Upregulation of microRNA-450 inhibits the progression of lung cancer *in vitro* and *in vivo* by targeting interferon regulatory factor 2

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Abstract. MicroRNAs (miRNAs) are a class of non-coding RNAs that play pivotal roles in human lung cancer development. The majority of studies have focused on either non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC). In the present study, we investigated a plausible mechanism of action of miR-450 in these types of lung cancer. We found that the level of miR-450 was decreased in lung cancer cell lines, as well as in solid tumors. As exemplified in the H510A (SCLC) and H2291 (NSCLC) cells, transfection with lentivirus carrying miR-450 upregulated miR-450 expression and significantly attenuated lung cancer cell proliferation and invasion, as well as the growth of implanted tumors. Interferon regulatory factor 2 (IRF2) was also verified to be a direct target of miR-450 in lung cancer cells. The overexpression of IRF2 in the H510A and H2291 cells abrogated the inhibitory effects of miR-450 on lung cancer cell proliferation and invasion. Taken together, in this study, we identified a novel role of miR-450 in lung cancer. miR-450 targets IRF2 and thus suppresses lung cancer cell proliferation and invasion.

Introduction

Lung cancer has been reported to be among the most deadly cancers worldwide (1). Non-small-cell lung cancer (NSCLC) accounts for most primary lung cancer (2). Instead, the small-cell lung cancer (SCLC) is a distinct form of lung cancer with unique clinical and histological characteristics, representing ~15% of all new cases of lung cancer (2). The reported prognosis and five-year survival rates are terribly poor (1). In China, it has been shown that approximately over half a million people died from lung cancer each year (3).

The microRNAs (miRNAs) denote myriads of noncoding RNAs with short length which suppress gene expression by base paring to the 3'-untranslated region (3'-UTR) of targets (4,5). The miRNAs can either exist individually or as a cluster in the whole genome. Meanwhile, they can also reside in introns or exons of functional genes (6). To date, more than one thousand miRNAs have been defined. They can regulate multiple pathways associated with cancer by binding numerous target mRNAs (7). Although the detailed clues for the exact molecular mechanisms underlying lung cancer cell proliferation or metastasis are still elusive, recent researches have implied that miRNAs may play pivotal roles in lung cancer progression (6,8,9). For instance, miR-451 was shown to be lowered in NSCLC tissues while raised levels of miR-451 are capable of suppressing NSCLC proliferation by inhibiting ras-related protein 14 (RAB14) and triggering apoptosis (10). miR-31 was found to be an oncogenic miRNA through promoting NSCLC growth and repressing tumor suppressor genes such as large tumor suppressor 2 (LATS2) and PP2A regulatory subunit B α isoform (PPP2R2A) (11). Instead, miR-25 was reported to regulate SCLC progression by targeting CDK2 (12). The miR-34b/c has also been suggested to play an oncogenic role in SCLC (13). However, whether a single microRNA can play a role in both NSCLC and SCLC is largely elusive.

The interferon regulator factor 2 (IRF2) is a member of the interferon regulatory family and participates in various pathways related to tumorigenesis via modulating target gene expression (14). IRF2 was also reported to be the functional antagonist for IRF1 and regulate those genes with interferon regulatory element binding sites either as positive or negative factors (15). IRF2 has recently become an active area of research owing to its role in tumor progression (16,17). For example, IRF2 can inhibit p21 and promote proliferation of cells as shown in leukemogenesis (18). In some type of cancer cells, aberrant expression of IRF2 was correlated with tumor growth, invasion and stage (19). Meanwhile, reducing IRF2 can decrease the expression of cyclin D1 while elevating IRF2 expression can enhance the malignancy of ESCC cells (19,20). Some studies have implicated the role of IRF2 in tumor progression such as in pancreatic cancer and breast cancer although the exact molecular mechanisms remain to be clarified (21).
In this study, we first showed that downregulating miR-450 in both NSCLC and SCLC cells and human tumors. We then explored the functional roles of miR-450 in shaping malignant phenotypes of NSCLC such as proliferation and invasion. Furthermore, we investigated the targeting effect of miR-450 on IRF2 and direct regulatory roles of IRF2 in miR-450 induced NSCLC and SCLC growth inhibition. Our present study may extend current understanding on the epigenetic regulation in human lung cancer.

Materials and methods

Lung cancer cells and human samples. There were 7 lung cancer cell lines used in this study, H1703, SPC-A1, H510A, H1299, H920, H522 and H2291. The SPC-A1 cell line was purchased from the Shanghai Institute of Cell Biology (Shanghai, China). The other cell lines were all commercially available from the American Type Culture Collection (ATCC, Shanghai, China). All the cell lines were NSCLC, apart from H510A, which was SCLC. A control cell line, normal human bronchial epithelial cell line (NuLi-1) was obtained from ATCC. All the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 150 U/ml penicillin plus 150 µg/ml streptomycin (all from Sigma, Shanghai, China) in a culture chamber with 5% CO₂ at 37°C. Human lung cancer samples were surgically retrieved from patients registered at Shanghai General Hospital between October 2014 and April 2015. The tumor (T) tissues and corresponding adjacent non-tumor (ANT) tissues were all paired samples. All patients provided written informed consent for the use of their samples. All surgical and experimental procedures related with human subjects were approved by the Human Research and Ethics Committee of Shanghai General Hospital, Shanghai Jiaotong University School of Medicine.

Reverse transcription-quantitative RT-PCR (RT-qPCR). To evaluate the expression of miR-450 and IRF2 in lung cancer, RT-qPCR was carried out. In brief, total RNA was extracted from both the NSCLC and SCLC cell lines, and human samples using TRIzol reagent (Sigma), and reverse transcribed into cDNA using a SYBR Premix Ex Taq™ kit (Takara Bio, Inc., Shiga, Japan) following the manufacturer's instructions. To detect the gene for miR-450, a TaqMan miRNA qRT-PCR kit (Applied Biosystems, Foster City, CA, USA) was applied. For quantifying IRF2 mRNA, a SYBR-Green PCR Master mix kit (Applied Biosystems) was used. In both cases, GAPDH was used as an internal control. All reactions were performed using an ABI PRISM® 7000 Sequence Detection system (Applied Biosystems) following the manufacturer's instructions. Relative expression levels were shown which denote the fold change relative to the levels under control conditions.

MicroRNA-450 transfection. We used a lentiviral transfection system to ectopically upregulate miR-450 in the SCLC and NSCLC cell lines (H510A and H2291 cells, respectively). The lentivirus covering human miR-450 mimics (Lenti-miR-450), or a negative control miRNA (Lenti-C) were purchased from Sigma. The sequences were as follows: miR-450 sense, 5'-TTTTTGCCAGTGTTCTTCAATG-3' and antisense, 5'-GATATGCCACGGGTAGATT-3'; Lenti-C sense, 5'-CTC GCTTCTCGAGCACA-3' and antisense, 5'-AACGCTTTA CGAAGGTTCGT-3'. The transduction of lentivirus into the H510A and H2291 were performed using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h, the cultured medium was removed and RT-qPCR was used to confirm the efficiency of transfection.

Cell proliferation assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay (Sigma) was utilized to examine cell proliferation. In brief, 24 h following transfection, the H510A and H2291 cells were all suspended and plated in 96-well plates (10⁴ cells/well) for 5 days. Subsequently, 20 µl MTT solution (5 mg/ml) were added to the culture every 24 h and maintained for 4 h. Crystalline formazan was dissolved in 150 µl sodium dodecyl sulfate sodium salt (SDS, 15%) solution for 24 h. The optical density (OD) at 490 nm was evaluated using a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) following the instructions provided by the manufacturer.

Cell invasion assays. Chemotaxis 96-well Transwell assay (Qiangen, Inc., Valencia, CA, USA) was utilized to evaluate cell invasion. The upper chamber was first coated with Matrigel (Invitrogen, Shanghai, China) overnight. The lentivirus-transfected H510A or H2291 cells were suspended and plated into the upper chamber (10⁴ cells/well) in RPMI-1640 medium (Sigma) without serum. The lower chambers were filled with RPMI-1640 medium plus additional 2% fetal bovine serum (FBS). After 24 h of incubation, all upper chambers were replenished and the cells migrating into lower chambers were fixed with 5% PFA and immunostained with crystal violet. The results of Transwell assay were subsequently quantified using a Leica inverted microscope fluorescent microscope (DM-IRB; Leica Microsystems GmbH, Wetzlar, Germany). The invasive capability was determined by counting the total number of invaded cells under each experimental condition and normalized to the number for the control condition.

In vivo implantation of tumors. The H510A cells were transduced with the lentiviruses for 8 h and then cultured for an additional 24 h. The cells were then re-suspended and 10⁶ cells were implanted subcutaneously into the rear flanks of nude mice. The nude mice were obtained from Model Animal Research Center (Nanjing, China). In total, 12 mice (age, 4-6 weeks; average weight, 16.1 g) were used in this study (i.e., 6 in the Lenti-C and 6 in the Lenti-450 group). The animal experiments and procedures were approved and reviewed by the Ethics Committee of Shanghai General Hospital, Shanghai Jiaotong University School of Medicine. The in vivo volume of tumors was determined weekly (length x width x height). After 6 weeks, all mice were sacrificed by an overdose of sodium amobarbital for Ki-67 immunostaining (Sigma).

Dual-luciferase reporter assay. To identify potential targets of miR-450, we utilized several miRNA Targets prediction tools, such as TargetScan (www.targetscan.org) PicTar (pic.tar.mdc-berlin.de) and miRDB (www.mirdb.org). The IRF2 gene was amplified from a human lung cDNA library and verified. The 3'-UTR of IRF2 with predicted binding sites to hsa-miR-450 was cloned into the XbaI immediately downstream of Renilla luciferase reporter.
luciferase reporter plasmid phRL-TK (Promega, Madison, WI, USA) leading to the wild-type IRF2 luciferase reporter plasmids (IRF2 3' UTR WT). The binding site of hsa-miR-450 on the IRF2 3'-UTR was also mutated by a Quick-Change™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutated IRF2 3'-UTR was then incorporated into phRL-TK to create the mutated IRF2 luciferase reporter plasmid (IRF2 3' UTR MUT). In 293T cells (obtained from the Shanghai Institute of Cell Biology), transfection with Lenti-miR-450 with IRF2 3' UTR (WT), IRF2 3' UTR (MUT) or an empty Renilla luciferase reporter plasmid (control) was performed for 36 h. The relative luciferase units (RLU) were then measured by a dual-luciferase reporter assay (Promega) following the manufacturer's instructions.

**Western blot analysis.** The H510A and H2291 cells were harvested with cell lysis buffer containing 15% glycerol and 2% (Sigma). The protein extracts (100 µg each) were dissolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were then incubated with antibodies against human IRF2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4˚C overnight, and HRP-conjugated secondary antibodies (1:103) at 20˚C for 1.5 h. β-actin was used as an internal control. The blots were visualized with an enhanced chemiluminescence film system (Amersham Pharmacia Biotech, Shanghai, China).

**IRF2 overexpression assay.** Whole sequences of IRF2 were cloned into a recombinant plasmid eukaryotic expression plasmid pcDNA3.1 (Sigma) to create the IRF2 overexpression plasmid, pcDNA3.1/IRF2, following the manufacturer's instructions. The transfection of pcDNA3.1/IRF2 and an empty pcDNA3.1 plasmid pcDNA3.1(+) into H510A and H2291 cells were performed with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were re-suspended and plated in 96-well plates.

**Statistical analysis.** All experiments were performed at least in triplicate. The results are presented as the mean ± standard error. Survival was evaluated with Kaplan-Meier curves and compared using a log-rank test. Statistical differences were measured using a Student's t-test using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and a value of P<0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-450 expression is decreased in lung cancer cells and correlates with survival.** In the present study, we first used RT-qPCR to demonstrate the downregulation of miR-450 in established lung cancer cell lines in vitro, as well as in human samples in vivo. The results revealed that miR-450 was downregulated in the 7 lung cancer cell lines examined, H510A, H1703, SPC-A1, H522, H1299, SK-MES-1, H920 and H2291 compared with the normal human bronchial epithelial cell line (NuLi-1) (Fig. 1A; P<0.01). We also demonstrated that miR-450 expression was significantly decreased in the tumor (T) tissues than in the corresponding non-tumor (ANT) tissues in the 11 patients with lung cancer (Fig. 1B; P<0.01). In addition, we also found that the intrinsic expression of miR-450 exhibited a significant correlation with the survival rates of the patients with lung cancer (P=0.004; Fig. 1C). These results suggest that miR-450 is frequently downregulated in lung cancer and that its downregulation correlates with poor survival.

**Upregulation of miR-450 inhibits lung cancer cell invasion and proliferation.** We transfected the H510A and H2291 cells with lentiviruses in order to increase the expression of miR-450. After 24 h, the transfection efficiency was verified by RT-qPCR. The results revealed that the expression level of miR-450 was significantly increased by transfection with lentivirus containing miR-450 mimics (Lenti-miR-450) in both the H510A and H2291 cells compared with the control (Lenti-C) (Fig. 2A; P<0.05).
The H510A and H2291 cells transfected with lentivirus were re-suspended and plated in a 96-well plate for 120 h. Cell proliferation assay was carried out every 24 h to determine the effects of miR-450 upregulation on lung cancer cell growth. The results revealed that the upregulation of miR-450 significantly attenuated lung cancer cell proliferation in both the H510A and H2291 cells (Fig. 2B; P<0.05). We also used a Transwell assay to examine the effects of miR-450 upregulation on lung cancer cell invasion. The results revealed that transfection with miR-450 mimics markedly reduced cell invasion (Fig. 2C, left panel). Quantification also confirmed that the upregulation of miR‑450 reduced the invasive capabilities of the H510A and H2291 cells by >50% (Fig. 2C, right panel; P<0.05). Taken together, these data suggest that the upregulation of miR-450 inhibits the proliferation and invasion of lung cancer cells.

Upregulation of miR-450 inhibits the growth of implanted lung tumors in vivo. As we found that the upregulation of miR-450 inhibits lung cancer cell, we wished to determine whether miR-450 has a similar effect on lung tumor growth in vivo. Thus, we transfected the H510A cells with Lenti-miR-450 or Lenti-C for 24 h. The cells were then re-suspended (the final cell number is 10^6) and implanted subcutaneously into the rear flanks of nude mice. The sizes of the lung tumors, including length, width, and height, were measured each week and the total tumor volumes were determined. The results revealed that the growth capacity of the implanted lung tumors was significantly attenuated by the upregulation of miR-450 (Fig. 3A; P<0.05). Six weeks after implantation, the lung tumors were extracted and Ki-67 immunostaining was then performed. The results revealed
that Ki-67 staining was substantially decreased with the upregulation of miR-450 (Fig. 3B).

miR-450 directly targets IRF2 in NSCLC. To identify potential targets of miR-450, we utilized several miRNA/targets prediction tools, such as TargetScan (www.targetscan.org) PicTar (pictar.mdc-berlin.de) and miRDB (www.mirdb.org) (Fig. 4A). The cross-verification of several databases indicated that the oncogene, IRF2, may be a promising candidate (Fig. 4A and B). We then performed dual-luciferase reporter assays using the 293T cells. The results revealed that miR-450 can target luciferase plasmids with an intact or WT IRF2 3' UTR and significantly reduced the RLU, whereas miR-450 had little effect on the luciferase plasmid containing a mutation (MUT) in the IRF2 3' UTR (Fig. 4C; P<0.05). We then paid more attention to two established NSCLC cell lines, H510A and H2291 cells, using western blot analysis and RT-qPCR to evaluate the profiles of IRF2 in the cells transfected with Lenti-miR-450. The results revealed that in both the H510A and H2291 cells, the protein level of IRF2 was markedly downregulated by the upregulation of miR-450 (Fig. 4D). The results of RT-qPCR confirmed this finding and showed that the expression of IRF2 in the H510A and H2291 cells was also downregulated by the upregulation of miR-450 (Fig. 4E; P<0.05). Taken together, our data from dual-luciferase reporter assay, western blot analysis and RT-qPCR suggest that IRF2 may be the direct downstream target of miR-450 in both NSCLC and SCLC cells.

Upregulation of IRF2 restores the malignant phenotypes of lung cancer cells following transfection with miR-450 mimics. We then conjectured that IRF2 may directly affect the miR-450-mediated inhibition of lung cancer cell proliferation and invasion. To verify this hypothesis, we constructed a mammalian plasmid to ectopically overexpress IRF2 (pcDNA3.1/IRF-2) in the H510A and H2291 cells. The efficiency of transfection was confirmed by RT-qPCR (Fig. 5A; P<0.05). We then performed a dual transfection assay. First, the H510A and H2291 cells were transfected with Lenti-miR-450.
After 24 h, the cells were transfected with either pcDNA3.1/IRF2 or the empty control plasmid pcDNA3.1(+) for an additional 24 h. A 5-day proliferation assay was then performed. The results revealed that IRF2 overexpression effectively restored lung cancer cell proliferation, which was initially attenuated by the upregulation of miR-450 in both the H510A and H2291 cells (Fig. 5B; P<0.05). Transwell assay also revealed that IRF2 overexpression significantly increased the number of invading cells into the lower chambers for both the H510A and H2291 cells (Fig. 5C, left panel). The invasive capability which was originally inhibited by miR-450 upregulation, was elevated by >200% with the overexpression of IRF2 (Fig. 5C, right panel; P<0.05). Therefore, all these results suggest that IRF2 directly mediates the regulatory effects of miR-450 on the proliferation and invasion of both NSCLC and SCLC cells.

Discussion

The miRNAs are small and non-coding RNAs, which serve as key regulators of gene expression by directly targeting mRNAs for translational repression. Deregulated miRNAs have been reported to be associated with the malignant progression of numerous types of cancer (22). These miRNAs may play regulatory and pivotal roles in the pathogenesis of
The role of miRNAs in lung cancer development has been an active area of research. However, the majority of studies have mainly focused on either SCLC or NSCLC. In a previous study, Du et al argued that miR-337-3p can sensitize NSCLC cells to taxanes by targeting STAT3 and RAP1A, whereas the inhibition of miR-139-5p decreases SCLC cell viability through an unknown target (29). Furthermore, a recent miRNA profiling study aimed to distinguish NSCLC from SCLC by identifying unique miRNA expression patterns (30). However, few reports have investigated whether a single miRNA species can play a concordant role in both NSCLC and SCLC. In the current study, we found that miR-450 can mediate the tumor suppression of both SCLC and NSCLC cells by targeting IRF2. The overexpression of miR-450 inhibits the proliferation of both H510A and H2291 cell lines. Therefore, our study may instead unravel a novel facet of miRNA regulation in cancer development.

We further demonstrated that the transcription factor, IRF2, serves as a direct molecular target of miR-450 (Fig. 4). Upon miR-450 upregulation, not only lung cancer cell proliferation or invasion was inhibited, but IRF2 was also downregulated. That prompted us to further investigate whether IRF2 is directly involved in the regulation of miR-450 in lung cancer. Our IRF2 overexpression experiment partially confirmed this hypothesis, showing that the miR-450-induced inhibition on lung cancer cell proliferation and invasion was substantially restored by IRF2 overexpression. IRF1 and IRF2 have been shown to be upregulated by type I and type II interferons (31). In return, IFN-α and -β can also be regulated by IRF family members (31). IRF1 has been reported to inhibit viral infection, particularly HCV (32). However, IRF2 was identified as a transcriptional repressor and can compete with the role of IRF1 (33). More importantly, IRF2 can also function as an oncogenic regulator. The deregulation of IRF2 has been reported in numerous tumor types. For example, Cui et al demonstrated that IRF2 was significantly upregulated in primary pancreatic cancer cells and correlated with a poor survival (34). Connett et al further argued that the level of IRF2 was maintained in tumor tissues and was associated with malignancy (35). In addition, it was also shown that IRF2 is involved in the negative feedback in the IFN-γ pathway and has been implicated in tumor progression (36). In a more recent study, IRF2 was directly implicated in gastric cancer by modulating p53 expression (16). Therefore, miR-450 may target a universal oncogenic factor and exert its tumor suppressive role. Whether miR-450 is involved in other signaling pathways and plays undetermined roles remains to be clarified in future studies.

In conclusion, in this study, we established for the first time, to the best of our knowledge, a functional association between miR-450 and human lung cancer. The upregulation of miR-450 expression exerts tumor suppressive effects on lung cancer. IRF2 was identified as the downstream target of miR-450 in lung cancer as it can counteract the effects of miR-450-induced tumor inhibition. The miR-450 and IRF2 signaling pathway should be further investigated in depth to uncover the epigenetic regulation of miRNAs in human lung cancer. This may help establish more elaborate targets in tumor intervention.

References


