ORIGINAL RESEARCH

MIR-34C REGULATES THE PROLIFERATION AND APOPTOSIS OF LUNG CANCER CELLS

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Xianliang Jiang, MD, Ming Li, MA, Li Ke, MA

Department of Thoracic Surgery, The First Affiliated Hospital of University of Science and Technology of China, Hefei, China

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ABSTRACT

Purpose: As miR-34c acts as a tumor suppressant for multiple cancers, the purpose of this study was to investigate that role that miR-34c plays in the proliferation and apoptosis of lung cancer.

Methods: The expression of miR-34c in 600 patients with lung cancer was quantitatively analyzed with real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) technology and correlated to clinical pathological parameters. The CCK-8 analysis and flow cytometry were carried out to detect cell proliferation and apoptosis in miR-34c-mimic transfected cell lines. Moreover, the regulation of miR-34c to interleukin-6 (IL-6) in cell lines was detected by western blot, qRT-PCR and dual-luciferase reporter assay.

Results: The expression of miR-34c was downregulated in lung cancer compared with adjacent normal tissues. The expression level of miR-34c was linked to stromal invasion. Furthermore, overexpressing miR-34c played an active role in effectively inhibiting cell proliferation and inducing apoptosis. In addition, a significant inverse relationship was exhibited between the expression of miR-34c and IL-6 in tumor tissues.

Conclusion: At the molecular level, IL-6 can be used as a direct target of miR-34c in the treatment of lung cancer cells and miR-34c can be used as an effective biomarker and therapeutic target for lung cancer.

Correspondence to: Xianliang Jiang, xianliangjiang@163.com

INTRODUCTION

For decades, lung cancer has represented the primary cause of cancer-related deaths worldwide. Of all types of lung cancer, approximately 85% are non-small cell lung cancer including adenocarcinomas, large cell carcinomas, and squamous cell carcinomas. As the most frequently seen isoform of non-small cell lung cancer, lung adenocarcinoma develops and evolves through a series of genetic and epigenetic alterations in lung epithelial cells [1]. Only 15% of patients with lung cancer have an overall five-year survival rate, and this varies with the different stages of the disease; from 50% of patients with local tumors to 25% of patients with local disease, and 5% of patients with distant metastasis. Accordingly, metastatic spread contributes to the majority of lung cancer mortality rather than the primary tumor itself. As metastatic diseases account for approximately 90% of all cancer mortalities, it is essential to make an in-depth study of the molecular mechanism of cancer cell metastasis [2, 3].

MicroRNAs are non-coding RNA molecules (~22 nt) that can regulate the expression of genes at post-transcriptional levels. Over 50% of the activity in all protein-coding genes is regulated by microRNAs, and disorders of these small molecules are common features of the pathogenesis of many diseases, including cancer [4-6]. The miR-34 family encompasses three tumor-suppressor miRs that are frequently downregulated in human malignant tumors, including miR-34a, miR-34b and miR-34c, and share a common primary transcript. The miR-34 is an active contributor in the p53 tumor suppressor network, the expression of which is directly induced by p53 as a response to DNA damage and carcinogenic stress [7-10]. Several known oncogenes involved in cell cycle conversion, proliferation and apoptosis have been identified as direct targets for miR-34s, including BCL2, NOTCH1 and MYC [11-14]. In addition, miR-34s have been identified as direct inhibitors of epithelial-to-mesenchymal transformation (EMT) and metastasis [15-21]. Low expression of miR-34 has been shown to predict the distant metastasis formation in various cancers, including carcinomas

of the liver, breast and colon [22-28]. Although studies have reported that miR-34c can target eIF4E [29], PAC1 [30] and HMGB1 [31] genes to inhibit the proliferation and migration of lung cancer, whether miR-34c can also be targeted for the treatment of lung cancer remains unknown. More comprehensive and in-depth studies of the relationship between miR-34c and lung cancer are necessary. Lung cancer has the highest incidence of malignant tumors in China. The present study was conducted to ascertain how miR-34c works in lung cancer and to provide evidence on the regulatory role of miR-34c through *in vitro*.

MATERIALS AND METHODS

Preparation of clinical specimen

A total of 600 specimens were collected from the First Affiliated Hospital of the University of Science and Technology of China from 2014 to 2019. The patients were 27 to 70 years of age. Radiotherapy and chemotherapy were not performed before surgery but all patients with lung cancer underwent histopathological diagnosis. Patients with a history of other tumors or hematological malignancies, or with other lung diseases (such as chronic obstructive pulmonary disease) or lung infections were excluded. Only patients with epidermal growth factor receptor (EGFR) mutations were included in the study.

Informed consent of each patient was provided, and this study was approved by the Ethics Committee in the First Affiliated Hospital of University of Science and Technology of China (No. ChiCTR2000050158). All tissue samples were quickly frozen in liquid nitrogen immediately after collection and stored at -80°C until use. The clinicopathological characteristics of 600 patients were presented as follows in Table 1. The TNM staging standard was established by the International Union Against Cancer (UICC) in 2015.

EGFR mutation detection

Genomic DNA was extracted from fresh tumor specimens using the TIANamp genomic DNA kit (Tiangen Biotech, Beijing, China). The cases were analyzed by ARMS, according to the protocol of the ADx EGFR Mutations Detection Kit (Amoy Diagnostics, Xiamen, China). The assay was carried out using an MX3005P real-time polymerase chain reaction (PCR) system (Stratagene, La Jolla, USA).

Cell culture

Four cell lines were purchased from the China Center for Type Culture Collection (Wuhan, China), including human lung adenocarcinoma cells (A549 and 95-D). Human normal lung epithelial cells (BEAS2B) and human lung normal cells (LL24) were cultured in Dulbecco's

modified eagle medium (DMEM, Thermo Fisher Scientific, Waltham, USA) with 10% fetal bovine serum (Thermo Fisher Scientific). Among them, LL24 and BEAS2B were taken as the control. All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air.

Transfection

The miR-34c mimics or negative control mimics were synthesized by Guangzhou RiboBio (Science City, China). Twenty-four hours before transfection, A549 cells were seeded in 24-well plates at a concentration of 2×10^5 cells in 500 µL non-antibiotic medium. Transfection was conducted when the cell confluence reached 60% - 80%. miR-34c mimic or negative control mimic (20 pmol) was diluted with 50 µL Opti-MEM (Thermo Fisher Scientific) and mixed well. Cells were transfected with the 100 µL transfected mixture (Lipofectamine TM 2000 and miR-34c mimic diluent) (Thermo Fisher Scientific) and the cell plate was cultured in a 37°C, 5% CO₂ incubator. The transfected cells were harvested in 24 hours after transfection for downstream assays. The miR-34c mimic sequence was 5'-AGGCAGUGUAGUUAGCUGAUUGC-3' and the negative control mimic was 5'-CGGUACGAUCGCGCGGGAUAUC-3'. The transfection efficiency of miR-34c mimic was evaluated by real-time PCR assay (additional details on assay results available from the authors).

RNA extraction and miRNA detection

The RNA was extracted from lung cancer tissue and cell lines (A549, 95D, BEAS2B and LL24) by applying the RNeasy Mini test kit (74104, Qiagen, USA) to analyze the expression of miR-34c. All procedures were performed following the manufacturer's instructions. Absorbance was measured at 260/280 nm with BioPhotometer (Eppendorf, Germany) and the concentration and quality of the extracted RNA were evaluated (OD260/OD280 was between 1.7 and 2.0). mRNA was reversely transcribed using the ABI HC cDNA kit (Thermo Fisher Scientific) and miRNA using the miScript II RT Kit (Qiagen). The expression level of miR-34c was subsequently detected by using the TaqMan MicroRNA assay kit (4427975, ABI, USA). The method of $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression. The sequence of quantitative PCR primers was as follows:

miR-34c-F, 5'-GCTGCTGTAGGCAGTGTAGTTAG-3';

miR-34c-R, 5'-CTCAACTGGTGTCTGGAGTC-3';

U6-F, 5'-CTCGCTTCGGCAGCACAC-3'; and U6-R, 5'-AAC-

GCTTCACGAATTTGCGT-3'.

Cell viability assay

All tests were carried out according to the manufacturer's instructions. In brief, the A549 cells treated with control or miR-34c mimics were spread in 96-well plates, and then a 10 µL CCK-8 solution was added to each

well. The specimens were incubated for one hour, and the absorbance was measured at 490 nm. Each group was repeated three times, and each test was performed three times.

Scratch assay

A549 cells with a density of $5\text{--}10 \times 10^5$ cells/mL were seeded in a 24-well plate (500 μL per well), cultured with DMEM containing 10% fetal bovine serum, and then incubated for 16–24 h to form a monolayer of cells. A 10 μL pipette tip was used to make a “one” scratch on the monolayer cells, washed three times with PBS, incubated for 24 h, and refreshed with DMEM medium containing 10% fetal bovine serum following incubation of 24 h. Images by a scratch wound assay were captured with an inverted microscope after being transfected with miR-34c or miR-control mimics for 36 h. The software Image J was applied to detect the wound healing rate. Each experiment was repeated at least three times.

Determination of apoptosis

A549 cells were harvested by the utility of trypsin digestion 72 h after transfection. The Annexin V-Alexa apoptosis assay kit (Fcmacs, China) was utilized to detect cell apoptosis. The cells were resuspended with 100 μL of the labelling solution, incubated for 10–15 min at room temperature in the dark, centrifuged at 1,000 rpm for 5 min. The resulting pellet was washed once with incubation buffer. The SA-FLOUS solution was added to the suspended cells, and cells were then incubated for 20 min at 4°C in the dark with constant shaking. A flow cytometer (BD Biosciences, USA) was used at an excitation wavelength of 488 nm; with a emission wavelength at 515 nm to detect Alexa fluorescence. An excitation wavelength at 535 nm and a emission wavelength at 615 nm were used to detect propidium iodide.

Western blot

Protein was extracted from each sample with RIPA lysis buffer. The protein sample was centrifuged at $15,000 \times g$ for 20 min at 4°C and the homogenate supernatant was withdrawn. Protein quantification was performed using the bicinchoninic acid method. A total of 50 μg of protein were used in each sample, which was separated and transferred by 10% SDS-PAGE gel electrophoresis. After electrophoresis, the protein binding was transferred to the polyvinylidene difluoride membrane, which was washed with Tris buffer saline, sealed with 5% skim milk powder for 1 h, and incubated with primary antibodies. The samples were then rinsed twice with Tris buffered saline Tween and incubated with a horseradish peroxidase-coupled secondary antibody for 1 h at room temperature. The following antibodies used: IL-6 (BM0286,

IGEE, China), 1:500; β -actin (BMC026, IGEE), 1:2000; and HRP goat anti-rabbit IgG (BMS014, IGEE), 1:1000.

Detection of a target gene by dual-luciferase reporter assay

Through the use of Starbase (<http://starbase.sysu.edu.cn/agoClipRNA.php?source=mRNA>), IL-6 was predicted to be a miR-34c target gene. The fragment of wild type 3'-UTR of IL-6 containing the miR-34c binding sites or mutant 3'-UTR of IL-6 was synthesized and cloned into the pMIR-report luciferase vector. To detect and validate miR-34c target genes, cultured cells were seeded into 12-well plates at a density of 1×10^5 cells/well.

The IL-6 gene in a wild type or mutated 3'-UTR vector was co-transfected with pRL-TK and the oligonucleotides (miR-34c or control mimics). The cells were collected 24 h later, and luciferase activity was determined with the Dual-Luciferase® Reporter Assay Kit (E1910, Promega, USA).

Transwell

A 24-well Transwell plate with 8 μm pore polycarbonate membrane inserts (Corning Costar, Cambridge, USA) was employed to assess the invasive capacity of A549 cells. Cells in the logarithmic growth phase were diluted with serum-free DMEM medium until a concentration of 5×10^4 cells/200 μL was reached. The upper chamber of Transwell was pre-covered with Matrigel (BD356230, Corning). A total of 100 μL of diluted cell suspension was added to the upper chamber, while 600 μL of medium containing 10% fetal bovine serum was added to the lower chambers. After incubation for 24 h at 37°C, the invasive cells attached to the lower surface of the membrane were fixed with 4% paraformaldehyde, stained using 10% crystal violet for 15 min, and then photographed using a microscope.

Statistical analysis

All data were analyzed with SPSS 20.0 software (SPSS, USA) and presented as mean $\pm$ SD. Analysis of variance (ANOVA) and two-tailed t-tests were used for statistical comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Correlation between miR-34c expression and clinical pathological parameters

To determine the expression level of miR-34c in lung cancer tissues, 600 cases of lung cancer tissues were analysed by qRT-PCR. The expression of miR-34c in the lung cancer tissue was found to be significantly decreased in

TABLE 1.
CORRELATION OF MIR-34C EXPRESSION WITH THE CLINICAL CHARACTERISTICS OF LUNG CANCER PATIENTS.

Characteristics	Case	miR-34c expression		P value
		High expression (%)	Low expression (%)	
Subgroup				0.8102
SCLC	80	41 (51.3)	39 (48.7)	-
NSCLC	520	259 (49.8)	261 (50.2)	-
Age (years)				0.5136
<65	296	152 (51.4)	144 (48.6)	-
≥65	304	148 (48.7)	156 (51.3)	-
Sex				0.0590
Male	260	136 (52.3)	124 (47.7)	-
Female	340	154 (45.3)	186 (54.7)	-
Smoking habit				0.1208
No	396	189 (47.7)	207 (52.3)	-
Yes	204	111 (54.4)	93 (45.6)	-
Pathology				0.5605
Adenocarcinoma	462	228 (49.4)	234 (50.6)	-
Squamous cell carcinoma	138	72 (52.2)	66 (47.8)	-
TNM staging				0.4140
I, II	290	150 (51.7)	140 (48.3)	-
III, IV	310	150 (48.4)	160 (51.6)	-
EGFR mutation spot				0.6311
19 del	203	105 (51.7)	98 (48.3)	-
20 ins	70	34 (48.6)	36 (51.4)	-
L858R	184	99 (53.8)	85 (46.2)	-
L861Q	69	31 (44.9)	38 (55.1)	-
T790M	19	8 (42.1)	11 (57.9)	-
G719X	24	9 (37.5)	15 (62.5)	-
S768I	31	14 (45.2)	17 (54.8)	-
Tumor size (cm)				<0.0001
<5	322	239 (74.2)	83 (25.8)	-
≥5	278	61 (21.9)	217 (78.1)	-
Stromal invasion				<0.0001
<2/3	293	244 (83.3)	49 (16.7)	-
≥2/3	307	56 (18.2)	251 (81.8)	-
Lymph node metastasis				0.3690
Yes	295	153 (51.9)	142 (48.1)	-
No	305	147 (48.2)	158 (51.8)	-

advanced lung cancer with either larger tumor size or more stromal invasion. Through analysis of the correlation between the miR-34c expression and the clinical pathological parameters, we found that the expression of miR-34c in lung cancer cases was linked to tumor size and stromal invasion ($P<0.05$). No remarkable difference was found in the factors of subgroup, age, sex, smoking habit, TNM staging, EGFR mutation spots, pathological type and lymph node metastasis (Table 1).

miR-34c expression decreased and affected cell viability in lung cancer cell lines

Expression of miR-34c was evaluated in the lung cancer cell lines (A549 and 95-D). The expression of miR-34c in A549 and 95D cells was significantly lower than that in the control group (Figure 1A). Because the expression of miR-34c was the lowest in A549 cells, these cells were selected to transfect miR-34c or control mimics for subsequent experiments. Overexpression of miR-34c in A549 cells was confirmed by qRT-PCR. The cell viability in the miR-34c treatment group was reduced compared with the control group (Figure 1B).

miR-34c induced cell apoptosis and inhibited A549 cell migration

The effect of miR-34c on apoptosis of lung cancer cells was measured by flow cytometry. miR-34c overexpression induced significantly higher levels of apoptosis in A549 cells (Figure 2). The results of the scratch assay and Transwell assay revealed that the cells transfected with the miR-34c mimic developed a slow migration (Figure 3A and B). The wounds in the control group (but not the miR-34c mimic group) were mostly healed 36 h after the scratch, suggesting that miR-34c inhibited A549 cell migration. One limitation here is that we added 10% fetal bovine serum to the culture medium, which may affect the cell vitality.

miR-34c directly targeted IL-6 in lung cancer cells

Potential target genes of miR-34c were searched using the software TargetScan 6.2 and IL-6 was identified to be the target for miR-34c. Meanwhile, the expression of IL-6 was significantly decreased following overexpression of miR-34c (Figures 4A, B). Furthermore, the dual-luciferase reporter assay showed that miR-34c significantly inhibited the WT 3'-UTR of IL-6 luciferase activity in lung cancer cells, whereas it failed to regulate Mut 3'-UTR (Figure 4C), which demonstrated that IL-6 acted as a target gene of miR-34c.

DISCUSSION

Recent studies have indicated that miRNA participates in the progression of various cancers, including lung can-

FIGURE 1.
MIR-34C DECREASED AND AFFECTED CELL VIABILITY IN LUNG CANCER CELL LINES.

A) Detection of miR-34c expression in A549, 95-D, BEAS2B and LL24. Different letters represent significant differences ($p < 0.05$). B) CCK8 assay.

FIGURE 2.
MIR-34C INDUCED CELL APOPTOSIS OF A549 CELLS.

A) Cell apoptosis was detected by flow cytometry. B) Statistical results of cell apoptosis. ## represents $p < 0.01$.

cer, and in the regulation of multiple target gene expressions involved in this process. Increasingly, evidence has shown that aberrant miRNA expression plays an important role in regulating cancer development and progression [2, 3]. Studies have also suggested that miRNA disorders may serve as therapeutic targets in a variety of cancers, including lung cancer [2]. Accordingly, the study of miRNA expression and function in lung cancer may contribute to the development of treatment strategies for refractory lung cancer. The present study demonstrates down-regulation of miR-34c in lung cancer tissues.

The overexpression of miR-34c may inhibit cell proliferation and induce apoptosis of A549 cells, and this overexpression was identified as a potential inhibitor of lung cancer progression in the present study. However, further research is necessary to investigate the mechanism of miR-34c on lung cancer progression *in vivo*.

The results of the present study are consistent with numerous previous studies. The inhibitory effect of miR-34c on proliferation of lung cancer cells has been verified by directly regulating the expression of oncogenes or tumor suppressors. For instance, eIF4E [29], PAC1 [30] and HMGB1 [31] genes were identified as target genes of miR-34c. We found a significant downregulation of IL-6 in A549 cells with miR-34c overexpression. Additionally, IL-6 was shown to be the target gene of miR-34c. However, there could be more target genes of miR-34c involved in lung cancer progression than those currently discovered. In the future, more research is expected to draw a more comprehensive control network diagram for lung cancer progression.

The present research revealed that a reduction in miR-34c expression was associated with poorer outcomes in clinical and pathological characteristics of lung cancer cases. These results indicated that the expression of miR-34c was significantly decreased in advanced lung cancer tissues, which was identical to those of previous studies. Taken together, this research suggests that miR-34c functions as a potential tumor suppressor in lung cancer.

Previous studies have reported that EMT also functions as an important marker of tumor metastasis. As EMT is triggered by exposure

FIGURE 3.
MIR-34C INHIBITED THE MIGRATION OF A549 CELLS.

A) Scratch assay. B) Transwell assay of cell migration.

FIGURE 4.
MIR-34C DIRECTLY TARGETED IL-6 IN LUNG CANCER CELLS.

A) Western blot was used to detect the expression of IL-6 protein. B) Statistical result of western blot data. C) Data from dual-luciferase reporter assay. # represents $p < 0.05$, ## represents $p < 0.01$.

of epithelial cell lines to IL-6, inactivation of IL-6 may inhibit EMT in cancer cells [32-34]. Additionally, our results showed that IL-6 served as a direct target for miR-34c in lung cancer cells.

In summary, the present study demonstrated that miR-34c was downregulated in the development of lung cancer, and that miR-34c inhibited cell migration and induced apoptosis by regulating IL-6. These results provide a potential therapeutic role of miR-34c in lung cancer and offer evidence that miR-34c may be used as an effective biomarker and therapeutic target for lung cancer in the future.

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