluated in NSCLC tissues. Our data showed that the expression level of miR-132 was significantly reduced in NSCLC tissues with lymph node metastasis and NSCLC cells. The migration and invasion capability, EMT-related markers expression and TGFβ1/Smad2 expression were increased in miR-132 inhibited NSCLC cells compared to miR-132 NC cells, and the opposite results were

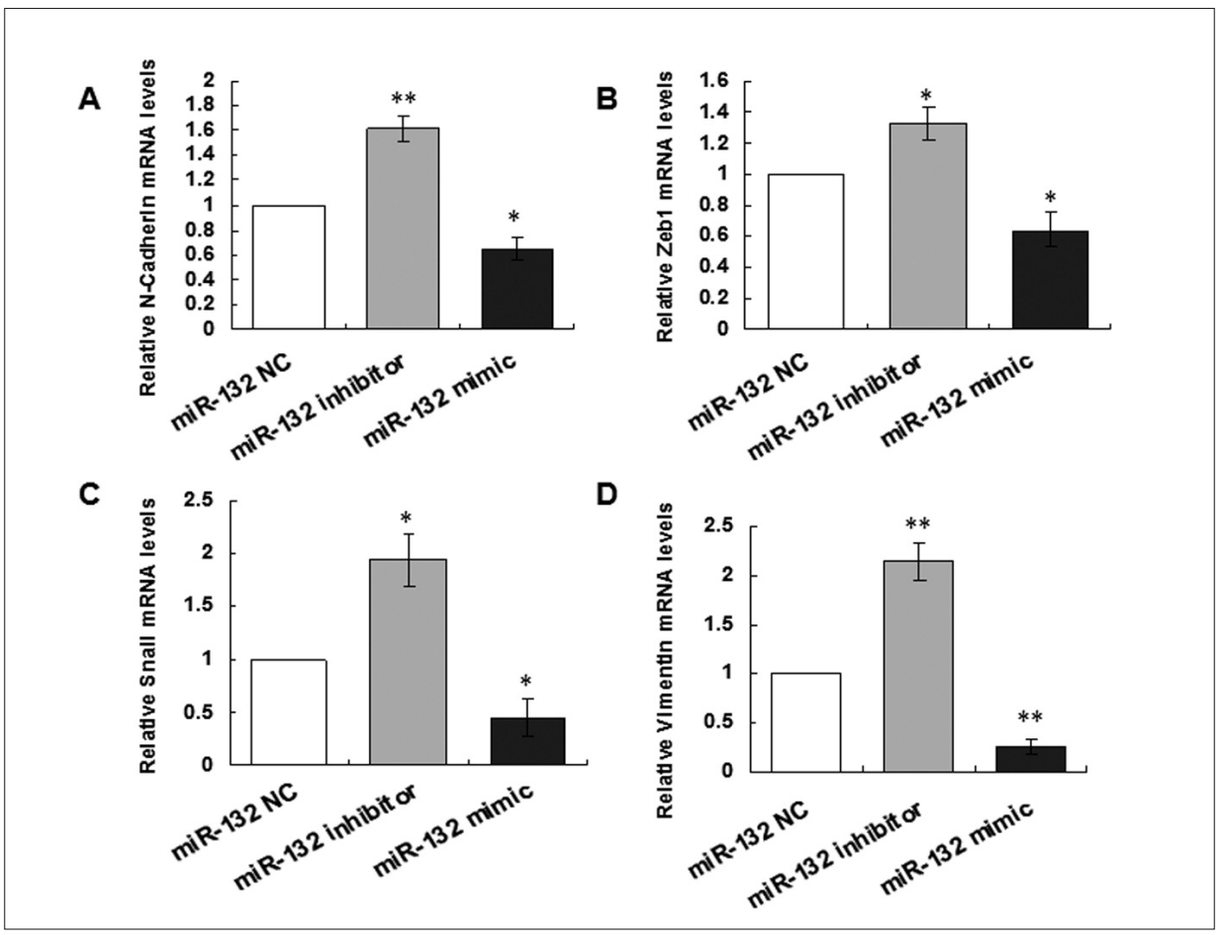

Figure 3. The expression of EMT-related markers. The mRNA level of mesenchymal markers N-Cadherin and Vimentin was elevated in miR-132 inhibited cells and was decreased in miR-132 overexpression cells (***A*** and ***D***). The expression tendency of EMT-related transcription factors Zeb1 and Snail was the same as N-Cadherin and Vimentin in transfection cells (***B*** and ***C***). *$p < 0.05$; **$p < 0.01$.

observed in miR-132 overexpression NSCLC cells. We found a negative correlation between the expression of SMAD2 and miR-132 in NSCLC tissues.

Tumor metastasis is known as a common cause of lethality in cancer including NSCLC. Patients with metastasis in NSCLC often have a poor prognosis and high mortality[15]. Therefore, the identification of biomarkers for NSCLC with metastasis was very important. In our study, we found miR-132 was significantly reduced in the NSCLC with lymph node metastasis. And cancer cells metastasis capability was increased when inhibiting miR-132 expression. The result was consistent with previous studies[14,16] and it suggests the down-regulation of miR-132 may be considered as a specific marker for early progression and prognosis of NSCLC with lymph node metastasis.

EMT is one of the major molecular mechanisms promoting cancer metastasis[17]. In the EMT process, the epithelial cells gain the morphology of the mesenchymal cells and gene expression pattern[18]. MicroRNAs can induce EMT in cancer cells in many studies: Jiang et al[19] reported miR-218 can inhibit EMT and invasion in cervical cancer; Li et al work[20] revealed that miR-139-5p was significantly correlated with the metastasis potential and drug resistance of colon cancer cells by affecting EMT; MiR-30a is an important miRNA modulating EMT and cisplatin sensitivity in gastric cancer cells[21]; MiR-181a upregulation is associated with EMT in ovarian cancer cells[22]; MiR-145 and miR-203 can inhibit TGF-β-induced EMT in NSCLC cells[23]. In our study, we first found the expression of mesenchymal cell markers N-Cadherin, Zeb1, Snail and Vimentin were suppressed in

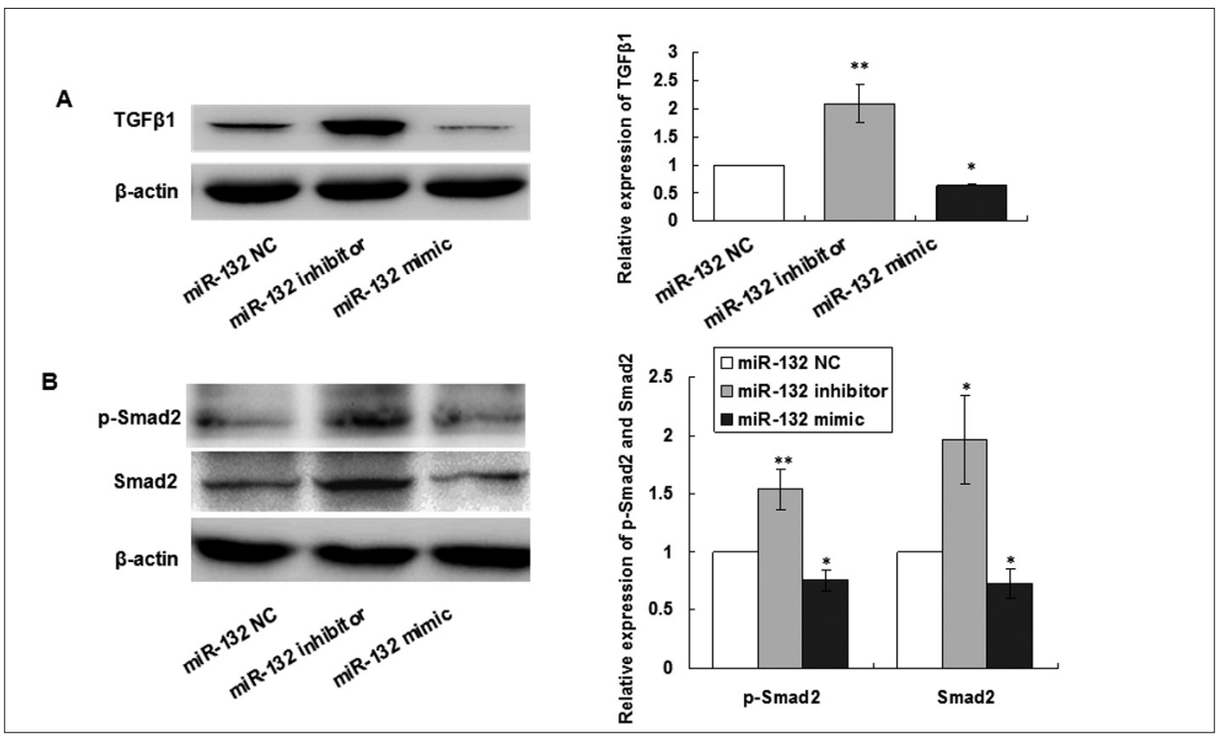

Figure 4. TGFβ1/Smad2 signaling was active in miR-132 inhibited cells. The protein level of TGFβ1 was up-regulated in miR-132 inhibition cells while it was down-regulated in miR-132 mimic cells ***(A)***. Expression of p-Smad2 and smad2 was increased in miR-132 inhibition cells and was decreased in miR-132 overexpression cells, compared to miR-132 NC cells ***(B)***. *$p < 0.05$; **$p < 0.01$.

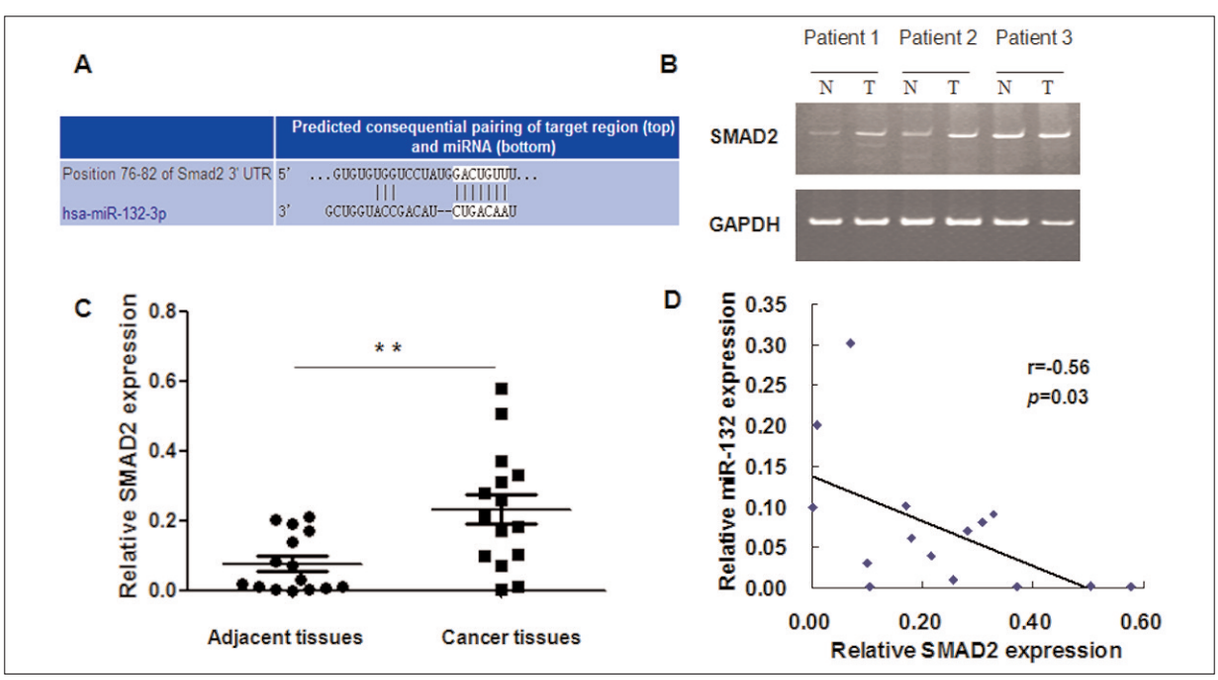

Figure 5. The reverse expression of SMAD2 and miR-132 in NSCLC tissues. The prediction of combine state between miR-132-3p and the 3′ UTR of Smad2 on the website of www.targetscan.org ***(A)***. Semi-quantitative results showed the expression of SMAD2 was increased in the tumor tissue compared to in the matched normal tissues in NLCLC patients ***(B)***. The mRNA expression of SMAD2 in NSCLC tissues was significantly increased in NLCLC tissues compared to in normal tissues ***(C)***. Correlation between SMAD2 and miR-132 expression was negative by Pearson's correlation analysis (r = -0.56, $p < 0.05$) ***(D)***. *$p < 0.05$; **$p < 0.01$.

miR-132 overexpression NSCLC cells, that is, miR-132 can inhibit EMT in NSCLC.

TGF-β1/Smad signaling is an important regulating pathway of EMT and TGF-β1 induced EMT plays an important role in NSCLC cell invasion[23-25]. Based on the EMT-related markers results, we further explored the TGF-β1/Smad2 signaling in NSCLC cells to clarify the mechanism of EMT-inducing by miR-132. TGF-β1, Smad2 and p-Smad2 were increased in miRNA-132 down-regulated NSCLC cells. As one target of miRNA-132, we observed a significant reverse correlation between SMAD2 and miR-132 levels in NSCLC tissues and the results consistent with theoretical speculation. Thus, Smad2 and phosphorylation of Smad2 may be regulated by the expression of miR-132, then the expression of N-Cadherin, Zeb1, Snail and Vimentin was induced, which would promote EMT and NSCLC metastasis. Therefore, our results suggest that miR-132 inhibits the EMT in NSCLC metastasis partly at least via TGFβ1/Smad2 signaling pathway.

Conclusions

In this study, we discovered that miR-132 functioned as a metastasis suppressor in NSCLC and may provide a strategy for targeting miR-132/TGFβ1/Smad2 in the treatment of metastatic NSCLC. These findings are necessary to be confirmed in a larger of NSCLC tissue samples with metastasis. Although Smad2 is one of the predicted targets of miR-132 and we observed the inverse correlation between miR-132 and SMAD2 expression in NSCLC specimens, however, whether miR-132 was able to interact with the 3′-UTR of Smad2 directly in NSCLC cells should be verified by luciferase reporter assay. MiR-132/TGFβ1/Smad2 signaling pathways responsible for NSCLC metastasis also need to be elucidated *in vivo* study in future studies.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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