Research Article

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MiR-223-3p regulates cell viability, migration, invasion, and apoptosis of non-small cell lung cancer cells by targeting RHOB

Abstract: Non-small cell lung cancer (NSCLC) is the most common type of lung cancer with a high fatality rate in men and women worldwide. Recently, microRNAs (miRNAs) have been reported to be diagnostic biomarkers and therapeutic targets in NSCLC. MiR-223-3p was proved to act as a promoter in NSCLC progression. However, the regulatory mechanism of miR-223-3p in NSCLC remains little known. This study aimed to explore the regulatory mechanism between miR-223-3p and its target gene Ras homolog family member B (RHOB) in NSCLC. The mRNA level of miR-223-3p and RHOB was measured by quantitative reverse transcription PCR. Furthermore, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Flow cytometry was conducted to analyze cell apoptosis. Transwell assays and wound healing assay were employed to examine migration and invasion. The target relationship between miR-223-3p and RHOB was predicted by starBase online database and verified by dual-luciferase assay. The protein level of RHOB was tested by western blot. Our data suggested that miR-223-3p was upregulated in NSCLC tissues and cell lines and high level of miR-223-3p contributed to a poor survival in NSCLC patients. Knockdown of miR-223-3p exerted inhibitory effects on NSCLC cell viability, migration, and invasion and promotion effect on cell apoptosis. Furthermore, RHOB was directly targeted by miR-223-3p and constrained NSCLC progression. Moreover, knockdown of RHOB rescued the effect of anti-miR-223-3p on NSCLC progression. In vivo experiments indicated that miR-223-3p deletion suppressed tumor growth. MiR-223-3p could regulate the NSCLC cellular processes through targeting RHOB.

Keywords: NSCLC, miR-223-3p, RHOB, progression

1 Introduction

Lung cancer is one of the most common cancers with a high fatality rate worldwide [1,2]. There were 2.1 million new lung cancer patients in 2018 [3]. Approximately 80–85% of lung cancers are non-small cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma [4]. More importantly, though surgical resection and stereotactic ablative radiotherapy were the main treatments to cure the NSCLC in recent decades, the 5-year survival rate is less than 15% [5,6]. Therefore, it is important to explore the molecular regulatory mechanisms of NSCLC for further therapeutic progression.

MicroRNAs (miRNAs) are a class of small noncoding RNAs with a length of about 22 nt and negatively regulate the gene expression by binding to the 3′-untranslated region (3′-UTR) of target miRNAs [7]. Recently, many studies have indicated that miRNAs played important roles in various human cancers [8]. MiR-223-3p, located on Xq12, was reported to be involved in the regulation of several cancers. For instance, miR-223-3p expression was associated with a high risk for progression in adenocarcinoma patients [9]. A previous study indicated that miR-223-3p could inhibit cancer cell aggressiveness by targeting genes involving in bladder cancer pathogenesis [10]. Moreover, miR-223-3p was found to be significantly upregulated and might be a diagnostic biomarker and therapeutic target in...
NSCLC [11–13]. However, although miR-223-3p inhibition served as a suppressor in NSCLC progression [14], the underlying mechanism was still largely unknown.

miRNAs exerted effects through regulating Ras homolog family member B (RHOB) which was demonstrated to be an emerging class of “non-oncogene addiction” factor that is critical for maintenance of malignant phenotypes in human cancers [15]. RHOB is a major membrane androgen receptor effector regulating actin cytoskeleton and apoptosis in various tumor cells [16]. Although RHOB expression was inversely correlated with disease progression in several epithelial cancers, accumulating evidence suggested that RHOB might promote malignant phenotypes in certain cancer types, such as glioblastoma and lung adenocarcinoma [15,17]. Moreover, RHOB was proved to be involved in NSCLC progression by regulating cell proliferation and invasion [18]. starBase predicted that RHOB contained the potential binding sites of miR-223-3p. A previous study demonstrated that miR-223 decreases hypoxia-triggered vascular remodeling in pulmonary arterial smooth muscle cells by regulating RHOB [19]. However, the regulatory network of miR-223-3p/RHOB axis in NSCLC remains undefined.

In this study, we aimed to explore the biological functions and underlying mechanism of miR-223-3p in the pathogenesis and development of NSCLC.

2 Materials and methods

2.1 Samples and cell culture

A total of 46 pairs of NSCLC tissues and corresponding adjacent normal tissue samples were collected from 46 NSCLC patients in the Lanzhou University Second Hospital. The patients participated in this study did not receive any treatment before surgery. NSCLC tissues and normal tissues were resected and kept in liquid nitrogen until RNA extraction. The clinicopathologic features of these patients are presented in Table 1.

NSCLC cell lines (A549, PC-9, SK-MES-1, and H1299) and lung bronchus epithelial cells (16HBE) used in this study were acquired from Bnbio (Beijing, China). 16HBE, PC-9, SK-MES-1, and H1299 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA). A549 cells were cultured in F-12K medium (Gibco). The medium used to culture the cells was supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

| Characteristics | Patients (N = 46) | Tissue miR-378 | p  |
|-----------------|------------------|---------------|----|
|                 |                  | Low | High |    |
| Sex             | Male             | 31  | 10   | 21 | 0.172 |
|                 | Female           | 15  | 6    | 9  |       |
| Age             | <60              | 20  | 7    | 13 | 0.611 |
|                 | ≥60              | 26  | 11   | 15 |       |
| Histology       | Adenocarcinoma   | 26  | 9    | 17 | 0.231 |
|                 | Squamous cell carcinoma | 17  | 7    | 10 |   |
|                 | Others           | 3   | 2    | 1  |   |
| Lymph node metastasis | Negative   | 28  | 15   | 13 | 0.034 |
|                 | Positive         | 18  | 8    | 10 |   |
| Tumor size      | <5 cm            | 32  | 14   | 18 | 0.301 |
|                 | ≥5 cm            | 14  | 6    | 8  |   |

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance with the tenets of the Helsinki Declaration, and has been approved by the Clinical Ethics Committee of Lanzhou University Second Hospital.

2.2 Transfection

The miR-223-3p inhibitor (anti-miR-223-3p) and its negative control miR-NC inhibitor (anti-NC), miR-223-3p mimic (miR-223-3p) or the negative control (NC), RHOB overexpression vector pcDNA-RHOB (RHOB) and
pcDNA empty plasmid (pcDNA), small interfering RNA (siRNA) against RHOB (si-RHOB), and its negative control non-specific control siRNA (si-NC) were purchased from GeneCopoeia (Guangzhou, China). These oligos or plasmids were transfected into SK-MES-1 or H1299 cells using Lipofectamine 3000 (Invitrogen) and harvested after 48 h for further experiments. Cells at 50% confluence were transfected and plated onto six-well plates (GeneCopoeia Inc., Rockville, MD, USA) in accordance with the manufacturer’s recommendations.

2.3 RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

RNA was extracted from NSCLC tissues or cell lines using TRizol reagent (Invitrogen). Subsequently, RNA was reversely transcribed by a Reverse Transcription Kit (Toyobo, Osaka, OSA, Japan). Next, qPCR was performed on an ABI 7500 (Applied Biosystems, Foster, CA, USA). MiR-223-3p expression was normalized to U6, while β-actin was used as an internal control for RHOB expression. All the procedures were conducted according to the manufacturer’s instructions. This assay was independently repeated three times.

2.4 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

SK-MES-1 and H1299 cells were seeded into 96-well plates (Corning Costar, Corning, NY, USA) at a density of $2 \times 10^3$ cells per well and incubated for 24 h prior to transfection. Then, 20 μL of MTT (Sigma, St. Louis, MO, USA) solution (5 mg/mL) was added to each well at indicated times and cultured for another 4 h at 37°C. After discarding supernatant, intracellular formazan crystals were dissolved by 200 μL of dimethyl sulfoxide (DMSO; Sigma) in each well. Cell viability was determined by measuring the absorbance at 490 nm on a Multiskan Ascent 354 microplate reader (Thermo Labsystems, Waltham, MA, USA). This assay was independently repeated in triplicate.

2.5 Cell apoptosis assay

Transfected SK-MES-1 and H1299 cells were treated with two fluorescent dyes in accordance with the manufacturer’s recommendations: fluorescein isothiocyanate–annexin V and propidium iodide. Finally, the cells were analyzed by using a flow cytometer (FACScan; BD Biosciences, Franklin Lakes, NY, USA). This experiment was independently repeated in triplicate.

2.6 Transwell assay

Cell migration and invasion of SK-MES-1 and H1299 cells were detected by transwell assay. For invasion assay, 1 $\times 10^5$ cells re-suspended in 100 μL of DMEM containing 0.1% FBS were placed into the upper chamber, which was pre-coated with Matrigel (BD Biosciences). DMEM (500 μL) containing 15% FBS was added into the lower chamber. After a 12-h incubation, cells on the upper membrane were gently removed using cotton swabs. The invaded cells were stained with 0.1% crystal violet, photographed, and counted under an optical microscope (Olympus, Tokyo, Japan). For migration assay, the procedures were similar, except that the upper chamber was not coated with Matrigel. This experiment was independently repeated three times.

2.7 Wound healing assay

For wound healing assay, cells were seeded into six-well plates. A 200 μL pipette tip was used to generate an artificial wound at 12 h post-culture. Then, the cells were cultured for 48 h and the wound closure was observed and photographed. The migrated distance was deemed to represent the migration rate. This assay was carried out in triplicate.

2.8 Western blot assay

Total protein was isolated from SK-MES-1 and H1299 cells, as well as from tumor tissues, by using lysis buffer containing two protease inhibitors (phenylmethylsulfonyl fluoride and ribonuclease inhibitor; Abcam, CA, USA). After quantification by the Bradford method, 40 μg protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to 0.22 μm nitrocellulose membranes (Sigma). Then, the membranes were first blocked by nonfat dried milk for 2 h. Next, the membranes were incubated with primary antibody against RHOB or β-actin. Subsequently, the membranes were incubated with secondary antibodies (HRP conjugated) at room temperature for 2 h. Protein bands were visualized using
the ECL procedure (Amersham Biosciences, USA) and analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA). All antibodies were purchased from Abcam (Cambridge, MA, USA). In addition, β-actin antibody was used as a control. This experiment was performed in triplicate.

2.9 Dual-luciferase reporter assay

The 3’-UTR of RHOB containing putative binding site between miR-223-3p and RHOB or its mutation was amplified and inserted into the luciferase gene in pGL3 vectors (Promega Corporation, Fitchburg, WI, USA), named as RHOB-WT and RHOB-MUT. Before transfection, H1299 cells were seeded into 24-well plates for 24 h. Then, H1299 cells were co-transfected with 50 ng RHOB-WT or RHOB-MUT and 50 nM miR-NC, miR-223-3p, anti-NC, or anti-miR-223-3p using Lipofectamine 3000 (Invitrogen). After 48 h, the luciferase activity was examined using the Dual-Luciferase Reporter Assay Kit (Promega). This experiment was conducted in triplicate.

2.10 Xenograft tumor model

H1299 cells (2 × 10⁶ in 200 μL) transfected with anti-miR-223-3p or anti-NC were subcutaneously injected into the mice. The tumors were monitored and measured every 7 days after injection. After 35 days, all mice were sacrificed and tumors were removed and weighed. Tumor volume was calculated by the following formula: tumor volume = length × width²/2. Then, the tumor tissues were frozen in liquid nitrogen immediately after collection and stored at -80°C until use.

Ethical approval: The research related to animals’ use has been complied with all the relevant national regulations and institutional policies for the care and use of animals, and has been approved by the Institutional Animal Ethics Committee of Lanzhou University Second Hospital.

2.11 Statistical analysis

GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the data. Moreover, the data were presented as mean ± standard deviation, and each experiment was conducted in triplicate. Differences between two groups were examined by Student’s t-test, and differences among three or more groups were analyzed via one-way analysis of variance. Spearman’s rank test was employed to verify the correlation between expression of RHOB and miR-592 in human NSCLC specimens (n = 46). In addition, Kaplan–Meier curves with log-rank tests were used to calculate the 5-year survival rate for overall survival (OS). P < 0.05 indicated the statistically significant difference.

3 Results

3.1 MiR-223-3p was highly expressed in NSCLC tissues and cell lines and associated with poor survival in NSCLC patients

To illustrate the function of miR-223-3p, the relative expression of miR-223-3p in NSCLC tissues and adjacent normal tissues, as well as in four NSCLC cell lines and normal cell lines, was measured by qRT-PCR. The result showed that miR-223-3p expression in NSCLC tissues (n = 46) and four cell lines was significantly higher than that in normal tissues (n = 46) and 16 HBE cell line (Figure 1a and c). Hence, SK-MES-1 and H1299 cells with more obvious difference were chosen for the next experiments. For further evaluating the miR-223-3p function in NSCLC, the short OS rate was analyzed and calculated by the Kaplan–Meier curves. The result proved that the patients with low expression of miR-223-3p (n = 30) had higher OS compared with the ones with high expression of miR-223-3p (n = 16) (Figure 1b). The above data showed that miR-223-3p was upregulated in NSCLC tissues and cells and associated with a poor prognosis in NSCLC patients.

3.2 Knockdown of miR-223-3p repressed cell viability, migration, and invasion, but promoted cell apoptosis in NSCLC cell lines

To further elucidate the role of miR-223-3p in NSCLC, SK-MES-1 and H1299 cells were transfected with anti-NC or anti-miR-223-3p for 48 h. As shown in Figure 2a, miR-223-3p expression was dramatically decreased in SK-MES-1 and H1299 cells transfected with anti-miR-223-3p compared with anti-NC groups. Moreover, cell viability in SK-MES-1 and H1299 cells of anti-miR-223-3p groups was significantly declined compared to that of anti-NC groups from 48 h (Figure 2b). The cell apoptosis rate of SK-MES-1 and H1299
was tested by qRT-PCR that miR-223-3p directly targeted RHOB, the online starBase bioinformatics tool was used to predict the interaction region. There was a conserved interaction binding site for miR-223-3p in the 3'-UTR of RHOB (Figure 3a). The target relationship was further illustrated by dual-luciferase assay. The luciferase activity of RHOB-WT was prominently repressed by miR-223-3p mimics, but elevated by anti-miR-223-3p. No apparent change was observed in the luciferase activity of RHOB-MUT (Figure 3b). Moreover, the protein level of RHOB was elevated by miR-223-3p downregulation in H1299 and SK-MES-1 cells (Figure 3c). These results concluded that RHOB was directly targeted by miR-223-3p.

3.3 MiR-223-3p directly targeted RHOB

In order to explore the relationship between miR-223-3p and RHOB, the online starBase bioinformatics tool was used to predict the interaction region. There was a conserved interaction binding site for miR-223-3p in the 3'-UTR of RHOB (Figure 3a). The target relationship was further confirmed by dual-luciferase assay. The luciferase activity of RHOB-WT was prominently repressed by miR-223-3p mimics, but elevated by anti-miR-223-3p. No apparent change was observed in the luciferase activity of RHOB-MUT (Figure 3b). Moreover, the protein level of RHOB was elevated by miR-223-3p downregulation in H1299 and SK-MES-1 cells (Figure 3c). These results concluded that RHOB was directly targeted by miR-223-3p.

3.4 Overexpression of RHOB inhibited NSCLC cell progression

To further elucidate the role of RHOB in NSCLC, the expression and the functional role of RHOB were explored. RHOB expression was aberrantly higher in NSCLC tissues than that in normal tissues (Figure 4a). RHOB expression was negatively correlated with miR-223-3p expression ($r^2 = -0.888, P < 0.0001$) (Figure 4b). Furthermore, RHOB expression was significantly increased in H1299 and SK-MES-1 cells transfected with RHOB compared with the pcDNA groups (Figure 4c). Moreover, upregulated RHOB strikingly inhibited the cell viability in both SK-MES-1 and H1299 cells (Figure 4d). Cell apoptosis of the two lines was greatly enhanced by RHOB overexpression (Figure 4e). Cell migration and invasion were notably decreased in RHOB groups compared to pcDNA groups (Figure 4f–h). These findings illustrated that RHOB overexpression constrained NSCLC progression.

3.5 Knockdown of RHOB partially reversed the suppressive effect of miR-223-3p deletion on NSCLC progression

In order to determine whether miR-223-3p regulated NSCLC progression through targeting RHOB, si-RHOB and anti-miR-223-3p were co-transfected into SK-MES-1 and H1299 cells to examine RHOB expression, cell viability, apoptosis, migration, and invasion in NSCLC cells. Western blot assay indicated that silenced miR-223-3p remarkably promoted RHOB protein expressed in SK-MES-1 and H1299, while the promoted impact was reversed by knockdown of RHOB (Figure 5a). Furthermore, the cell viability was repressed by knockdown of miR-223-3p, but co-transfection with si-RHOB weakened the repressive effect (Figure 5b). As exhibited in Figure 5c, silencing of RHOB effectively abolished the enhancement of cell apoptosis induced by anti-miR-223-3p in SK-MES-1 and H1299 cells. Moreover, the anti-miR-223-3p-mediated repressive effects on cell migration and invasion were distinctly restored by si-RHOB (Figure 5d–f). In conclusion, si-RHOB...
Figure 2: Anti-miR-223-3p had a suppressive influence on NSCLC progression. SK-MES-1 and H1299 cells were transfected with control, anti-NC, or anti-miR-223-3p. (a) The miR-223-3p expression of transfected cells was measured by qRT-PCR ($n = 3$). (b) The cell viability of the two cell lines was examined by MTT assay ($n = 3$). (c and d) Flow cytometry was used to test cell apoptosis ($n = 3$). (e–g) Transwell assays and wound healing assay were applied to examine cell migration and invasion ($n = 3$). *$P < 0.05$. 
rescued the repressive effects of anti-miR-223-3p on NSCLC progression.

3.6 Knockdown of miR-223-3p restrained the NSCLC xenograft tumor model growth in vivo

For the sake of investigating the effect of miR-223-3p on NSCLC progression in vivo, the xenograft tumor model was utilized. For the tumor volume and weight, the tumors of mice injected with cells transfected with anti-miR-223-3p were distinctly decreased in contrast to anti-NC groups (Figure 6a and b). Moreover, miR-223-3p expression was remarkably downregulated, while RHOB protein expression was markedly upregulated in tumors of mice injected with silenced miR-223-3p cells (Figure 6c and d). These results indicated that knockdown of miR-223-3p repressed tumor growth in vivo.
4 Discussion

MiRNAs were proved to participate in the progression of various cancers, including NSCLC. For example, miR-144-5p had an enhancive effect on NSCLC cell radiosensitivity via targeting ATF2 [20]. Moreover, miR-185 also acted as a tumor suppressor by targeting AKT1 in NSCLC [21]. Another previous study reported that miR-5702 suppressed proliferation and invasion in NSCLC via post-transcriptional suppression of ZEB1 [22]. Furthermore, miR-605 promoted cell proliferation, migration, and invasion in NSCLC by directly targeting LATS2 [23]. Besides, miR-223-3p was proved to function as an oncogene in NSCLC [11]. Consistent with the previous studies, we observed that miR-223-3p was increased in NSCLC tissues and cell lines compared with adjacent normal tissues and the normal lung cell line. Besides, we analyzed the relationship between the expression of miR-223-3p and clinicopathologic factors of NSCLC patients. Clinical data showed that miR-223-3p expression was

Figure 4: The effect of RHOB on NSCLC progression was explored. (a) RHOB expression in NSCLC tissues (n = 46) and normal tissues (n = 46) was measured by qRT-PCR. (b) The correlation between expression of miR-223-3p and RHOB was analyzed via Spearman’s rank test. (c–g) SK-MES-1 and H1299 cells were transfected with pcDNA or RHOB. (c) RHOB protein expression in transfected SK-MES-1 and H1299 cells was measured by western blot assay (n = 3). (d) Cell viability was measured by MTT assay (n = 3). (e) Cell apoptosis was examined by flow cytometry assay after transfection (n = 3). (f–h) Cell migration and invasion of transfected cells were tested through transwell assays and wound healing assay (n = 3). *P < 0.05.
closely associated with lymph node metastasis. Moreover, Kaplan–Meier survival curves disclosed an association between high miR-223-3p expression and the OS in NSCLC patients. Knockdown of miR-223-3p significantly suppressed cell viability, migration, and invasion and promoted the cell apoptosis in vitro, as well as inhibited tumor growth in vivo by directly targeting RHOB.

RHOB, a member of the Ras homolog gene family and GTPase, could regulate intracellular signaling pathways by interfacing with EGFR, RAS, and PI3K/Akt/mammalian target, and RHOB expression was associated with an increased propensity for tumorigenesis [24]. It was reported that RHOB was significantly downregulated in NSCLC tissues [14,25]. In this research, we also found that RHOB was distinctly downregulated in NSCLC tissues and cell lines. Moreover, miR-19a had a promotion effect on bladder cancer cell invasion and epithelial-to-mesenchymal transition by targeting

**Figure 5:** si-RHOB recovered the constrained effects of anti-miR-223-3p on NSCLC progression. SK-MES-1 and H1299 cells were transfected with anti-NC, anti-miR-223-3p, anti-miR-223-3p + siRNA, or anti-miR-223-3p + si-RHOB. (a) Western blot assay was conducted to analyze the RHOB protein level in transfected SK-MES-1 and H1299 cells (n = 3). (b) MTT assay was employed to test the cell viability of NSCLC cells (n = 3). (c) Flow cytometry was used to examine cell apoptosis of treated cells (n = 3). (d–f) Cell migration and invasion of transfected cells were measured by transwell assays and wound healing assay (n = 3). *P < 0.05.
RHOB [26]. In our research, we elucidated that RHOB was directly targeted by miR-223-3p and knockdown of miR-223-3p could repress the NSCLC cell progression via targeting RHOB.

This research demonstrated the relationship between miR-223-3p and RHOB, revealing a novel mechanism regulating NSCLC cell viability, migration, invasion, and apoptosis. Moreover, these results suggested that miR-223-3p deletion exerted an inhibition effect on NSCLC progression, providing a new insight into NSCLC treatment.

In conclusion, we observed that miR-223-3p might be a tumor oncogene in NSCLC cells through repressing RHOB expression, providing a target for therapeutic intervention of this process. However, NSCLC is a complicated cancer, and the novel therapeutic target for the treatment of NSCLC must be further confirmed by in vivo experiments and clinical experiments.

Conflict of interest: The authors state no conflict of interest.

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