Abstract. MicroRNAs (miRNAs) play critical roles in the development and progression of various cancers, including non-small-cell lung cancer (NSCLC). Studies have suggested that miR-330-5p is involved in the progression of several cancers. However, the role of miR-330-5p in NSCLC remains unclear. We investigated the effect on and mechanism of miR-330-5p in the progression of NSCLC. We found that miR-330-5p was significantly downregulated in NSCLC tissues and cell lines as detected by real-time quantitative polymerase chain reaction (RT-qPCR). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bromodeoxyuridine (BrdU), colony formation and cell cycle assays showed that overexpression of miR-330-5p markedly inhibited cell growth. Annexin V-FITC/PI and caspase-3 activity assays showed that overexpression of miR-330-5p significantly promoted cell apoptosis of NSCLC cells. Bioinformatics analysis and dual-luciferase reporter assays confirmed NIN/RPN12 binding protein 1 (NOB1) as a target gene of miR-330-5p. RT-qPCR and Western blot analysis showed that overexpression of miR-330-5p inhibited the expression of NOB1 as well as cyclin D1 and cyclin-dependent kinase 4 in NSCLC cells. Moreover, overexpression of NOB1 markedly reversed the miR-330-5p-mediated inhibitory effect on NSCLC cell growth. Correlation analysis showed that miR-330-5p expression was inversely correlated with NOB1 mRNA expression in NSCLC tissues. Taken together, our results indicate that miR-330-5p inhibits NSCLC cell growth through downregulation of NOB1 expression. Our study suggests that miR-330-5p may serve as a potential therapeutic target for the treatment of NSCLC.

Introduction

Lung cancer is the leading cause of cancer-associated deaths worldwide (1). The majority of lung cancers are non-small cell lung cancer (NSCLC), which accounts for approximately 85-90% of all lung cancers (1). Although treatment strategies have been advanced over the last few decades, the overall survival rate remains extremely poor, with a 5-year overall survival of <15% (2,3). However, the molecular pathogenesis of NSCLC remains elusive. Therefore, it is crucial to gain a better understanding of the mechanisms underlying NSCLC carcinogenesis to help development of novel and promising diagnostic markers and therapeutic targets.

MicroRNAs (miRNAs) are small non-coding RNA molecules that can target the 3'-untranslated region (3'-UTR) of target mRNA to inhibit protein translation (4,5). miRNAs have emerged as critical regulators in many diseases, including cancer (6). Aberrantly expressed miRNAs have been suggested to harbor diagnostic, prognostic and therapeutic implications in cancer (7,8). miRNAs play an important role in various life activities, including development, metabolism, differentiation,
proliferation and apoptosis (9). To date, various miRNAs have been found to be dysregulated in NSCLC and participate in the tumorigenesis of NSCLC (10,11). Therefore, miRNAs may serve as promising therapeutic targets in NSCLC treatment.

In recent years, the N111/RPN12 binding protein 1 homologue (NOB1) has been recognized as an oncogene in many types of human cancers (12,13). NOB1 is located on chromosome 16q22.1 and consists of nine exons and eight introns encoding a 50 KDa protein which is a subunit of the 26 S proteasome (14). NOB1 regulates protease functions and RNA metabolism (15). NOB1 is expressed in various tissues and organs and is mainly distributed in the nucleus (14,15). However, the dysregulation of NOB1 is associated with tumorigenesis in various cancers, including hepatocellular carcinoma (16), glioma (17) and prostate cancer (18). High expression of NOB1 is associated with cancer cell proliferation, migration and invasion (18,19). Studies have shown that high expression of NOB1 in NSCLC is related with tumor node metastasis stage, histopathological grade and lymph node metastasis (20,21). Silencing of NOB1 inhibits NSCLC cell proliferation in vitro and in vivo (6,22). Therefore, NOB1 may serve as a potential therapeutic target for NSCLC.

Previous studies have documented the critical role of miR-330-5p in regulating cell proliferation, migration and invasion of many cancer types (23,24). However, the potential role of miR-330-5p in NSCLC has still not been documented. In the present study, we investigated the potential role of miR-330-5p in NSCLC. Our results suggest that miR-330-5p inhibits NSCLC cell growth by downregulating NOB1 expression. Our study suggests that miR-330-5p is a tumor suppressor in NSCLC and has the potential to be a therapeutic target for NSCLC treatment.

Materials and methods

**Patient specimens.** NSCLC tumor samples and paired adjacent normal lung tissue samples were obtained from twelve NSCLC patients undergoing primary surgical resection at the Second Affiliated Hospital of Medical School, Xi'an Jiaotong University. The resected tissues were immediately frozen in liquid nitrogen at -80°C. The patients that participated in this study provided signed informed consent. This study was approved by Institutional Human Experiment and Ethics Committee of the Second Affiliated Hospital of Medical School, Xi'an Jiaotong University and was conducted in accordance with the Helsinki Declaration.

**Cell lines.** Human NSCLC cell lines including A549, H1299, H1975 and NCI-H1264, and the normal human lung cell line BEAS-2B were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum plus with 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA). Cells were routinely grown in a humidified atmosphere containing 5% CO2 at 37°C.

**Cell transfection.** The miR-330-5p mimics and negative control (NC) were purchased from GenePharma (Shanghai, China) and transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cDNA of NOB1 without 3'-UTR was inserted into pcDNA3 plasmid that was transfected into cells using Lipofectamine 2000 (Invitrogen).

**Real-time quantitative polymerase chain reaction (RT-qPCR).** Total RNA from tissues or cells was extracted using TRIzol reagent (Invitrogen) as per the manufacturer's instructions. First-strand cDNA was synthesized using the MMLV Reverse Transcriptase (for mRNA detection) or miRcute miRNA cDNA kit (Tiangen, Beijing, China). RT-qPCR was performed with the SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA) and specific primers in the ABI7500 Real-time PCR system (Applied Biosystems). GAPDH and U6 served as internal controls. Data were calculated using the 2-∆∆Ct method. Fold changes in gene expression were obtained by normalization to the control group.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** Cells were plated into 96-well plates at a density of 1x10^5 cells/well and cultured overnight. After transfection with miR-330-5p mimics for 72 h, cells were treated with 20 µl of MTT (5 mg/ml; Sigma) for 4 h. The formazan crystals were solubilized in 200 µl of dimethyl sulfoxide (Sigma). The absorbance at 490 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

**Bromodeoxyuridine (BrdU) assay.** The BrdU assay was performed using a BrdU cell proliferation assay kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Briefly, cells were plated into 96-well plates at a density of 1x10^5 cells/well and transfected with miR-330-5p mimics for 48 h. Then, cells were treated with 10 µl of BrdU solution for 2 h followed by incubation with 150 µl of denaturing solution. Afterwards, peroxidase conjugated anti-BrdU (1:1,000) was added and incubated for 90 min at room temperature. The end of the incubation period, the solution was discarded and cells were washed with 200 µl of washing buffer. Finally, 100 µl of substrate solution was added and incubated for 15 min. The absorbance at 450 nm was measured with a microplate reader (Bio-Rad).

**Annexin V/PI apoptosis assay.** Apoptosis was detected using an Annexin V-FITC/PI kit (Beyotime Biotechnology, Haimen,)
cells were transfected with miR-330-5p mimics for 48 h and then harvested. Cells were digested by trypsin and washed with PBS. Cells were re-suspended in 500 µl of binding buffer containing 1.25 µl of Annexin V-FITC and 10 µl of PI for 20 min in the dark. Cells were then assessed by a BD flow cytometer (BD Biosciences).

Caspase-3 activity assay. Caspase-3 activity was detected using a caspase-3 activity assay (Beyotime Biotechnology). Briefly, after cells were transfected with miR-330-5p mimics for 48 h, cells were harvested, lysed and incubated with Ac-DEVD-pNA for 2 h at 37˚C. The absorbance at 405 nm was determined using a microplate reader (Bio-Rad).

Dual-luciferase reporter assay. The 3'-UTR of NOB1 containing the wild-type or mutant binding sites of miR-330-5p were inserted into the pmirGLO vector (Promega, Madison, WI, USA) following by transfection into A549 and H1299 cells along with miR-330-5p mimics using Lipofectamine 2000 (Invitrogen). After 48 h, the relative luciferase activity was detected by a dual-luciferase assay kit (Promega).

Western blot analysis. Total protein was extracted using RIPA buffer and the protein concentration was measured using a BCA kit (Beyotime Biotechnology). An equal amount of protein (50 µg) was loaded onto a sodium dodecyl sulfate polyacrylamide gel for separation. The separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Boston, MA, China) according to the manufacturer's instructions.
USA). The membrane was then blocked using 5% non-fat milk for 1 h at 37°C followed by incubation with primary antibodies (anti-NOB1 and anti-GAPDH; Abcam, Cambridge, UK) with appropriate concentrations at 4°C overnight. Then, the membrane was probed with horseradish peroxidase conjugated secondary antibodies (1:1,000, Beyotime Biotechnology) for 1 h at 37°C. The immunoreactive proteins were developed by using enhanced chemiluminescence system (Millipore). The intensity of the protein blots on the membrane was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Data analysis. All results were depicted as means ± standard deviation and the statistical analyses were conducted by SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA). Differences were analyzed by Student's t-test or one-way analysis of variance with a Bonferroni correction. Correlation analysis were performed by Spearman's correlation analysis. Differences were regarded as statistically significant at P<0.05.

Results

miR-330-5p is downregulated in NSCLC tissues and cell lines. To investigate the potential role of miR-330-5p on the progression of NSCLC, we first examined the expression of miR-330-5p expression in NSCLC tissues by RT-qPCR. The results showed that miR-330-5p was significantly decreased in NSCLC tissues compared with adjacent normal lung tissues (Fig. 1A). Moreover, we detected miR-330-5p expression in NSCLC cell lines including A549, H1299, H1975 and NCI-H1264. The results showed that miR-330-5p was markedly decreased in NSCLC cell lines compared with the normal lung cell line BEAS-2B (Fig. 1B). Taken together, these results suggest that miR-330-5p may be involved in the development and progression of NSCLC.

Overexpression of miR-330-5p inhibits cell growth of NSCLC cells. To investigate the exact biological function of miR-330-5p in NSCLC, we overexpressed miR-330-5p in A549 and H1299 cells by transfection with miR-330-5p mimics. We then detected the effect of miR-330-5p overexpression on cell growth of NSCLC cells by MTT, BrdU, colony formation and cell cycle assays. The MTT assay showed that overexpression of miR-330-5p significantly reduced the viability of A549 and H1299 cells (Fig. 2A). The BrdU assay showed that miR-330-5p overexpression markedly inhibited the proliferation of A549 and H1299 cells (Fig. 2B). Moreover, the colony-forming capacity of A549 and H1299 cells was also significantly decreased by miR-330-5p overexpression (Fig. 2C). In addition, overexpression of miR-330-5p significantly induced cell cycle arrest in G0/G1 phase (Fig. 2D). Overall, these results suggest that miR-330-5p suppresses NSCLC cell growth.

Overexpression of miR-330-5p promotes apoptosis of NSCLC cells. To further investigate the biological function of miR-330-5p in NSCLC, we assessed the effect of miR-330-5p overexpression on NSCLC cell apoptosis by Annexin V/PI apoptosis assay and caspase-3 activity assay. The Annexin V/PI apoptosis assay showed that overexpression of miR-330-5p significantly induced apoptosis in NSCLC cells (Fig. 3A). Furthermore, caspase-3 activity was markedly increased by...
miR-330-5p overexpression (Fig. 3B). These results suggest that miR-330-5p promotes apoptosis in NSCLC cells.

**NOB1 is a target gene of miR-330-5p in NSCLC cells.** To investigate the underlying mechanism of miR-330-5p in regulating NSCLC cell growth, we performed bioinformatics analysis to predict the potential target genes of miR-330-5p. Interestingly, we found that NOB1 was a potential target gene of miR-330-5p (Fig. 4A). To investigate whether miR-330-5p directly binds to NOB1 3'-UTR, we performed dual luciferase reporter assays. The results showed that luciferase activity in the luciferase reporter vector containing the NOB1 3'-UTR was significantly reduced by overexpression of miR-330-5p (Fig. 4B and C). However, this inhibitory effect of miR-330-5p overexpression on luciferase activity was abolished by mutating binding sites in the NOB1 3'-UTR (Fig. 4B and C).

Furthermore, we detected a direct regulatory effect of miR-330-5p on NOB1 expression by RT-qPCR and western blot analysis. The results showed that the mRNA (Fig. 5A and B) and protein (Fig. 5C and D) expression levels of NOB1 were significantly decreased with miR-330-5p overexpression in A549 and H1299 cells. Taken together, these results indicate that NOB1 is a target gene of miR-330-5p in NSCLC cells.

**Overexpression of miR-330-5p inhibits the expression of cyclin D1 and cyclin-dependent kinase 4 (CDK4).** Previous studies have reported that NOB1 regulates NSCLC cell growth associated with regulating cyclin D1 and CDK4 (22). Considering the effect of miR-330-5p on NOB1 expression, we speculated that miR-330-5p may regulate the expression of cyclin D1 and CDK4. To test this hypothesis, we measured the effect of miR-330-5p overexpression on mRNA expression of cyclin D1 and CDK4 by RT-qPCR. The results showed that the expression of cyclin D1 (Fig. 6A) and CDK4 (Fig. 6B) was significantly reduced by miR-330-5p overexpression, implying that miR-330-5p regulates the expression of cyclin D1 and CDK4.

**miR-330-5p inhibits NSCLC cell growth by targeting NOB1.** To further investigate whether miR-330-5p inhibits NSCLC cell growth by targeting NOB1, the pcDNA3-NOB1 vector and miR-330-5p mimics were cotransfected into A549 and H1299 cells. The results showed that NOB1 expression was significantly increased in cells cotransfected with the pcDNA3-NOB1 vector and miR-330-5p mimics compared with cells transfected with miR-330-5p mimics only (Fig. 7A). Moreover, the inhibitory effect of miR-330-5p on NSCLC cell growth was significantly reversed by NOB1 overexpression. (Fig. 7B and C). NOB1 overexpression also markedly abrogated miR-330-5p-induced NSCLC cell apoptosis (Fig. 7D). Collectively, these results suggest miR-330-5p suppresses cell growth and induces apoptosis by inhibiting NOB1 in NSCLC cells.

**miR-330-5p expression is inversely correlated with NOB1 mRNA expression in NSCLC tissues.** To further confirm the relevance of miR-330-5p/NOB1 in the progression of NSCLC,
we analyzed the correlation between miR-330-5p expression and NOB1 mRNA expression in NSCLC tissues by RT-qPCR. The results showed that NOB1 mRNA was highly expressed in NSCLC tissues (Fig. 8A). Correlation analysis showed that miR-330-5p expression was inversely correlated with NOB1 mRNA expression in NSCLC tissues (Fig. 8B). The data indicate that decreased miR-330-5p expression may contribute to the increased expression of NOB1 in NSCLC.

Discussion
Increasing evidence suggests that miRNAs play important roles in the development and progression of NSCLC by regulating oncogenes or tumor suppressive genes (25-27). However, the precise roles of miRNAs in NSCLC remains largely unknown. In this study, we demonstrated that miR-330-5p was a novel NSCLC-associated miRNA. We
found that miR-330-5p was decreased in NSCLC and overexpression of miR-330-5p inhibited NSCLC cell growth. Our study indicates that miR-330-5p may play an important role in the development of NSCLC.

In recent years, miR-330-5p has been reported as an important regulator in many pathological processes. Dysregulated miR-330-5p is found in peripheral whole blood of bipolar disorder patients (28). miR-330-5p is involved in regulating plasticity, learning and memory (29). miR-330-5p is highly upregulated in bone marrow-derived human mesenchymal stem cells during cellular senescence (30). miR-330-5p regulates depigmentation through targeting tyrosinase (31). Studies have shown that miR-330-5p inhibits proliferation keratinocytes by targeting protein disulfide-isomerase A3 (32) and signal recognition particle receptor α (33). miR-330-5p inhibits cell proliferation and invasion of cutaneous malignant melanoma cells through the downregulation of tyrosinase and protein disulfide-isomerase A3 (34). miR-330-5p represses pancreatic cancer cell proliferation, migration and invasion as well as sensitizes pancreatic cancer cells to gemcitabine through targeting mucin 1 (24). miR-330-5p strongly inhibits the expression of T cell immunoglobulin and mucin domain-3 in acute myeloma leukemia cells (35). Downregulated miR-330-5p expression has been found in colorectal cancer; miR-330-5p overexpression represses colorectal cancer development by targeting integrin α5 (36). Moreover, miR-330-5p has been reported as an important modulator of neoadjuvant chemoradiotherapy sensitivity in esophageal adenocarcinoma (37). These studies suggest a tumor suppressive role of miR-330-5p. In this study, we demonstrated that miR-330-5p was decreased in NSCLC and overexpression of miR-330-5p inhibited NSCLC cell growth, further supporting a tumor suppressive role of miR-330-5p.

NOB1 has been found to be highly expressed in many types of cancers, including ovarian cancer (13), papillary thyroid carcinoma (17), glioma (12), prostate cancer (38), colon cancer (39) and oral squamous cell carcinoma (40). Silencing of NOB1 by short hairpin RNA (shRNA) or small interfering RNA (siRNA) exhibits considerable antitumor effects (12,13,39-42). Studies have shown that high expression of NOB1 in NSCLC is related to histopathological grade, tumor node metastasis stage and lymph node metastasis (20,21). High expression of NOB1 is associated with a poor early response to cisplatin-based chemotherapy in advanced NSCLC patients (43). Simultaneously determining the expression of NOB1 and right open reading frame kinase 2 can improve the diagnostic rate in early stages of NSCLC (44). Knockdown of NOB1 by shRNA has been shown to inhibit cell proliferation and tumor growth in NSCLC, both in vivo and in vitro (6). Lentivirus-mediated gene silencing of NOB1 inhibits cell growth and induced cell cycle arrest associated with inhibition of cyclin D1 and CDK4 (22). These findings indicate that NOB1 may serve as a promising target for NSCLC treatment. In this study, we identified NOB1 as a target gene of miR-330-5p. We showed that inhibition of NOB1 by miR-330-5p overexpression repressed NSCLC cell growth and the expression of cyclin D1 and CDK4. We also showed that NOB1 mRNA expression was conversely correlated with miR-330-5p expression in NSCLC tissues. Decreased expression of miR-330-5p may contribute to increased expression of NOB1 in NSCLC and may be associated with NSCLC tumorigenesis.

In addition to shRNA and siRNA, miRNAs can be used for modulating NOB1 expression. Several studies have reported that miR-326 functions as a tumor suppressor by targeting NOB1 and inhibiting NOB1 expression in glioma (45), colorectal cancer (46) and gastric cancer (47). Downregulated miR-646 correlates with tumor metastasis of clear cell renal carcinoma by promoting NOB1 expression (48). miR-192 inhibits the tumorigenicity of prostate cancer cells by targeting NOB1 (49). Moreover, Huang et al have reported that miR-139-3p targets NOB1 to induce apoptosis and inhibit metastasis in cervical cancer (50). Our study suggests that miR-330-5p is a novel regulator of NOB1. miR-330-5p may serve as a potential target for the development of mirNA-based therapy for NSCLC treatment by targeting NOB1.

In summary, our results show that miR-139-5p inhibits NSCLC cell growth by targeting NOB1. Decreased miR-330-5p expression was correlated with high expression of NOB1 in NSCLC, implying that miR-330-5p/NOB1 plays an important role in the development and progression of NSCLC. Our study suggests that miR-330-5p is a candidate tumor suppressor in NSCLC. Inhibiting NOB1 via miR-330-5p...
may serve as a promising therapeutic strategy for the treatment of NSCLC.

References


