MicroRNA-23b inhibits cell viability and migration but promotes apoptosis in non-small cell lung cancer cells in vitro

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Abstract: Background: Lung cancer is a major public health problem worldwide and currently its effective therapies are still imperfect. MicroRNA-23b (miR-23b) was reported to modulate various cancer pathological conditions. Our study was aimed to reveal the potential effect of miR-23b on non-small cell lung cancer (NSCLC) cells and its possible mechanism. Methods: NSCLC cells line A549 were transfected with miR-23b mimic, miR-23b inhibitor or control, the miR-23b expression was monitored by quantitative polymerase chain reaction (qPCR). The cell viability, migration and apoptosis of A549 cells were respectively measured by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), modified two-chamber migration assay, and flow cytometry. Western blot was used to detect the phosphorylation levels of phosphatidylinositol 3-kinase (PI3K), AKT (Protein Kinase B) and glycogen synthase kinase-3 beta (GSK3β) in transfected cells. Results: In A549 cells, miR-23b was effectively overexpressed and suppressed (P < 0.001). We observed that miR-23b overexpression significantly suppressed A549 cells viability (P < 0.05 or P < 0.01) and migration (P < 0.001), but enhanced apoptosis (P < 0.001). In contrast, miR-23b suppression displayed the contrary results (P < 0.05, P < 0.01 or P < 0.001). In addition, we found that miR-23b overexpression downregulated the phosphorylation levels of PI3K, AKT, and GSK3β in A549 cells, whereas miR-23b suppression could upregulate them. Conclusion: MiR-23b overexpression suppressed A549 cells viability and migration, but enhanced apoptosis through the PI3K/AKT/GSK3β pathway. Overexpression of miR-23b has potential as a novel therapeutic strategy in NSCLC treatment.

Keywords: microRNA-23b, non-small cell lung cancer, cell viability, migration, apoptosis, PI3K/AKT/GSK3β pathway

Introduction

Lung cancer is one of malignant tumors with highest mortality in the human race. Non-small cell lung cancer (NSCLC) is one of the most common types of lung cancer and also the leading cause of death among all cancers because of very low survival rate after treatment and the unlikely event of surgical resection. This tumor often presents at the advanced stage, which hinders effective treatment [1, 2]. There were multiple researches have been carried out to prevent, anticipate, and diagnose this cancer [3-5]. However, smaller lesions will inevitably lead to poses diagnostic challenges and more effective therapies needed at earlier stages [6]. Nowadays, a variety of novel molecular methods have been investigated to find effective treatments for NSCLC [7]. Nevertheless, to date only limited response and survival benefit has been shown, in part because of a lack of understanding in the carcinogenesis of NSCLC [8].

MicroRNAs (miRNAs) are small non-coding RNA molecules capable of regulating expression of over 50% of protein-coding genes. It has rapidly emerged as potential biomarkers showing possibility to assist the clinical management of lung cancer [9]. MiRNAs are involved in the pathogenesis of lung diseases such as cystic fibrosis, lung cancer, asthma and etc [10]. It has been demonstrated to be relevant to lung cancer development, epithelialmesenchymal transition and response to therapy [11]. A lot of researches certified that miRNAs can affect the migration, invasion and apoptosis of NSCLC cell [12, 13]. A better understanding of the involve-
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The overexpression of miRNAs in pathogenesis of this disease could result in the development of new therapeutic and diagnostic tools. Recently, miR-23b has emerged as a promising new cancer biomarker but its accurately role in NSCLC has not been established yet [14, 15].

In the present study, we investigated the role of miR-23b in NSCLC cells viability, migration and apoptosis. We observed that overexpression of miR-23b altered A549 cell progresses in vitro. Subsequently, we verified the protein levels changes of phosphatidylinositol 3-kinase (PI3K), AKT (Protein Kinase B) and glycogen synthase kinase-3beta (GSK3β) to reveal the possible molecular mechanisms of miR-23b in NSCLC cells. Our finding may offer a partial understanding about miR-23b roles in NSCLC and provide evidences about that miR-23b might be used in NSCLC treatment as novel gene therapeutic target.

Materials and methods

Cell culture and transfection

The NSCLC cells line A549 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; Hyclone, Logan, UT) medium supplemented with 10% heat-inactivated feral bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY) in a humidified CO₂ incubator at 37°C [16].

A549 cells were transfected with miR-23b mimic, miR-23b inhibitor, or control (GenePharma, Shanghai, China), respectively. The transfection was performed by using Lipofectamine 3000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. After 48 h of transfection, cells were selected and established a stable cell line as described previously for the further analysis [17].

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted with Trizol (Invitrogen, USA) and reversely transcribed by MultiScribe RT kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer’s instructions. All reverse transcriptase reactions were run in a master cycler gradient (Eppendorf, Germany). Fast START Universal SYBR Green Master (ROX) (Roche, USA) was used for qPCR analysis according to the manufacturer’s instructions. The qPCR was performed in triplicate, including no template controls. MiR-23b expressions were normalized to U6 expressions using 2⁻ΔΔCT method [18]. All primers were synthesized by GenePharma (Shanghai, China). The data were analyzed with Real-Time StatMiner (Integromics).

Cell viability assay

MiR-transfected cells were seeded at 2 × 10³ cells/well in 96-well plates. After 1-4 days of incubation, 20 µl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) were added into each well and incubated for another 4 h at 37°C. Then, 150 µl dimethylsulfoxide (DMSO; Sigma, USA) was added and the plates were shaken for 10 min. The absorbance was measured at 570 nm (OD₅₇₀) with a microplate reader (Bio-Rad Laboratories, Hercules, CA, US).

Cell migration assay

Cell migration was determined by using a modified two-chamber with 8.0 µm pore membranes (Greiner 662638). MiR-transfected cells were suspended in 200 µl of serum-free culture medium, and were added into the upper compartment of 24-well transwell culture chamber. Add 600 µl complete medium to the lower compartment, then incubated at 37°C for 12 h. After that, cells were fixed with 4% methanol (NIST, USA) for 30 mim, non-transferred cells were removed from the upper surface of the filter carefully with a cotton swab. Traversed cells in the lower were stained with 0.1% crystal violet for 20 min and counted using an vertical microscope (Leica Microsystems, Wetzlar, Germany).

Cell apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells using Annexin V-FITC/PI apoptosis detection kit (Beijin Biosea Biotechnology, Beijing, China) according to manufacturer’s recommendations. MiR-transfected cells were collected and suspended in 200 µl of binding buffer containing 10 µl Annexin V-FITC and 5 µl of PI, then incubated at room temperature for 30 min in the dark. Apoptotic cells were measured with flow cytometer (Beckman Coulter, USA) [19].
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Western blot analysis

Cell lysates were extracted by lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitor (Roche, Guangzhou, China) from miR-transfected cells. Proteins were resolved over 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for 1 h using 5% non-fat dry milk and probed with the respective primary antibodies (all 1:1,000). Then the blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz biotech) and detected using enhanced chemiluminescence (ECL) Western Blotting Substrate (Thermo Scientific) and autoradiograph. The protein loading was normalized using antibody to glyceraldehyde-3-phosphohde dehydrogenase (GAPDH). The data were analyzed using Image Lab (Bio-Rad) software. The following antibodies were used in this study: PI3K (ab86714), phosphorylated PI3K (p-PI3K, ab182651), AKT (ab8805), phosphorylated AKT (p-AKT, ab38449), GSK3β (ab32391), phosphorylated GSK3β (p-GSK3β, ab75745) and GAPDH (ab9485) (Abcam, Cambridge, MA, USA).

Statistical analysis

Results were expressed as the mean ± standard deviation (SD), and statistical analyses were performed using a one-way analysis of variance (ANOVA) or two-tailed paired-sample t-test in GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). A P value of < 0.05 was considered statistically significant.

Results

MiR-23b overexpression reduced cell viability and migration of A549 cells

In order to investigate the role of miR-23b in NSCLC, A549 cells were respectively transfected with miR-23b mimic, miR-23b inhibitor, or control. We observed that miR-23b was effectively overexpressed or suppressed (P < 0.001) in A549 cells after transfection (Figure 1). Then, cell viability was examined by MTT assay after cultured for 1-4 days. The results shown in Figure 2A indicated that miR23b overexpression caused a significant decrease in cell viability at 2 (P < 0.05), 3 (P < 0.01), and 4 days (P < 0.01) as compared to control group. MiR-23b suppression could significantly promote cell viability at the same time point (P < 0.05 or P < 0.01). The results from migration assay (Figure 2B) showed that cell migration was significantly decreased by miR-23b overexpression (P < 0.001), while was increased by miR-23b suppression (P < 0.001) compared with the control group. Collectively, these results indicated that miR-23b might affect cell viability and migration in NSCLC cells.

Overexpression of miR-23b promoted A549 cells apoptosis

The results of apoptotic cells classified by flow cytometry were shown in Figure 3A and 3B. We observed that the apoptotic cell rate was significantly enhanced (P < 0.001) in miR-23b mimic transfected cells, while miR-23b suppression group showed the opposite result (P < 0.01). Hence, the miR-23b might be involved in NSCLC cells apoptosis.

MiR-23b overexpression influenced on A549 cells by regulating PI3K, AKT and GSK3β

Activated PI3K, AKT and GSK3β are associated with differentiation and proliferation of cells in various types of cancers including lung cancer [20-23]. To define the molecular mechanism of the miR-23b effect on NSCLC cells, we detected the protein levels of PI3K, AKT and GSK3β in miR-transfected cells by western blot analysis.
As shown in Figure 4A and 4B, the phosphorylation level of these three proteins were significantly downregulated by miR-23b overexpression (P < 0.05 or P < 0.01) when compared with
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Discussion

Researchers have reported that altered miRNAs expression contributed in carcinogenesis and was implicated in cancer cell proliferation and apoptosis [24]. MiRNAs might function as regulatory molecules and acted as tumor suppressors or oncogenes [25]. Nowadays, plenty of miRNAs have been found to affect NSCLC cell invasion, induct apoptosis and etc. by its own post-transcriptional regulation, self-regulation of expression, epigenetic regulation and anti-tumor properties [26, 27]. Nora et al. have reported that miR-15a/miR-16 were implicated in cell cycle control and likely contribute to the tumorigenesis of NSCLC [28]. In the current study we presented in vitro data suggesting a possible role for miR-23b in NSCLC cells viability and migration inhibition and apoptosis induction. More importantly, the protein expression levels of p-PI3K, p-AKT and p-GSK3β in A549 cells were all downregulated by transfected with miR-23b mimic.

Figure 4. MiR-23b downregulated the expression of p-PI3K p-AKT, and p-GSK3β in A549 cells. Cell lysates from miR-23b mimic and miR-23b inhibitor transfected cells were analyzed for expression of p/t-PI3K, p/t-AKT, and p/t-GSK3β using specific antibodies. These blots were reprobed with an antibody to GAPDH for comparison of protein loading in each lane. A. The protein level of p/t-PI3K, p/t-AKT, and p/t-GSK3β GAPDH expression in miR-23b treated A549 cells were detected by western blot. B. Densitometric analyses of these proteins were done by using Image Lab software and shown. Data were represented as mean ± SD. MiR-23b, microRNA-23b; PI3K, phosphatidylinositol-3-kinase; p-PI3K, phosphorylated-PI3K, p-AKT, phosphorylated-AKT; GSK3β, Glycogen synthesis kinase 3 beta; p-GSK3β, phosphorylated- GSK3β; GAPDH, glyceraldehyde-3-phosphohate dehydrogenase; SD, standard deviation. *, P < 0.05; **, P < 0.01.

MiR-23b which belongs to the miR-23b-27b-24-1 cluster (9q22.32), has been described as a pleiotropic modulator in different organs especially with regard to cancer development [29]. MiR-23b has been verified play important roles in cancer progression, and have dual role in carcinogenesis, both as a tumor suppressor and as an oncogene [30]. For examples, miR-23b promoted tumorigenesis of breast cancer cells [31], inhibited carcinogenesis in prostate cancer cells [32] and inhibited migration and metastasis in colorectal cancer cells [33]. It also has been verified miR-23b suppressed lymph node metastasis in breast cancer [34], blocked gastric cancer progression [33] and etc. Previous studies indicated that miR-23b was significant amplification in NSCLC, and it was a potential oncogene for the genesis and progression of NSCLC [35]. But the exactly effects of miR-23b on NSCLC cells activity has not been deeply explored. In our studies, we measured biological functions of miR-23b on A549 cells in vitro. Our results indicated that miR-23b overexpression could significantly inhibit cell viability and migration, while induce apoptosis in A549 cells. Therefore, miR-23b might exert tumor suppressor function in NSCLC cells by regulating cell viability, migration and apoptosis.
In order to reveal the underlying molecular mechanisms by which miR-23b affected NSCLC cells activity, the protein expression levels of p-PI3K, p-AKT, and p-GSK3β were assessed. We found that expression levels of these three proteins were decreased significantly in miR-23b mimic transfected cells. Among these kinases, PI3K and AKT were the important kinases in the PI3K/AKT signaling pathway. The PI3K/AKT signaling pathway plays an essential role in maintaining tumor cell proliferation and apoptosis [36]. GSK3β is one of the downstream negatively regulated targets of PI3K/AKT pathway, which could induce apoptosis via an apoptosis protein of B cell lymphoma/leukemia-2 (Bcl-2) family myeloidcellleukemia-1 (Mcl-1) phosphorylation and also participate in diverse cellular processes including proliferation, differentiation, motility and survival [37, 38]. Thus our study indicated that miR-23b might affect the cell progresses in A549 cells via regulating the PI3K/AKT/GSK3β pathway.

In conclusion, we found that miR-23b was the major mediator of cell viability, migration and apoptosis in vitro, and this might be related with PI3K/AKT/GSK3β signaling pathway. Further exploration need to be focused on the precise role of miR-23b in the other types of NSCLC cells.

Disclosure of conflict of interest

None.

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References


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