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Effects of MicroRNA-10b on lung cancer cell proliferation and invasive metastasis and the underlying mechanism

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ABSTRACT

Objective: To study the influence of MicroRNA-10b on proliferation and invasion of human low metastatic lung cancer cell 95-C and its mechanism. **Methods:** Lipofectamine MicroRNA-10b eukaryotic expression plasmid was transfected into 95-C. The experiment group was divided into blank control group, empty vector transfected group and MicroRNA-10b transfected group. Real time quantitative RT-PCR was used to detect the expression of MicroRNA-10b and KLF4mRNA expression. Proliferations of cells were detected by cell proliferation assay, invasion of the detected the cell Transwell experiments, the expression of KLF4 protein was detected in Western blotting cells. **Results:** The proliferation rate of MicroRNA-10b plasmid transfection group increased significantly after transfection, invasion and migration ability enhancement, by comparison, there are statistically significant differences in the blank control group and negative control group ($P<0.05$); the expression of MicroRNA-10b plasmid transfection group KLF4 protein decreased, the difference was statistically significant ($P<0.05$); reduce the expression of MicroRNA-10b plasmid transfection group KLF4mRNA, but no significant differences ($P>0.05$). **Conclusions:** MicroRNA-10b may promote proliferation and invasion of 95-C cells by down regulating the expression of KLF4 protein.

1. Introduction

Lung cancer is a common malignant tumor with a high mortality. Searching for an effective diagnosis and treatment method of lung cancer is of great clinical significance[1]. There is evidence that microRNA (miRNA) shows significant effect on tumor cell proliferation and invasive metastasis[2]. In this study, we transiently transfected lung cancer cell line 95-C with miRNA-10b eukaryotic expression plasmid using liposome technology and investigated the effects of miRNA-10b on human lung cancer cell line 95-C proliferation and invasive metastasis and the underlying mechanism.

2. Materials and methods

2.1. Cell lines and reagents

Lowly metastatic human lung cancer cell line 95-C was selected. The following reagents were used: RPMI 1640 culture medium; fetal bovine serum (FBS); miRNA-10b eukaryotic expression plasmid; liposomal transfection reagents Lipofectamine®2000 and TRIzol; Transwell chamber; Krüppel-like factor 4 (KLF4) antibody; SYBRGreen PCR reagent kit; Cell Counting Kit-8 (CCK-8); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.2. Cell culture

Lowly metastatic human lung cancer cell line 95-C was cultured in Dulbecco's Modified Eagle's Medium (DMEM; high glucose) containing 10% FBS at room temperature in a 5% CO_2 incubator.

2.3. Cell transfection

Cells in the logarithmic phase were transfected as follows. Cells incubated in the 6-well plate were divided into three groups: blank control, negative control and miRNA-10b transfection group. Cells in the blank control group were not subjected to cell transfection. Cells in the negative control group were transfected with empty plasmid (NC sequence:

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TTC TCC GAA CGT GTC ACG T). Cells in the miRNA-10b group were transiently transfected with miRNA-10b eukaryotic expression plasmid using Lipofectamine® 2000 Transfection Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA).

Precisely, at 1 d prior to cell transfection, cells were inoculated at a density of 30%-50% overnight. Culture medium one was prepared by fully mixing 250 μL Eagle's minimal essential medium and 5 μL lipofectamine® 2000 Transfection Reagent and placing the mixture at room temperature for 5 min. Culture medium two was prepared by fully mixing 250 μL opti-MEM and 5 μL siRNA and leaving the mixture at room temperature for 20 min. After getting rid of culture medium, cells were cultured with serum- and antibiotics-free culture medium and then transfected with 500 μL of liposome and siRNA solution in a 37℃ and 5% CO_2 environment. After 4-6 h transfection, the culture medium was replaced with PBS containing 10% FBS. After 24 h transfection, the transfection efficacy was observed through the use of fluorescence microscope.

2.4. Real time quantitative RT-PCR analysis

After 48 h transfection, three groups of cells were collected and total RNA was extracted according to instruction provided by Taqman microRNA Reverse Transcription Kit and quantified by spectrophotometry. After reverse transcription, cDNA was synthesized from an RNA template. Then miRNA-10b level in the sample was detected by SYBRGreen quantitative PCR technique. The specific primers used in detection of miRNA-10b level were as follows: miRNA-10b upstream primer: 5′GGA TAC CCT GTA GAA CCG AA3′, downstream primer: 5′CAG TGC GTG TCG TGG AGT 3′; internal reference U6 upstream primer: 5′TGG GGT TAT ACA TTG TGA GAG GA3′, downstream primer: 5′GTG TGC TAC GGA GTT CAG AGG TT3′; KLF-4mRNA upstream primer: 5′AAG AAT CCA AAC CCA AGC3′, downstream primer: 5′CGA ATT TCC AGC CAC ATC3′; internal reference GAPDH upstream primer: 5′TCT GAT TTG GTC GTA TTG GG3′, downstream primer: 5′GGA AGA TGG TGA TGG GAT T3′. RT-PCR was performed at 95℃ for predenaturation for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60℃ for 45 s. The Ct value of each sample, GAPDH and internal reference U6 was obtained. Then the relative level of miRNA-10b and KLF-4mRNA was calculated. Each experiment was repeated for 3 times.

2.5. Detection of cell proliferation

After digested with trypsin, each group of cells were diluted into 1-5 ×10^4 cells/mL. A 100 μL aliquot of cells from each group were cultured in a 96-well plate and divided into an experimental group and a control group. Each kind of cells were inoculated into 3 parallel wells, 1-5×10^3 cells per well. A 100 μL culture medium was designed as a control group. All cells were cultured at 37 ℃ overnight. CCK-8 and serum-free DMEM were fully mixed at a proportion of 1:10 and then added to the tested wells, 100 μL per well. Then cells were incubated in a 37 ℃ 5% CO_2 environment for 1 h. Absorbance at 450 nm was determined and cell growth curves were plotted.

2.6. Transwell invasion assays

At 24 h prior to Transwell invasion assay, culture medium in each group was replaced with serum-free DMEM culture medium. Before cell inoculation, 24-well plates and Transwell chambers were soaked with PBS. Cells in each group were digested with trypsin, washed with serum-free DMEM, and then prepared into cell suspension with 1% FBS-containing DMEM. Subsequently, cells were inoculated into PBS-soaked Transwell chambers at a density of 1×10^5 cells/mL, 0.5 mL per chamber. 0.75 mL DMEM culture medium containing 10% FBS was added to the 24-well plate in the bottom chamber. Three parallel wells were designated for each group. Then the cells were incubated at 37℃ for 48 h. After addition of 1 mL of 4% formaldehyde solution into each well, cells were incubated at room temperature for 10 min. After getting rid of fixing solution, cells were washed once with PBS, stained with 0.5% crystal violet solution, 1 mL per well, for 30 min, washed three times with PBS, and then dried in the air. The non-migrating cells in the chamber were removed through the use of cotton buds. Finally, the cells in the field of view were observed and counted under 200×magnification.

2.7. Statistical analysis

All data were statistically processed using SPSS15.0 software. Measurement data were compared between groups using t test and expressed as mean ± SD. Numeration data were compared between groups using chi-square test. A level of $P<0.05$ was considered statistically significant.

3. Results

3.1. miRNA-10b expression in transfected cells

After 48 h transfection, the mean RQ value of miRNA-10b in each group was determined by real time quantitative PCR. Compared with blank control and negative control groups, miRNA-10b expression was significantly increased in the miRNA-10b transfection group ($P<0.05$). There was no significant difference in miRNA-10b expression between blank control group and negative control group ($P>0.05$; Figure 1).

3.2. Cell proliferation after transfection

Through cell growth curves, the rate of cell growth in the miRNA-10b transfection group was significantly higher than in the blank control and negative control groups ($P<0.05$; Figure 2).

3.3. Transwell invasion assay results

A significantly higher number of cells migrating through the Transwell membrane in the miRNA-10b transfection group than in the blank control and negative control groups ($P<0.05$), and there was no significant difference between blank control and negative control groups ($P>0.05$). This suggests that the invasive capability of miRNA-10b eukaryotic expression plasmid-transfected 95-C cells was increased (Figure 3).

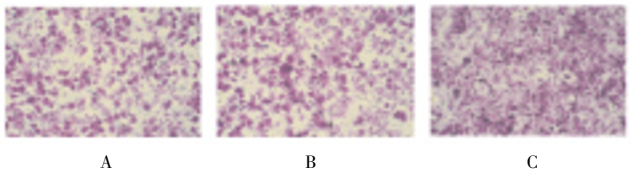

Figure 3. Invasive ability of 95-C over expressed MiRNA-10b determined by Transwell (200×).

3.4. Intracellular KLF4 protein expression after transfection

After 48 h transfection, intracellular KLF4 protein expression was detected by western blot analysis. Intracellular KLF4 protein expression in the miRNA-10b transfection group (0.861±0.057) was significantly lower than in the blank control and negative control groups (1.477±0.048, 1.541±0.078; $P<0.05$), however, there was no significant difference between blank control and negative control groups ($P>0.05$; Figure 4).

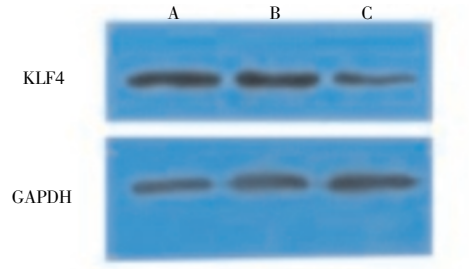

Figure 4. The expression of KLF4 protein in each group after transfection.

3.5. Intracellular KLF4 mRNA expression after transfection

After 48 h transfection, the mean RQ value of miRNA-10b in each group was determined by real time quantitative PCR. Intracellular KLF4 mRNA expression in the miRNA-10b transfection group was slightly decreased than in the blank control and negative control groups, but there was no significant difference between three groups ($P>0.05$; Figure 5).

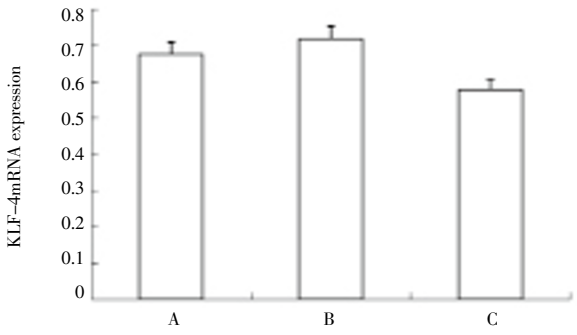

Figure 5. RT-PCR analysis of KLF-4mRNA expression in each group after transfection.

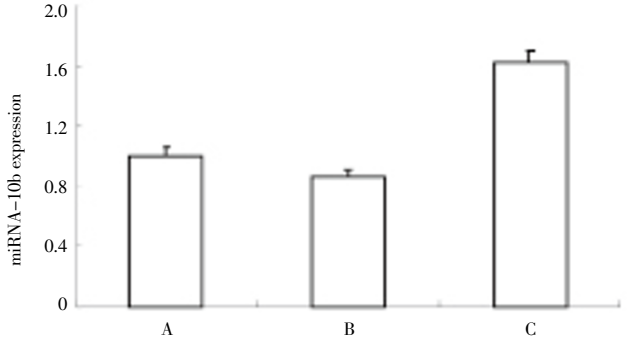

Figure 1. RT-PCR analysis of miRNA-10b expression after transfection.

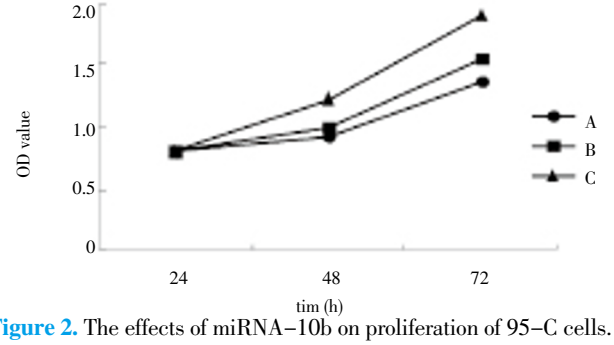

Figure 2. The effects of miRNA-10b on proliferation of 95-C cells.

4. Discussion

4.1. Lung cancer

Lung cancer is highly malignant and has a high morbidity and mortality in China. At present, surgical removal is most commonly used to treat lung cancer in the clinic, because which can remarkably prolong patient's survival. But most patients have developed advanced lung cancer at admission, so the treatment efficacy is not satisfactory. Therefore, searching for an effective diagnosis and treatment method is of important clinical significance[3].

4.2. miRNAs

miRNAs are a class of small, non-coding single-strand RNA molecules[4]. miRNA is encoded by endogenous gene and consists of around 22 nucleotides. It has been documented that more than half of miRNAs are located in tumor-related genomic regions, fragile sites, and the regions of loss of heterozygosity and amplification[5]. This suggests that miRNA likely exhibits cancer-inhibitory effects.

miRNA-10b, located on Hox gene clusters, can maintain the proliferation of normal tissue and produce great effects on the proliferation and invasive metastasis of diverse malignant tumor cells[6]. Results from this study showed that 95-C cells expressing miRNA-10b were created by transfecting miRNA-10b plasmid into 95-C cells. Through cell proliferation tests, the cells with increased miRNA-10b expression proliferated fast. Through Transwell invasion assay, after miRNA-10b plasmid transfection, the invasive capacity of 95-C cells was increased. This result suggests that MiRNA-10b overexpression can increase the invasive capacity of 95-C cells.

4.3. miRNA-10b and lung cancer cells

The matching degree of miRNA and its target site will produce great influence on miRNA functions[7,8]. If the matching between miRNA and the miRNA3′UTR or coding region of target gene is not complete, then miRNA translation will be inhibited, which further influences protein expression; complete complementary pairing will lead to the degradation of target gene[9-11]. A previous study reported that in esophageal cancer, the mRNA of KLF4 gene is a direct and functional target of miRNA-10b and miRNA-10b promotes esophageal cancer cell invasion and metastasis by downregulating target gene KLF4 expression[12]. KLF4 is a gut-enriched Krüppel-like factor 4 or an epithelial zinc finger-type transcription factor, belongs to the KLF family and is highly expressed in the digestive tract and epithelial cells, but its expression is downregulated in various tumor types[13,14]. KLF4 gene transfection can inhibit tumor cell growth and invasive metastasis in vivo, which occurs possibly due to G1/S cell cycle arrest through inhibiting the expression of extracellular matrix protein, cysteine-rich acidic matrix-associated protein, upregulating p21 expression or downregulating cyclin D1 expression. In this study, at 48 h after miRNA-10b transfection, KLF4 protein level in the cells overexpressing miRNA-10b was decreased as shown by western blot analysis and KLF4 mRNA expression in the cells overexpressing miRNA-10b was also decreased (taking GAPDH mRNA as an internal reference), as shown by real time quantitative PCR detection. But there were no significant differences in KLF4 mRNA expression between three groups. These findings suggest that miRNA-10b exerts post-transcriptional inhibition of KLF4 mRNA expression.

A single miRNA can target multiple genes. miRNA-10b possibly exerts its regulatory role through the synergistic effects of its multiple target genes[3]. The biological functions of miRNA-10b in tumor cells are attributable to a series of related target genes. KLF4 plays an important role in lung cancer cell proliferation and invasive metastasis. Results from this study showed that KLF4 is a functional target gene of miRNA-10b. Whether KLF4 is a direct target gene of miRNA-10b remains uncertain because no corresponding luciferase reporter system was established, which needs to be further investigated in future studies.

Taken together, results from this study showed that miRNA-10b can promote 95-C cell proliferation and invasive metastasis possibly through downregulating KLF4 protein expression. miRNA-10b is likely to become a potential target for treatment of non-small cell lung cancer. Silencing miRNA-10b gene expression holds great promise for improving the prognosis of patients with non-small cell lung cancer.

Conflict of interest statement

We declare that we have no conflict of interest

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