miR-154 suppresses non-small cell lung cancer growth in vitro and in vivo

XINGYU LIN1, ZHIGUANG YANG2, PENG ZHANG1 and GUOGUANG SHAO1

Departments of 1Thoracic Surgery and 2Anesthesiology, The First Hospital of Jilin University, Changchun, Jilin 130021, P.R. China

Received December 13, 2014; Accepted February 24, 2015

DOI: 10.3892/or.2015.3895

Abstract. miR-154 has been proven to act as a tumor suppressor in several types of tumors. However, its role in non-small cell lung cancer (NSCLC) remains unclear. Thus, the aim of this study was to investigate the effects of miR-154 on NSCLC tumorigenesis and development. Using real-time quantitative PCR (qRT-PCR), we analyzed expression of miR-154 at the transcriptional level in 40 NSCLC tumor tissues and matched adjacent normal tissues and the correlation with clinicopathological features of the patients. The miR-154 mimic was stably transfected into NSCLC A549 cells, and the effects of miR-154 on cancer cell proliferation, colony formation, cell cycle arrest, apoptosis, migration and invasion in vitro, and on the growth of in vivo xenografts were investigated. miR-154 expression levels were significantly downregulated in the NSCLC compared to the corresponding non-cancerous lung tissues (P<0.05), and decreased miR-154 expression was significantly associated with metastasis (P<0.001), larger tumor size (P<0.001) and advanced TNM stage (P<0.001). Furthermore, transfection of the miR-154 mimic into the NSCLC A549 cells was able to inhibit cell proliferation, colony formation, invasion and migration, and induce cell apoptosis and G0/G1 cell cycle arrest. Enforced expression of miR-154 also suppressed the growth of cancer cell xenografts in vivo. These findings indicate that miR-154 may become a potential target for miR-based therapy of NSCLC.

Introduction

Lung cancer is the most commonly diagnosed type of cancer and is one of the leading causes of tumor-related deaths among adults worldwide (1). Among all forms of lung cancer, non-small cell lung cancer (NSCLC) is the predominant type accounting for 75-80% of all cases (2). Despite great progress in early diagnosis and treatment strategies in recent decades, patients with NSCLC are normally associated with poor prognosis (2,3), with an estimated 5-year survival rate of <16% according to data from the International Association for the Study of Lung Cancer (IASLC). Therefore, it is of great significance to further our understanding of the molecular mechanisms underlying non-small cell lung carcinogenesis to develop better diagnostic and treatment methods, thereby improving the survival and quality of life of lung cancer patients.

microRNAs (miRNAs) are a class of small, well-conserved, non-coding, single-stranded RNAs 18-25 nucleotides in length (4). miRNAs can negatively regulate gene expression by binding partially and complementarily to the 3’-untranslated regions (UTRs) of target mRNAs at the post-transcriptional level, resulting in mRNA degradation or inhibition of translation (4-6). Emerging evidence has demonstrated that miRNAs are involved in various physiological, biological and pathological processes, such as, cell differentiation, proliferation, invasion and death (6,7). A growing body of evidence strongly suggests that the deregulation or dysfunction of miRNAs contributes to human carcinogenesis and cancer progression (5,7-10). miRNAs may function as either oncogenes or tumor suppressors based on the roles of their target genes (7). In terms of NSCLC, recent reports indicate that certain miRNAs play important roles in the pathogenesis of lung cancer, and may function as potential biomarkers for NSCLC diagnosis, progression, and as therapeutic tools (10,11).

Among the miRNAs correlated with carcinogenesis, miR-154 is one of importance. The miR-154 cluster, located in the human imprinted 14q32 domain (mouse chromosome 12F2), is a very conservative miRNA cluster in mammals (12). Dysregulation of miR-154 has been reported in various types of cancer, such as prostate (13), breast (14), colorectal (15) and thyroid cancer (16). Recently, several studies have demonstrated that miR-154 acts as a tumor suppressor and possesses inhibitory effects on cancer cell proliferation, migration and invasion (15,17,18). In lung cancer, miR-154 was found to be weakly expressed in cancerous tissues compared to normal lung tissues (19). However, to our knowledge, the correlation between miR-154 dysregulation and clinicopathological characteristics of NSCLC, and the functional attributes of miR-154 associated with NSCLC progression remain unclear.
Therefore, in the present study, we firstly examined the expression level of miR-154 in NSCLC cancer cell lines and cancer patients. Then, we transfected the miR-154 mimic into the A549 cells to investigate the functional role of miR-154 in regulating NSCLC proliferation, apoptosis, cell cycle arrest, migration and invasion, and tumor growth of xenografts in vivo. These studies contribute to improve our understanding of the underlying mechanisms of miR-154 regulation in NSCLC.

Materials and methods

Tissue samples. NSCLC samples and the corresponding adjacent tissues were collected from 40 patients that had undergone routine surgery at the First Hospital of Jilin University from April 2008 to June 2014. Normal lung tissues adjacent to the tumors were obtained 5 cm away from the lung tumor cells, and the lack of tumor cell infiltration was verified by pathological examination. All tissues were immediately snap frozen in liquid nitrogen, and stored at -80°C until RNA extraction. This study was approved by the Ethics Committee of Jilin University, and written informed consent was obtained from all study participants.

Cell culture. The lung cancer cell lines (A549, H1299, SPCA1 and H358) and normal lung cells (BEAS-2B) used in this study were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). All cell lines were cultured in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) with 10% fetal bovine serum (HyClone, Logan, UT, USA) and penicillin (100 U/ml). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

miRNA real-time RT-PCR analysis. Total miRNAs of the cultured cells, 40 surgically resected NSCLC tissues and the corresponding adjacent tissues were extracted using the miReasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity and concentration of the RNAs were determined by using a dual-beam ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). cDNA was synthesized with 5 ng of total RNA using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. All PCR reactions were detected by ABI 7900 Fast system (Applied Biosystems). Primers for miR-154 and U6 were obtained from GeneCopoeia (Carlsbad, CA, USA). The expression levels of U6 were used as the internal control. The relative quantification of miR-154 was presented as the fold-change after normalization to U6 RNA for the equation 2^-ΔΔCt in the Rotor-Gene 6000 Series software 1.7 (Qiagen).

Transient transfection. Oligonucleotide negative control (miR-control) and miR-154 mimics (hsa-miR-154 mimics) were purchased from GenePharma (Shanghai, China). A549 cells were seeded into 6-well plates and transfected with miR-154 or miR-control using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) at a final concentration of 100 nM. Transfection efficiency was monitored by qRT-PCR. Three independent replicated experiments were performed for each transfection.

Cell proliferation assay (Cell Counting Kit-8, CCK-8). Cell proliferation assay was performed using the CCK-8 method (Dojindo, Kunamoto, Japan). Briefly, ~6,000 miR-154 mimic or miR-control transfected cells were seeded into 96-well plates and then cultured. The viability of the cells was determined at the indicated times (0, 24, 48 and 72 h) according to the CCK-8 kit manufacturer's instructions. The absorbance of each well was read on a spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA) at 450 nm (A450). Three independent experiments were performed in quintuplicate.

Colony formation assay. Briefly, 24 h after transfection with the miR-154 mimic or miR-control, 1,000 cells were plated into each well of 6-well plates and incubated for 2 weeks at 37°C with 5% CO₂. Then, the cells were washed twice with PBS (0.01 M), with 4% paraformaldehyde for 10 min and counted after staining with 1% crystal violet for 30 min at room temperature. Colony numbers in each assay were quantified by using imaging software (Alpha Innotech, San Leandro, CA, USA) and the percentage of colony formation was calculated by adjusting control cells to 100%. The experiments were carried out at least 3 times.

Cell cycle assay. Briefly, 48 h after transfection with miR-154 mimic or miR-control, A549 cells were harvested and washed twice with PBS, and were fixed in ice-cold 70% ethanol for 48 h, washed by ice-cold PBS, and resuspended in 1 ml of PBS containing 1 mg/ml RNase and 50 µg/ml propidium iodide (PI; Sigma, St. Louis, MO, USA) at room temperature for 30 min. Cells were acquired using a BD LSR II flow cytometer and analyzed using Weasel 3.1 software (both from Becton-Dickinson, San Jose, CA, USA).

Apoptosis assay. Twenty-four hours after transfection, apoptosis in the cultured cells was evaluated by Annexin V staining and PI exclusion using the Annexin V-FITC Apoptosis Detection kit I (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Cells were acquired by a BD LSR II flow cytometer and were analyzed using BD FACSDiva 4.0 software (both from Becton-Dickinson).

In addition, the activities of caspase-3 and -8 were determined as an additional indicator of apoptosis using the caspases colorimetric protease assay kits (Millipore Corporation, Billerica, MA, USA) 48 h post-transfection. The relative caspase activity of the control blank group was referred to as 100%.

Wound-healing assay. To assess the effect of miR-154 on the cell migration of A549 cells, a wound-healing assay was performed. In brief, transfected A549 cells were seeded in a 6-cm dish with 1.5x10⁵ wells/dish and cultured for 24 h. The linear wound of the cellular monolayer was created by scratching the confluent cell monolayer using a plastic pipette tip. The monolayer of scratched cells was washed with PBS to remove debris. The images were respectively captured at 0-24 h after wound creation. All experiments were performed in triplicate.

Invasion assays. The invasion capacity of the A549 cells was performed in vitro using Transwell chambers (Corning,
In briefly, the cells were harvested 48 h post-transfection, and 5x10^4 cells with 200 µl of serum-free RPMI-1640 medium were seeded into the upper chamber of an insert coated with Matrigel (BD Bioscience, San Jose, CA, USA) following the manufacturer’s instructions. RPMI-1640 medium containing 10% FBS was added to the lower chamber. The cells were incubated for 48 h at 37˚C with 5% CO2. Then, the remaining cells on the upper membrane were removed using cotton swabs, whereas those that had invaded through the membrane were fixed in 90% alcohol and stained with 0.1% crystal violet. The number of invasive cells was counted in five randomly selected fields under a light microscope at a magnification, x200 (Olympus, Tokyo, Japan).

Western blot analysis. Proteins were extracted from the cultured cells 48 h after transfection, and were quantitated using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Invitrogen), blocked in 4% dry milk at room temperature for 1 h, and immunostained with primary antibodies at 4˚C overnight using mouse anti-human E-cadherin (1:1,000), N-cadherin (1:3,000), vimentin (1:1,500) and GAPDH (1:5,000) (all from Cell Signaling Technology, Danvers, MA, USA), and then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody at room temperature for 2 h. The blots were detected using a chemiluminescent detection system (Pierce ECL western blotting substrate; Thermo Fisher Scientific) and exposed using the Molecular Imager ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Protein levels were determined by normalization to GAPDH.

Lung cancer xenografts. Thirty female BALB/c mice 5-6 weeks old were provided by the Experimental Animal Center of Changchun Biological Institute (Changchun, China), and were maintained under specific pathogen-free (SPF) conditions. The protocol was approved by the Ethics Committees of Jilin University (Changchun, China).

A total of 2x10^6 stably overexpressing miR-154 or miR-control A549 or untreated A549 cells suspended in 100 µl of PBS were injected into the flanks of mice (n=10), respectively. Tumor volumes were calculated using the following formula: Volume (mm^3) = width^2 x length. Tumors were monitored weekly and extracted and weighed 35 days after inoculation. In addition, part of the tumors tissue was used to measure the miR-154 level by qRT-PCR as described above.

Statistical analysis. Statistical analyses were carried out using GraphPad Prism, version 5 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SD (standard deviation). The differences between the groups were analyzed by Student’s t-test when two groups were compared or by one-way ANOVA when more than two groups were compared. Patient survival curves were estimated by the Kaplan-Meier method. The significance level was set at P<0.05.

Results

Decreased expression of miR-154 in the NSCLC tissues.

To investigate the role of miR-154 in human NSCLC, we first detected the expression of miR-154 in NSCLC patient samples and corresponding adjacent tissues by qRT-PCR. The results showed that miR-154 was significantly decreased in the NSCLC samples compared to that in the normal non-cancerous lung tissue (n=40) (Fig. 1A). Patients were divided into 2 groups based on their miR-154 expression levels: those with less than or equal to the median of the miR-154 expression levels and those with more than the median of the miR-154 expression levels (median: 0.4514). Then the relationship between miR-154 expression levels and clinicopathological characteristics was analyzed. As shown in Table I, age and gender were not associated with the expression of miR-154, while larger tumor size (P<0.001), advanced TNM stage (P<0.001) and metastasis (P<0.001) were significantly associated with low miR-154 expression. These results indicated that miR-154 may be involved in the metastasis of patients with NSCLC.
The expression levels of miR-154 in human NSCLC cell lines A549, H1299, SPCA1 and H358, and normal lung cells (BEAS-2B) were detected by qRT-PCR. It was found that the miR-154 expression in four NSCLC cell lines was clearly downregulated compared to that in the normal lung cells (Fig. 1B). The A549 cell line, which possessed the lowest level of miR-154 expression among the four cell lines, was selected for further study.

We further investigated whether miR-154 expression has prognostic potential for overall survival (OS) of NSCLC patients. The Kaplan-Meier method showed that the survival rate of patients with high miRNA-154 expression was higher than that of patients with low miRNA-154 expression (P<0.001, Fig. 1C).

miR-154 inhibits cell proliferation and colony formation in the A549 cells. To investigate whether miR-154 mediates growth-suppressive effects, we transfected the miR-154 mimic into the A549 cells. Then real-time qRT-PCR was performed to determine whether the miR-154 mimic increased the miR-154 levels in the A549 transfectants. After 48 h of transfection, the miR-154 expression level was significantly increased in the cells transfected with the miR-154 mimic compared with that in cells transfected with the miR-control and the mock cells (P<0.05, Fig. 2A). CCK-8 assay showed that the cells transfected with the miR-154 mimic had a significantly lower proliferative index than cells transfected with the miR-control and the mock cells (P<0.05, Fig. 2B). Accordingly, colony formation analysis showed that the colony-forming efficiency of cells transfected with the miR-154 mimic was significantly lower than that of cells transfected with the miR-control and the mock cells (P<0.05, Fig. 2C).

miR-154 induces G1 cell cycle arrest and cell apoptosis in the A549 cells. To determine the effects of miR-154 on the cell cycle in the A549 cells, FACSScan flow cytometric assays were performed. After transfection with the miR-154 mimic for 48 h, the percentage of cells in the G0/G1 stage was higher than that of the miR-control group and mock group (P<0.05, Fig. 3A).

Next, we also assessed the role of miR-154 in A549 cell apoptosis. Cells transfected with the miR-154 mimic exhibited significantly induced cell apoptosis compared to the cells transfected with the miR-control and mock cells (P<0.05, Fig. 3B). In addition, we also analyzed the effects of miR-154 on caspase-3 and caspase-8 activity in the A549 cells. The activities of caspase-3 and caspase-8 were significantly increased in the cells transfected with the miR-154 mimic when compared with cells transfected with the miR-control and mock cells (P<0.05, Fig. 3C and D). These findings suggested that overexpression of miR-154 induced cell arrest at the G0/G1 stage and apoptosis of A549 cells.
Figure 3. Overexpression of the miR-154 induces cell cycle G1/G0 phase and apoptosis of A549 cells. (A) Analysis of the cell cycle distribution in the A549 cells was performed after transfection with either the miR-154 mimic or miR-control. (B) Cell apoptosis was determined in the A549 cells after transfection with the miR-154 mimic or miR-control. The activities of (C) caspase-3 and (D) caspase-8 were determined after transfection with either the miR-154 mimic or miR-control. *P<0.05 vs. Mock.

Figure 4. Overexpression of miR-154 inhibits cell migration and invasion of A549 cells. (A) Cell migration in the A549 cells was determined by wound healing assay after transfection with either the miR-154 mimic or miR-control. Phase micrographs of cells were captured at 0 and 24 h after monolayer wounding. (B) Relative migration of the A549 cells was quantified. (C) Cell invasion in the A549 cells was determined by Transwell assay after transfection with either the miR-154 mimic or miR-control. (D) Relative invasion of the A549 cells was quantified. *P<0.05 vs. Mock.
miR-154 inhibits cell migration and invasion in the A549 cells. We further studied the effects of miR-154 on cell migration and invasion using a wound healing/Transwell assay in the A549 cells with or without miR-154 mimic transfection. Results of the wound healing assay showed that the A549 cells transfected with the miR-154 mimic showed less migratory ability than that in the miR-control and mock groups at 24 h after wound creation (P<0.05, Fig. 4A and B).

We then investigated the effect of ectopic expression of miR-154 on the cell invasion in the A549 cells. Transwell matrix penetration (coated with Matrigel) assay showed that overexpression of miR-154 significantly inhibited cell invasion relative to the miR-control and mock groups (P<0.05, Fig. 4C and D). These findings demonstrated that miR-154 inhibits the migration and invasive ability of NSCLC cells in vitro.
Overexpression of miR-154 inhibits epithelial-to-mesenchymal transition (EMT). It has been shown that EMT plays a key role in the invasion of various types of cancer cells by the transformation of polarized and adherent epithelial cells into motile and invasive mesenchymal cells. To determine whether miR-154 affects molecular changes typical of EMT in the cell lines, the expression levels of mesenchymal markers, including N-cadherin and vimentin and the epithelial marker, E-cadherin, were examined in the A549 cells by western blot analysis 48 h after transfection with the miR-154 mimic. Upregulation of miR-154 resulted in increased E-cadherin expression, and decreased N-cadherin and vimentin expression (Fig. 5). This finding indicated that miR-154 contributes to the regulation of EMT marker expression in NSCLC cells in vitro.

Overexpression of miR-154 inhibits tumor growth in vivo. As the upregulation miR-154 had a functional role in regulating the NSCLC cells in vitro, we further investigated whether increasing the miR-154 expression has a similar antitumor effect on NSCLC tumor growth in vivo. Mice were transplanted with miR-154 overexpression or miR-control A549 cells, respectively. The tumors were extracted 5 weeks after implantation. The tumors were significantly smaller when miR-154 was downregulated (Fig. 6A). Quantification of the tumor size and weight confirmed that overexpression of miR-154 significantly reduced xenograft tumor volume (Fig. 6B) and tumor weight (Fig. 6C). To determine the miR-154 transfection activity in the nude mouse model, we also determined miR-154 expression in the xenograft tumors by qRT-PCR. The results revealed that miR-154 expression was upregulated in the xenograft tumors of the miR-154 group compared to the xenograft tumors of the miR-control group and untreated group (P<0.05, Fig. 6D). These results imply that upregulation of miR-154 inhibits tumor growth of NSCLC in vivo.

Discussion

Lung cancer is a malignant tumor that seriously threatens human health and affects the lives of millions (20). As estimated by the National Central Cancer Registry of China, in 2010, the lung cancer incidence and mortality were increased to 46.08/100,000 and 37.00/100,000, respectively (21). NSCLC is responsible for almost 80% of lung cancer-related deaths (1,2). To date, the highly complex molecular mechanisms underlying NSCLC carcinogenesis and progression remain relatively unclear. Therefore, it is of great significance to investigate the molecular and cellular mechanisms of lung cancer, which contribute to the identification of novel genetic or protein markers for accurate diagnosis and prediction of prognosis. In the present study, we firstly observed that miR-154 was downregulated in NSCLC compared with the adjacent non-cancerous tissues. Then, we also found that miR-154 expression was significantly correlated with aggressive clinicopathological features, such as metastasis (P<0.001), larger tumor size (P<0.001) and advanced TNM stage (P<0.001). Moreover, Kaplan-Meier analysis revealed that NSCLC patients with low miR-154 expression tended to have reduced OS. Finally, we found that upregulation of miR-154 expression in the A549 cells was able to reduce cell proliferation, invasion, and migration, and induce cell apoptosis and cell cycle arrest at the G0/G1 stage, as well as suppress tumor growth in vivo. To our knowledge, this is the first report regarding the clinical significance and functional attributes of miR-154 in NSCLC.

It has been shown that miR-154 acts as a tumor-suppressor in several types of cancer. Zhu et al demonstrated that forced expression of miR-154 suppressed prostate cancer cell proliferation, colony formation, invasion and migration, and promoted cell apoptosis (17,18). Subsequently, Gururajan et al demonstrated that elevated expression of miR-154 was observed in bone metastatic prostate cancer cell lines and tissues, and intracardiac inoculation (to mimic systemic dissemination) of miR-154 inhibitor-treated bone metastatic ARCaPM prostate cancer cells in mice led to decreased bone metastasis and increased survival (22). Xin et al showed that miR-154 was decreased in colorectal cancer (CRC) tissues and cell lines, and ectopic expression of miR-154 markedly suppressed cell proliferation and colony formation, migration and invasion in CRC cells (15). Consistent with these results, our study showed that overexpression of miR-154 markedly suppressed tumor growth of NSCLC in vitro and in vivo, suggesting that miR-154 exhibits a tumor-suppressor function in NSCLC.

It has been demonstrated that EMT plays an important role in the differentiation of multiple tissues and organs and in embryonic development as well as in the metastasis of cancer cells (23). EMT is characterized by a loss of cell-cell contact through the inhibition of epithelial markers, such as E-cadherin expression, and the acquisition of mesenchymal features via upregulation of mesenchymal markers, such as N-cadherin and vimentin (24,25). In NSCLC, it has been shown that restoration of E-cadherin expression markedly decreased the invasion/migration of tumors (26), while inhibition of N-cadherin reduced the proliferation and invasion of NSCLC (27). Importantly, recent studies showed that ectopic expression of miR-154 decreased the migratory and invasive capabilities of prostate cancer cells in vitro by regulation of EMT (17,22). In the present study, our results showed that upregulation of miR-154 resulted in increased E-cadherin expression and decreased N-cadherin and vimentin expression, suggesting that miR-154 can regulate EMT in NSCLC cells.

Recent research has identified several oncogenes as direct targets of miR-154, such as CCND2 (18), HMGA2 (17), TLR2 (15) and STAG2 (22). It is known that miRNAs execute their oncogenic or tumor-suppressive functions by regulating the expression of target genes. It has been reported that an average miRNA can have more than 100 targets (28). Therefore, we propose that miR-154 is involved in NSCLC progression via the regulation of multiple target genes.

In conclusion, our findings revealed that miR-154 expression was decreased in the NSCLC tissues and cell lines, and its expression level was correlated with TNM stage, tumor size, metastasis, and reduced overall survival. In addition, we also found that overexpression of miR-154 suppressed tumor growth of NSCLC in vitro and in vivo. Based on the multiple functions of miR-154 in the tumor growth of NSCLC, miR-154 may be considered as a diagnostic marker of NSCLC or a potential anticancer therapeutic target for NSCLC.
References


