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MicroRNA-99b inhibits NSCLC cell invasion and migration by directly targeting NIPBL

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide. microRNAs (miRNAs) have been confirmed as vital regulators of multiple tumors, including NSCLC. The aim of the current study was to explore the biological mechanisms of miR-99b in NSCLC progression.

PATIENTS AND METHODS: NSCLC tissues and adjacent matched human non-neoplastic lung tissues used in this study were collected from 50 cases of NSCLC patients. The expression of miR-99b and NIPBL in NSCLC tissues and cell lines (A549, NCI-H460, NCI-H1299 and SPC-A1) were determined by real-time-polymerase chain reaction (qRT-PCR). The NIPBL protein level was measured by Western blot. Dual-Luciferase reporter, Western blotting and qRT-PCR were carried out to verify the potential target of miR-99b. Transwell assay was used for investigating miR-99b effect on cell migration and invasion in NSCLC cells.

RESULTS: The results of qRT-PCR indicated that the expression of miR-99b was downregulated in the NSCLC tissues and cell lines. Overexpression of miR-99b could significantly inhibit the invasion and migration capacities in NSCLC cells. Furthermore, we also determined that NIPBL was a direct target of miR-99b. Additionally, we found NIPBL was implicated in the suppressive effects on NSCLC cell invasion and migration mediated by miR-99b.

CONCLUSIONS: In summary, miR-99b exerted anti-tumor functions in NSCLC via regulation of NIPBL, suggesting that miR-99b/NIPBL axis may be novel biomarkers for NSCLC treatments.

Key Words:
MicroRNA-99b, NSCLC, Invasion, Migration, NIPBL.

Introduction

As one of the most common malignancy all over the world, lung cancer has a very low survival rate and highest mortality[1]. Non-small cell lung cancer (NSCLC), accounting for 80% of all lung cancers, is the most common type of lung cancer[2]. Existed therapies for NSCLC patients mainly include surgery resection, radiotherapy and chemotherapy. Moreover, depending on the extent and progression of NSCLC patients, the above treatments can be applied alone or in combination[3]. Although great improvements have been obtained in the diagnosis and therapy for NSCLC patients, the 5-year survival rate is still unsatisfactory, and the prognosis remains poor[4]. Due to the tumor heterogeneities within histological subtypes, metastasis, recurrence and late disease presentation, the overall five-year survival rate of NSCLC patients is less than 18%[5,6]. Therefore, to improve the diagnosis, prognosis and drug-targeted therapies for NSCLC, it is essential to screen metastases-related genes and explore their underlying molecular mechanisms.

MicroRNAs (miRNAs/miRs) are a class of noncoding RNAs, 18-25 nucleotides (nt) in length, encoded by distinct genes and undergoing a sophisticated process to mature to evolutionarily conserved single-stranded forms[7]. MiRNAs are aberrantly expressed in multiple tumors by modulating the tumor progressions and acting as novel biomarkers for therapeutic intervention. MiRNAs regulate gene expressions via sequence-specific interactions with its 3'-UTRs, leading to degradation of mRNAs or translation inhibition[8]. MiRNAs regulate different kinds of biological processes, such as migration, invasion, differentiation, apoptosis and proliferation[9,10]. MiR-34a inhibited ovarian cancer cell proliferation via regulating HDAC1[11]; miR-1469 promoted cell apoptosis in lung cancer via targeting STAT5a[12]; miR-203 suppressed gastric cancer cell growth and metastasis via targeting Slug[13]. Therefore, investigating the underlying mechanisms of miR-99b in NSCLC is of great significance to identify novel treatment for NSCLC patients.

NIPBL (nipped-B-like protein), as a cohesion loading factor, was important in the interaction of cohesin and chromosomes. Components in

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the cohesin complex and cohesin regulatory proteins are frequently altered mutation levels in human cancers[14]. Mutations of NIPBL existed in about 60% of patients with Cornelia de Lange syndrome[15]. NIPBL frameshift mutations may affect the functional effect of cohesion complexes on the cell cycle[16]. NIPBL downregulation induced autophagy, apoptosis in breast cancer[17]. The function of NIPBL in NSCLC invasion and migration remains unclear and we speculated that NIPBL involved in NSCLC invasion and migration. In the current study, we identified the role of NIPBL and interpreted the potential mechanisms.

Patients and Methods

Patients and Samples

In accordance with WHO classification, 50 cases of NSCLC samples and the corresponding para-carcinoma tissue samples were obtained from Weifang People's Hospital between October 2015 and September 2017. All the NSCLC patients had not undergone any preoperative radiotherapy or chemotherapy. Samples were stored at 80˚C. Written informed consent was obtained from each patient. This investigation was approved by the Ethics Committee of Weifang People's Hospital. All experimental protocols were performed in accordance with the Declaration of Helsinki.

Cell Culture

Human NSCLC cell lines (A549, NCI-H460, NCI-H1299 and SPC-A1) and bronchial epithelium BEAS-2B cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37˚C, 5% CO_2.

Cell Transfection

MiR-99b mimics or inhibitor as well as the NIPBL vector or siRNA were obtained from GenePharma (Shanghai, China) and transfected into NSCLC cells by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufactures' proposals. Efficiencies of the transfected cells were analyzed by qRT-PCR after transfection of 48 h.

qRTPCR Analysis

TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was utilized for isolating RNA from NSCLC tissue specimens and cells. Subsequently, the RNA was reverse transcribed into cDNA by PrimeScript RT reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). Amplifications were carried out using a miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) and qRT-PCR was run on ABI 7900 RT-PCR Detection System (Applied Biosystems, Foster City, CA, USA). The expressions of miR-99b and NIPBL mRNA were normalized to U6 and GAPDH, respectively. The relative expression was determined by $2^{-\Delta\Delta Ct}$ method. The primers sequences were described in Table I.

Transwell Assays

Transwell chambers (8 μm pore size; Coning, Corning, NY, USA) coated with or without Matrigel (5 mg/ml, BD Biosciences, San Jose, CA, USA) were utilized to examine NSCLC cell invasion and migration capacities, respectively. NSCLC cells following transfections with miR-99b mimics or inhibitor were seeded into the upper transwell chambers, while NSCLC cells transfected with

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-99b forward	5'- ATCCAGTGCGTGTCGTG-3'
miR-99b reverse	5'-TGCTCAAGCTCGTGTCTGT-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'- AACGCTTCACGAATTTGCGT-3'
NIPBL forward	5'-GAAGTGGAGAAAGCCTGTGCC-3'
NIPBL reverse	5'-TTCTTCCTCCACATGAAAGCG-3'
GAPDH forward	5'-ACCTGACCTGCCGTCTAGAA-3'
GAPDH reverse	5'-TCCACCACCCTGTTGCTGTA-3'

U6: small nuclear RNA, snRNA; NIPBL: nipped-B-like protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

miR-99b+NIPBL siRNA or miR-99b+negative control siRNA were placed into the upper transwell chambers for rescue assays. In the meantime, medium including 10% FBS was added into the lower chambers. After incubation for 24 h, the cells that had traversed the membrane were subsequently fixed with formaldehyde (4%) and stained with crystal violet (0.1%), respectively. An inverted microscope (Olympus, Tokyo, Japan) was used to count the cells.

Western Blot

Total proteins were extracted from cultured cells by radio immunoprecipitation (RIPA) buffer (Beyotime, Jiangsu, China). Bicinchoninic acid assay (BCA) kit (Keygen, Nanjing, China) was utilized to measure the protein concentrations. Subsequently, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA). The PVDF was incubated with 5% skimmed milk for 2 h after incubation with the antibody 12 hours at 4˚C. The following primary antibodies were used: antibodies against NIPBL (1:1000; ab225908; Abcam, Cambridge, MA, USA) and GAPDH (1:1000; ab181603; Abcam, Cambridge, MA, USA). Followed by being washed with Tris-Buffered Saline and Tween-20 (TBST), the membranes were incubated with secondary antibody (1:5,000, ab6721; Abcam, Cambridge, MA, USA). GAPDH was used as an internal control. Protein was detected using enhanced chemiluminescence (ECL) kit.

DualLuciferase Reporter Analysis

The target gene of miR-99b was analyzed by Targetscan. NIPBL was determined as a candidate target of miR99b. The wild type (WT) NIPBL 3'UTR that had binding sites with miR-99b and mutual type (MUT) NIPBL 3'UTR were inserted into pmir-GLO vectors (Promega, Madison, WI, USA), followed by cotransfected into NSCLC cells with miR-99b mimics or miR control (NC). Luciferase abilities were determined by Dual-Luciferase reporter system (Promega, Madison, WI, USA).

Statistical Analysis

SPSS 17.0 (SPSS, Chicago, IL, USA) was applied to perform the data analysis. Student's *t*-test, ANOVA and Scheffe's post-hoc analysis were applied, where appropriate. Correlation between mRNA and miRNA were estimated using the Spearman's correlation. $p<0.05$ was regarded as significant difference.

Results

MiR-99b Was Downregulated and NIPBL Was Upregulated In NSCLC

We carried out qRT-PCR assays to determine the miR-99b and NIPBL expressions in NSCLC tissues and cell lines. The findings revealed that miR-99b expressions were prominently decreased in NSCLC tissues compared to normal tissues (Figure 1A). Additionally, we detected miR-99b expressions in NSCLC cell lines. As expected, miR-99b was significantly downregulated in NSCLC cells (A549, NCI-H460, NCI-H1299 and SPC-A1) compared to BEAS-2B cell lines (Figure 1B). Furthermore, we measured the NIPBL expressions in NSCLC cells and the results demonstrated significant increases in NSCLC cells (A549, NCI-H460, NCI-H1299 and SPC-A1) compared to BEAS-2B cells (Figure 1C). Moreover, we further analyzed the correlation between miR-99b and NIPBL expressions. MiR-99b was negatively correlated with NIPBL expressions in NSCLC tissues (Figure 1D). Moreover, we analyzed the clinical relevance of miR-99b expression with NSCLC clinicopathological characteristics. Results demonstrated that decreased miR-99b expression was closely correlated with lymph node metastasis and TNM stage of NSCLC patients (Table II).

MiR-99b Inhibited Cell Invasion and Migration of NSCLC

To explore the roles of miR-99b in NSCLC progression, a series of functional experiments was conducted. qRT-PCR was used to confirm the overexpression of miR-99b in A549 cells (Figure 2A). As shown in Figure 2B, overexpression of miR-99b remarkedly inhibited the invasion and migration of A549 cells. On the other hand, miR-99b inhibitor was transfected into SPC-A1 cells. MiR-99b inhibition was detected by performing qRT-PCR (Figure 2C). Moreover, the regulation of SPC-A1 cell invasion and migration mediated by miR-99b inhibition was confirmed by transwell assays. Downregulation of miR-99b promoted the invasion and migration of SPC-A1 cells (Figure 2D). Taken together, we deduced that miR-99b could suppress cell invasion and migration of NSCLC cells.

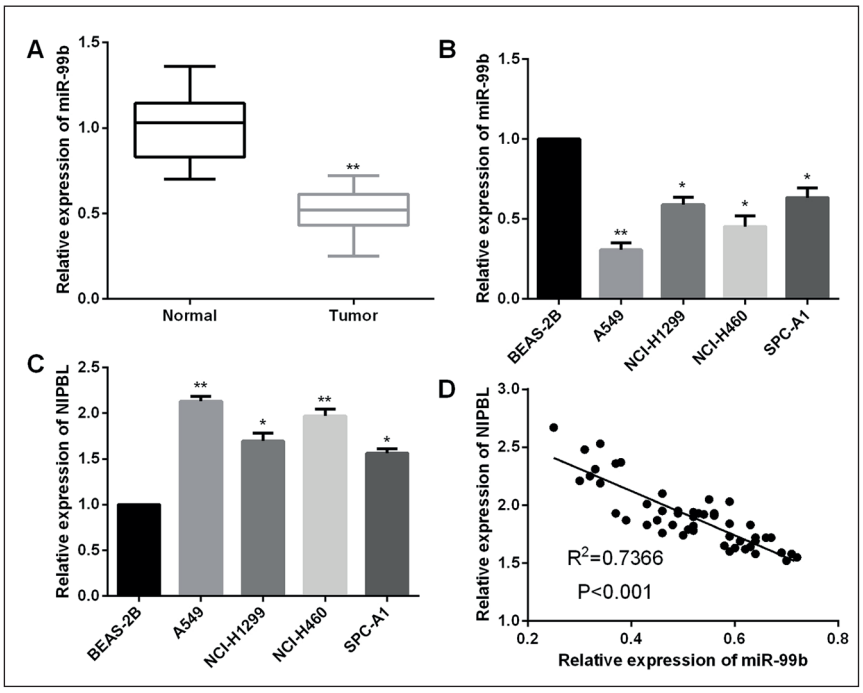

Figure 1. The expression of miR-99b and NIPBL in NSCLC. **A**, MiR-99b expressions in NSCLC tissues were measured using qRT-PCR. **B**, MiR-99b expressions in NSCLC cells (A549, NCI-H460, NCI-H1299 and SPC-A1) compared to BEAS-2B cell lines were measured using qRT-PCR. **C**, NIPBL expressions in NSCLC cells (A549, NCI-H460, NCI-H1299 and SPC-A1) compared to BEAS-2B cell lines were measured using qRT-PCR. **D**, A negative correlation between miR-99b expressions and NIPBL expressions in NSCLC tissues. *p<0.05, **p<0.01.

NIPBL Was Direct Target of MiR-99b

TargetScan (http://www.targetscan.org/vert_72/) was used for finding candidate targets of miR-99b and results showed that NIPBL may be possible target of miR-99b. Figure 3A showed the binding sites between miR-99b and NIPBL 3'UTR. Then, NSCLC cells were cotransfected with the Luciferase reporter vector containing WT or MUT NIPBL

Table II. Correlation of miR-99b expression with the clinicopathological characteristics of the NSCLC patients.

Clinicopathological features	Cases (n = 50)	miR-99b# expression		p-value
		High (n = 20)	Low (n = 30)	
Age (years)				0.2636
> 60	28	12	16	
≤ 60	22	8	14	
Gender				0.3324
Male	26	9	17	
Female	24	11	13	
Tumor size (cm)				0.1174
≥ 5.0	24	6	18	
< 5.0	26	14	12	
Lymph node metastasis				0.0031*
Yes	22	17	5	
No	28	3	25	
Histology				0.2107
Squamous cell carcinoma	24	11	13	
Adenocarcinoma	26	9	17	
TNM stage				0.0026*
I+II	23	15	8	
III+IV	27	5	22	
Smoker				0.3561
Yes	29	13	16	
No	21	7	14	

NSCLC: non-small cell lung cancer; TNM: tumor-node-metastasis. #The mean expression level of miR-99b was used as the cutoff. *Statistically significant.

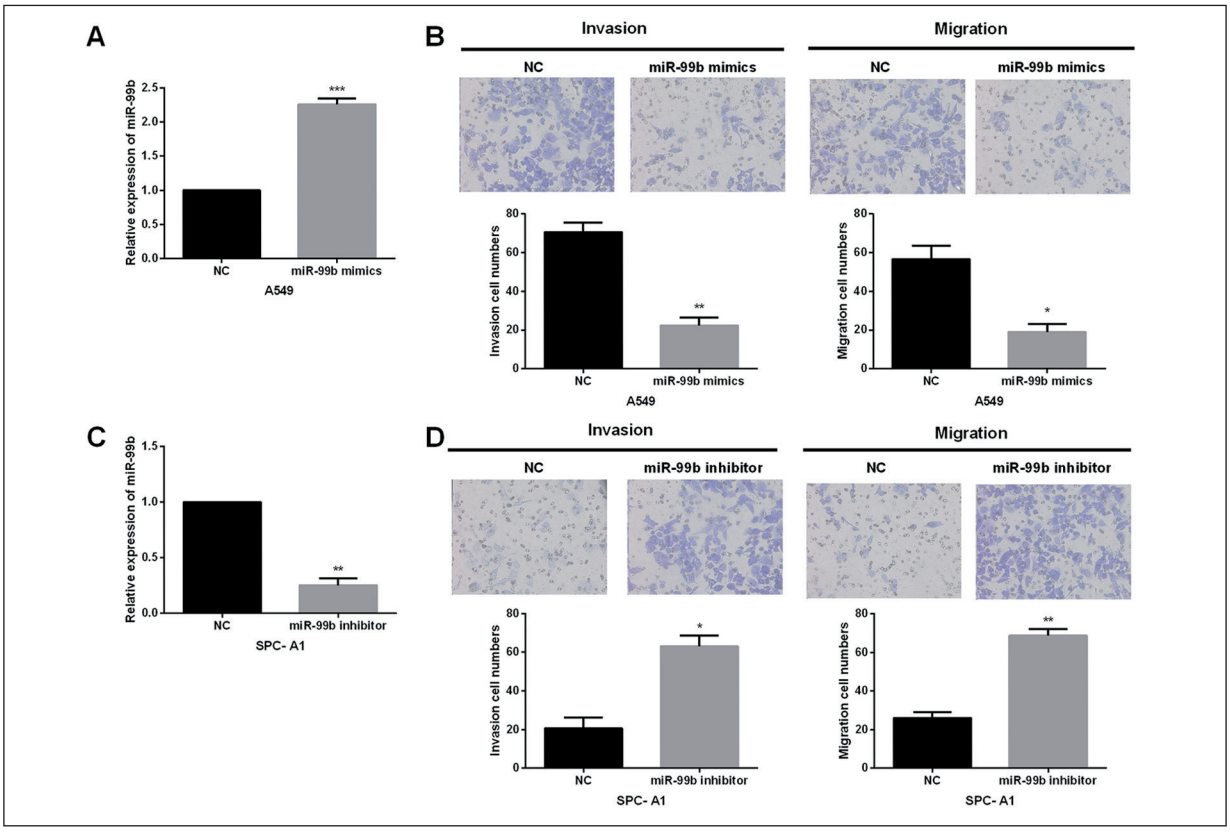

Figure 2. MiR-99b overexpression suppressed the invasion and migration of NSCLC cells. **A**, MiR-99b expressions in A549 cells with transfections of miR-99b mimics were measured using qRT-PCR. **B**, Cell invasion and migration were observed by transwell assays in A549 cells with transfections of miR-99b mimics (magnifications 100×). **C**, MiR-99b expressions in SPC-A1 cells with transfections of miR-99b inhibitor were measured using qRT-PCR. **D**, Cell invasion and migration abilities of SPC-A1 cells with transfections of miR-99b inhibitor (magnifications 100×). *p<0.05, **p<0.01, ***p<0.001.

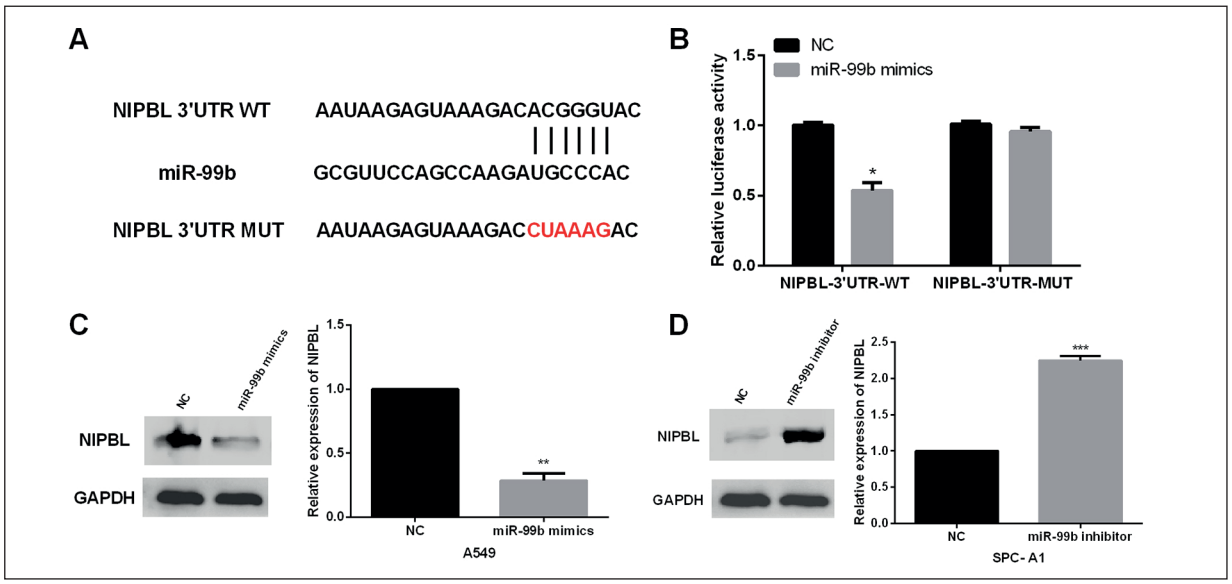

Figure 3. NIPBL was a direct target of miR-99b. **A**, Binding sites of miR-99b in NIPBL 3'-UTR. **B**, Luciferase activities of NSCLC cells cotransfected with luciferase reporters containing NIPBL-WT or NIPBL-MUT and miR-99b mimics. **C, D**, NIPBL expressions of NSCLC cells treated with miR-99b mimics or inhibitor. *p<0.05, **p<0.01, ***p<0.001.

and miR-99b mimics. In addition, miR-99b mimics significantly inhibited the Luciferase activity of NIPBL-WT, however, there are no changes in the luciferase activity of NIPBL-3'UTR-MUT (Figure 3B). Furthermore, we performed qRT-PCR and Western blot to verify whether NIPBL was the downstream target of miR-99b. Results indicated that miR-99b overexpression significantly reduced the NIPBL expressions in A549 cells (Figure 3C). On the other hand, we also found that miR-99b inhibition notably promoted the NIPBL expressions in SPC-A1 cells (Figure 3D). All above data suggested that NIPBL was a direct target of miR-99b.

NIPBL Was Involved In the Functional Effects of MiR99b on NSCLC

By having confirmed that NIPBL was directly targeted by miR-99b in NSCLC cells, next, we want to explore that NIPBL mediated the inhibitory functions of miR-99b in NSCLC cells. NIPBL overexpression vector was transfected into A549 cells, which had been transfected with miR-99b mimics. The results of qRT-PCR and Western blot indicated that NIPBL expression was significantly upregulated by NIPBL overexpression vector in miR-99b overexpressed A549 cells (Figure 4A). Subsequently, we performed transwell assays to verify that the effects of NIPBL/miR-99b on A549 cell invasion and migration. As expected, the NIPBL restoration in miR-99b overexpressed A549 cells notably reversed the suppressive effect of miR-99b on A549 cell (Figure 4B). In the meantime, we transfected NIPBL siRNA into miR-99b suppressed SPC-A1 cells and the transfection efficiencies were confirmed by performing qRT-PCR and Western blot (Figure 4C). Transwell assays demonstrated that

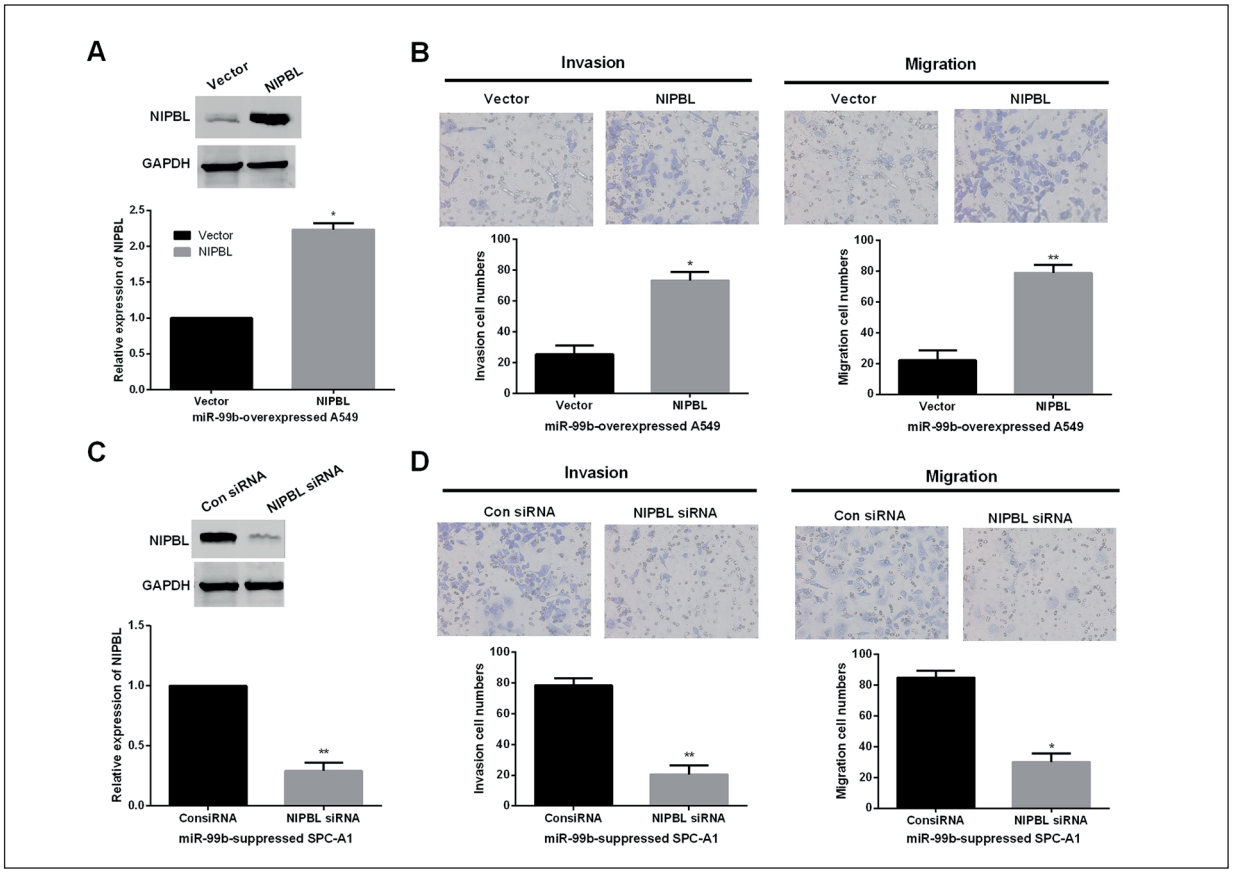

Figure 4. Alteration of NIPBL expressions affected the functions of miR-99b in NSCLC cell invasion and migration. **A**, NIPBL expressions in miR-99b overexpressed A549 cells which were cotransfected with NIPBL overexpressing vector were measured using qRT-PCR and Western blot. **B**, Invasion and migration capacities of A549 cells cotransfected with NIPBL vector and miR-99b mimics were determined by transwell assays (magnifications 100×). **C**, NIPBL expressions in miR-99b suppressed SPC-A1 cells which were cotransfected with NIPBL siRNA were measured using qRT-PCR and Western blot. **D**, Invasion and migration capacities of SPC-A1 cells cotransfected with NIPBL siRNA and miR-99b inhibitor were detected by transwell assays (magnifications 100×). *p<0.05, **p<0.01.

knockdown of NIPBL abrogated the functions of miR-99b inhibitor in SPC-A1 cell invasion and migration (Figure 4D).

Discussion

NSCLC remains one most lethal malignancy all over the world. NSCLC patients with NSCLC usually have poorer prognosis, especially for those who failed first-line systemic therapies. Therefore, there is an urgent need to identify novel biomarkers for diagnosis and prognosis of NSCLC patients. Increasing evidences have proved that miRNAs were related to tumorigenesis of NSCLC. Of note, miR-146b-5p exerted anti-tumor functions in NSCLC and functioned as a prognosis predictor[18]; Zhang et al[19] reported that miR-204 inhibited NSCLC via regulating ATF2; miR-1260b acted as oncogene in NSCLC via regulation of PTPRK. However, the mechanisms by which miR-99b modulates the invasion and migration in NSCLC are not well elucidated. In this study, we found a decreased expression of miR-99b in NSCLC and its low expression was closely associated with the prognosis of NSCLC patients. Moreover, restoration of miR-148b repressed NSCLC cell invasion and migration, while inhibiting expression of miR-148b enhanced NSCLC cell invasion and migration. More strikingly, NIPBL was determined as the direct target of miR-99b and it rescued miR-148b inhibitory effect on NSCLC metastasis.

Recently, it has been identified that miRNAs linked to the proliferation and metastasis of NSCLC. Kang et al[20] displayed that miR-612 suppressed the malignant development of NSCLC by targeting BRD4 through PI3K/Akt pathway. Feng et al[21] reported that miR-34b exhibited repression effect on NSCLC cell proliferation and promotion effect on cell apoptosis. MiR-99b has been indicated to be a common regulator of multiple developmental processes of carcinogenesis in various malignancies. According to Lukamowicz-Rajska et al[22] miR-99b-5p was down-regulated in clear cell renal cell carcinoma patients; Yang et al[23] found that miR-99b promoted hepatocellular carcinoma metastasis via inhibiting claudin 11 expressions and served as a prognostic biomarker. MiRNAs have drawn great attention in the diagnosis, prognosis and metastasis of tumors. We indicated miR-99b was significantly downregulated and inhibited cell invasion and migration in NSCLC. Consistent with our results, Du et al[24] reported that decreased miR-99b was found in NSCLC and miR-99b inhibited NSCLC cell growth by directly targeting FGFR3. Together with our findings, these data revealed that miR-99b functioned as tumor suppressor in NSCLC.

Metastasis-related deaths in tumors account for approximately 90% of total mortality[25]. Increasing evidence[26,27] has shown that miRNAs were involved in the proliferation and metastasis of tumors, including lung cancer. The mechanism of miRNAs altered gene expression by targeting their mRNA. Our bioinformatics analysis displayed that miR-99b and NIPBL have binding sites, and miR-99b could regulate the expression of NIPBL negatively. NIPBL was reported to take part in tumor progression and metastasis as the target of miRNAs. In particular, it served as the target of miR-187-3p in the regulation of infantile hemangioma[28]. Zheng et al[29] indicated the roles of NIPBL in various tumors, including NSCLC. These authors showed that increased NIPBL expressions conferred poor prognosis in NSCLC. The current study provided further evidence that NIPBL was significantly upregulated in NSCLC. In addition, we observed NIPBL directly targeted with miR-99b and it can regulate repressive functions of miR-99b in NSCLC invasion and migration. Of note, NIPBL could rescue the suppression effect of miR-99b on the metastasis of NSCLC.

Conclusions

We demonstrated that miR-99b was expressed at low levels in NSCLC tissues and cells. miR-99b overexpression repressed NSCLC cell invasion and migration. Furthermore, NIPBL was identified as direct target of miR-99b and partially regulated the suppressive functions in NSCLC cell invasion and migration mediated by miR-99b. This is the first time to show the miR-99b exerted anti-tumor effect in NSCLC cells by regulating NIPBL. MiR-99b/NIPBL axis may provide potential diagnostic and therapeutic strategy for NSCLC treatment. We revealed the important role of miR-99b in inhibiting progression of NSCLC, although further studies are required to confirm this.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of the Weifang People's Hospital. Signed written informed consents were obtained from the patients and/or guardians.

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