

Original Article

MicroRNA-140-3p inhibits proliferation, migration and invasion of lung cancer cells by targeting ATP6AP2

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Abstract: MicroRNAs are small noncoding RNA molecules that regulate gene expression at the post-transcriptional level. Compelling evidence reveals that there is a causative link between microRNAs deregulation and lung cancer development and metastasis. The aim of present study was to explore the function of miR-140-3p in the development and metastasis of lung cancer cell. Using real-time PCR, we detected the miR-140-3p expression of lung cancer tissues and its paired non-lung cancer tissue. Then, we evaluated the role of miR-140-3p in cell proliferation, invasion and migration using MTT, colony formation assay, Transwell invasion and Transwell migration assay in lung cancer cell lines. As a result, miR-140-3p expression level was lower in lung cancer tissues compared to adjacent normal lung cancer tissue. After miR-140-3p was upregulated in A549 or H1299 cells, cell proliferation, invasion and migration was notably attenuated. Furthermore, we identified ATP6AP2, which is associated with adenosine triphosphatases (ATPases), was a directly target of miR-140-3p in lung cancer cells. In conclusion, our data suggest miR-140-3p/ATP6AP2 axis might act as a potential therapeutic biomarker for lung cancer.

Keywords: Lung cancer, miR-140-3p, ATP6AP2, proliferation, invasion

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, and approximately 80% of lung cancers are classified histopathologically as non-small cell lung cancers. It has a high mortality and the five-year survival rate is about 15%, threatening the human health [1, 2]. The majority of patients have developed an aggressive form of the disease by the time of diagnosis, so the disease is often at late stage and even metastasizes to other organs, seriously troubling the doctors. So it is high time that to explore novel biomarkers for early detection and diagnosis.

microRNAs (miRNAs) are a classes of small, non-coding, endogenous single RNA molecules that play important roles in gene expression through binding to the 3'UTR of target gene mRNA, leading to mRNA cleavage or translational repression [3]. Numerous of studies show

that miRNAs participate in various biological processes, such as cell differentiation, cell growth, death and timing development [4, 5]. Aberrations in expression of microRNA are associated with different diseases like cancer. To the present, about 50% of miRNAs are located in cancer-related gene region or fragile region [6, 7]. For example, microRNA-99b acts as a tumor suppressor in non-small cell lung cancer by directly targeting fibroblast growth factor receptor 3 [8]. MicroRNA-7 Inhibits the Growth of Human Non-Small Cell Lung Cancer A549 Cells through Targeting BCL-2 [9]. Peng Guan et, al. using Meta-analysis for miRNA expression profiling between lung cancer tissues and normal tissues, they suggested that many miRNAs are deregulated in lung cancer, including miR-140 (miR-140-5p) and miR-140-3p [10]. Yuan et, al. have demonstrated suppresses tumor growth and metastasis of non-small cell lung cancer by targeting insulin-like

growth factor 1 receptor [11], however, the potential roles of miR-140-3p in lung cancer cells are still unclear.

In this study, we validated miR-140-3p was down regulated in lung cancer tissues compared to its paired adjacent normal tissues. And we also suggested that miR-140-3p played anti-proliferation and anti-metastasis roles in A549 and H1299 cells. We further identified a novel target of miR-140-3p, which would help to explore the regulation mechanism of miR-140-3p in lung cancer.

Materials and methods

Lung cancer tissue

Paired lung cancer and adjacent non-tumor lung tissues were obtained from 9 consecutive patients who underwent primary surgical resection of lung cancer with informed consent at the first hospital of Shanxi medical university, China. Surgically laser capture micro-dissected lung cancer and adjacent normal tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until total RNA was extracted.

Cell culture and transfection

All of the human lung cancer cell lines were cultured in RPMI1640 medium, supplemented with 10% fetal bovine serum (FBS) and 1% PS (100 U/ml penicillin, 100 µg/ml streptomycin). The cells were maintained in a humidified incubator with 5% CO₂ at 37°C. The miR-140-3p mimics (Sequence: 5'-uaccacagguagaaccacgg-3'), mimics control, were purchased from GenePharma Company (Shanghai, China). All the transfection was performed using LipofectamineTM 2000 (Invitrogen, USA) according to the manufacturer's protocol.

RNA isolation and real time PCR

The total RNA (including miRNA) was extracted by Trizol (Invitrogen, USA) according to the manufacturer's instructions. For miRNA reverse transcription (RT), special miR-140-3p RT primer was: 5'-gtcgtatccagtgcagggtccgaggtattcg-cactggatacgaccctgg-3' used and RNU6B (U6 small nuclear B non-coding RNA) was used as an internal control, while for the RT of total RNA, oligo (dT) was used as a common primer and GAPDH was used as an internal control.

The real time PCR was performed by the SYBR Green PCR master Mix (Applied Biosystems, USA) according to the following conditions: 95°C for 5 min followed by 40 cycles of amplification at 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec.

MTT assay and colony formation assay

The transfected cells were plated into 96-well plates at a density of 5000 cells/well. At 48 h after transfection, the cells were incubated with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h at 37°C. Then the cells were agitated with MTT solvent on an orbital shaker for 10 min avoiding light. The absorbance at 570 nm was measured using a Quant Universal Microplate Spectrophotometer (BioTek, Winooski, VT). For the colony formation assay, the cells were seeded into 12-well plate at a density of 200 cells per well after transfection. The medium was changed every three days. Approximately 10 days later, most of the cell clones contained more than 50 cells. The clones were washed with 1×PBS and stained with crystal violet for about 5 min. Finally the clones were taken pictures and counted. The colony formation rate = (number of clones)/(number of seeded cells) ×100%.

Transwell migration and invasion assays

The migration and invasion assays were performed using Transwell chamber (PIEP12R48, millipore, USA). For migration assay, the transfected cells were seeded into the upper chamber with serum-free medium (2.5×10⁴ cells), and the bottom of the chamber contained the RPMI1640 medium with 10% FBS. While for invasion assay, the chamber was coated with Matrigel, and the following steps were similar to migration assay. When the cells migrated or invaded for 20 h, the cells were fixed and stained with crystal violet. Then the migratory or invading cells were taken pictures under microscope (Hanrong company, Shanghai) and counted.

miRNA expression profile and target prediction

miRDB, mirorna.org, and Targetscan were used to predict the putative targets of miR-140-3p. And the expression of miR-140-3p was scanned from <http://mirnamap.mbc.nctu.edu.tw/>.

MicroRNA-140-3p targets ATP6AP2 in lung cancer

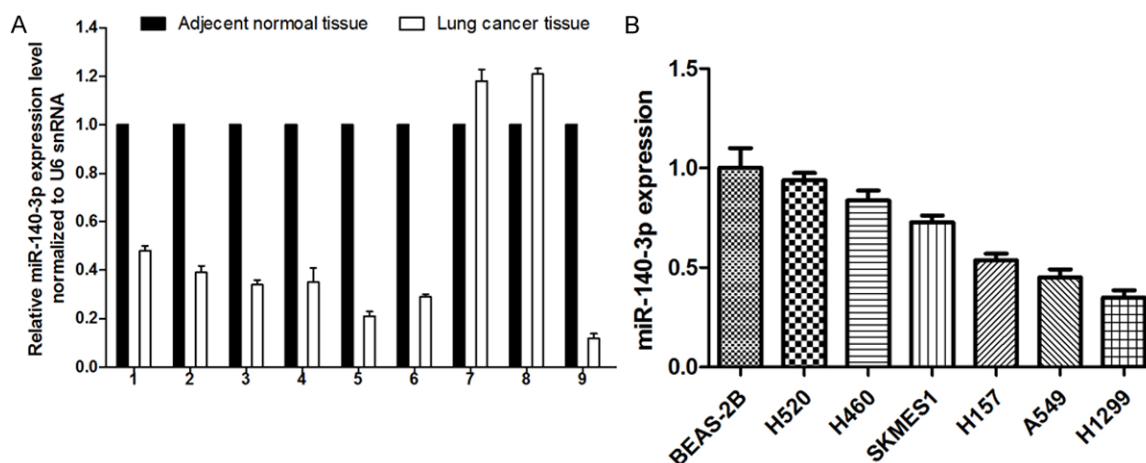


Figure 1. The expression of miR-140-3p is down-regulated in lung cancer. A. Quantitative analysis of the expression levels of miR-140-3p normalized to those of U6 by qRT-PCR in nine lung cancer tissues and its paired adjacent normal tissues. * $P < 0.05$. B. Quantitative analysis of the expression levels of miR-140-3p normalized to those of U6 by qRT-PCR in six lung cancer cell lines.

Fluorescent reporter assay

A549 cells or H1299 cells were cotransfected with miR-140-3p mimics or mimics control in a 48-well plate followed by the pGL3/Luciferase-ATP6AP2-3'UTR or pGL3/Luciferase-ATP6AP2-3'UTR mut. The cells were lysed 72 h later, and the proteins were harvested. The fluorescence intensities were detected with the F-4500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan).

Western blot

The cells were harvested at 48 h after transfection and lysed by RIPA buffer for 30 min at 4°C. 20 µg protein was loaded into 15% SDS-PAGE for analysis. The first antibody was rabbit polyclonal anti-ATP6AP2 (Sanying company, Wuhan, 1:100 dilution) and anti-GAPDH antibody (Number: ab9485, Abcam, USA, 1:1000 dilution). The secondary antibody was goat anti-rabbit IgG conjugated with HRP (horseradish Peroxidase) with a dilution of 1:1000. The bound antibodies were detected using ECL Plus Western Blotting Detection system (GE Healthcare). GAPDH was used as an internal control to normalize ATP6AP2 expression level.

Statistical analysis

All the data were shown as mean + SD and each group had a triplicate. The difference was determined by two-tailed students't-test and

$P < 0.05$ was considered statistically significant. * $P < 0.05$.

Results

miR-140-3p is down-regulated in human lung cancer specimen and cell lines

To explore the possible role of miR-140-3p in lung cancer development, we tested miR-140-3p expression in lung cancer tissues obtained from 9 patients by qRT-PCR. As shown in **Figure 1A**, 7 of 9 of carcinoma tissues showed reduced miR-140-3p expression with respect to normal counterparts. Then, we detected the expression of miR-140-3p in six lung cancer cell lines. As shown in **Figure 1B**, the relative expression levels for miR-140-3p in these NSCLC cells were significantly decreased compared with that of the normal cell line BEAS-2B., which is consistent with the specimen results. Together, these results suggest that miR-140-3p plays an important role in lung cancer development.

miR-140-3p inhibits proliferation of lung cancer cells

To determine the role of miR-140-3p in proliferation of lung cancer cells, MTT assays were performed, as showed in **Figure 2B**. Firstly, we used real time PCR to detect the efficient of miR-140-3p mimics in A549 and H1229 cells. As shown in **Figure 2A**, miR-140-3p mimics

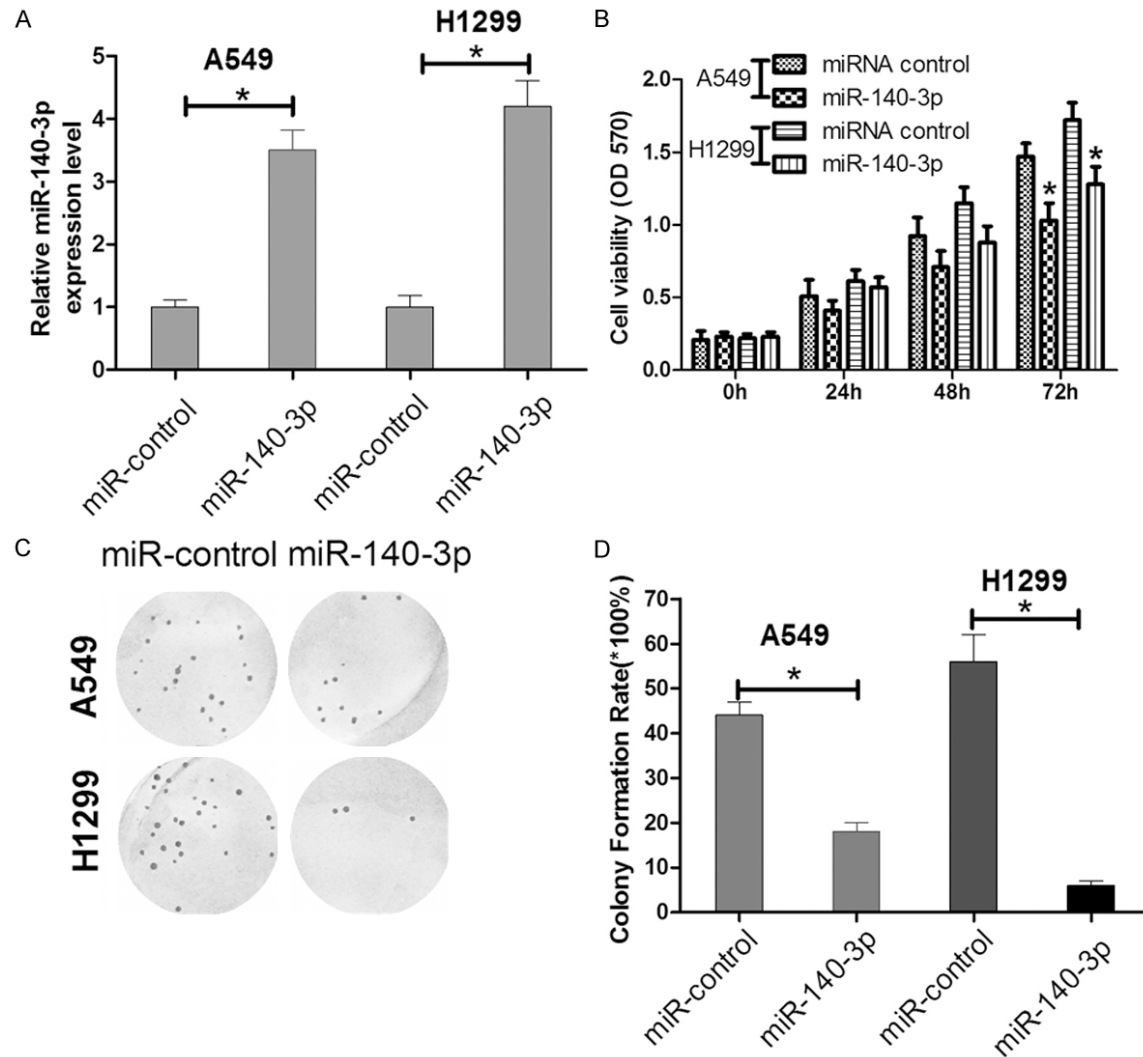


Figure 2. miR-140-3p inhibits proliferation of lung cancer cells. A. miR-140-3p effectively increasing miR-140-3p expression level, determined by real time PCR in A549 and H1299 cell lines, normalized to U6 RNA. B. Overexpression of miR-140-3p by miR-140-3p mimics affects the cell viability of A549 and H1299 cells determined by MTT assay at 24 h, 48 h and 72 h after transfection. All the histograms show normalized mean of OD570 from three independent experiments. *P<0.05. C, D. Overexpression of miR-140-3p by miR-140-3p mimics affects the growth capacity of A549 and H1299 cells determined by colony formation assay. All the histograms show normalized mean percentages of colony formation \pm SD from three independent experiments. *P<0.05.

could unregulate the expression of miR-140-3p about 3 and 4 times in the two lung cancer cell lines. Colony formation assays were used to further elucidate the effect of miR-140-3p on the growth of lung cancer cells. The colony formation rate of A549 cells transfected with miR-140-3p was reduced about 50% over that of the control group (Figure 2C, 2D). The same phenomenon was observed in H1299 cells. These results revealed that miR-140-3p inhibits the proliferation of human lung cancer cell lines.

miR-140-3p overexpression inhibits cell migration and invasion in A549 and H1299 cells

Migration and invasion abilities are two essential respects for cancer cell metastasis including lung cancer. We found that miR-140-3p reduced the migratory rate of both lung cancer cell lines by about 50% compared to the controls (Figure 3A, 3B). In consistent with migration, the cell invasion ability was also reduced by miR-140-3p in A549 and H1299 cells (Figure 4A, 4B). Taken together, these data suggest

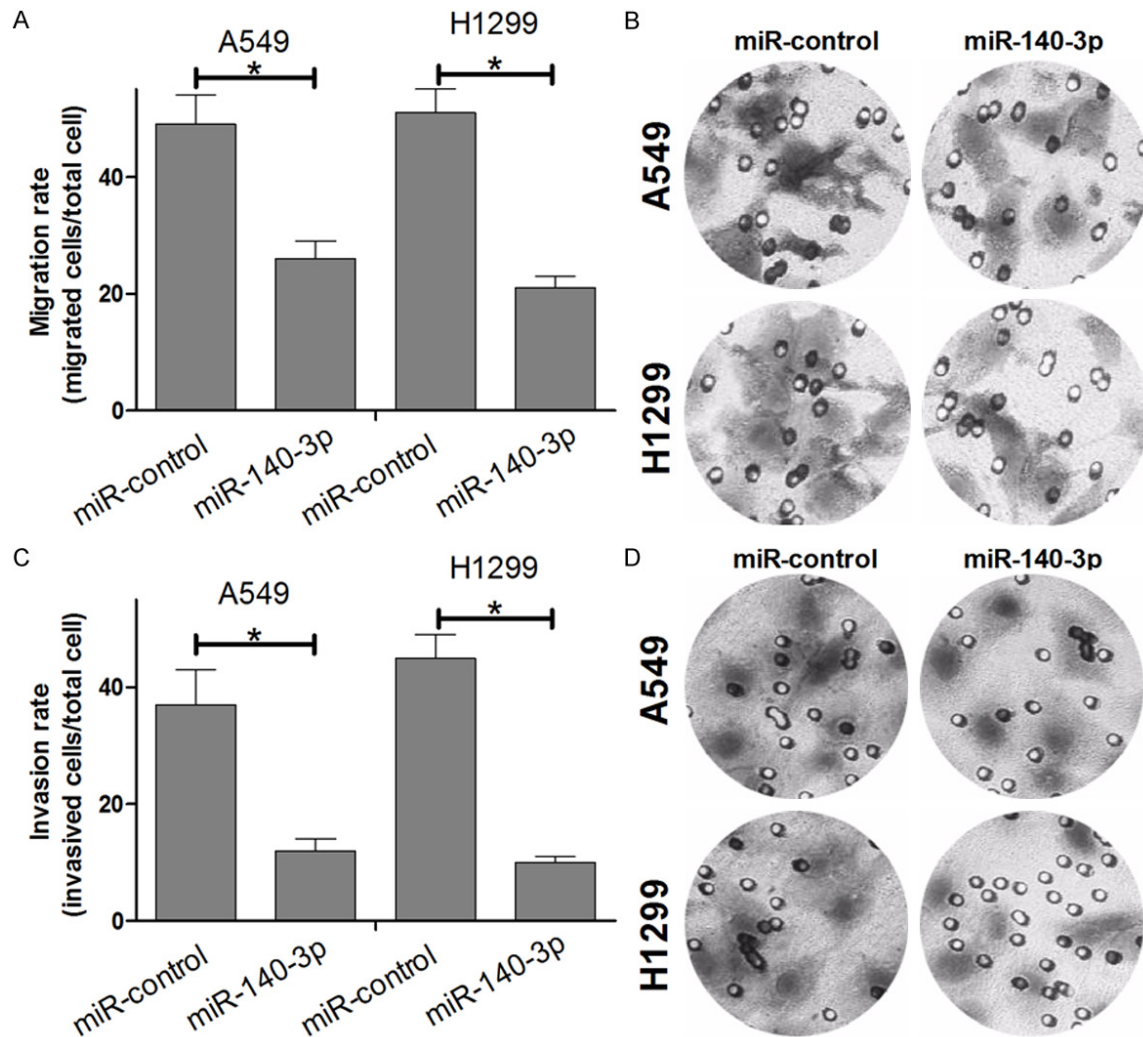


Figure 3. miR-140-3p inhibits lung cancer cells invasion and migration. A, B. Overexpression of miR-140-3p by miR-140-3p mimics affects the migration capacity of A549 and H1299 cells determined by Transwell migration assay. Representative pictures were captured at 48 h after transfection. All the experiments were test for three times. * $P < 0.05$. C, D. Overexpression of miR-140-3p by miR-140-3p mimics affects the invasion viability of A549 and H1299 cells determined by Transwell invasion assay. All the histograms show invasive cells per field from three independent experiments. * $P < 0.05$.

that miR-140-3p may play an anti-metastatic role in lung cancer metastasis.

ATP6AP2 is a direct target for miR-140-3p in A549 and H1299 cells

Three bioinformatics, Targetscan, miRanda and miRDB were used to predict the target genes for miR-140-3p, which mediated the anti-proliferation and anti-metastatic effects of miR-140-3p in A549 and H1299 cells. And we got five candidates (Figure 4A). Consensually considering the potential roles of target genes and the binding sites with miR-140-3p, (ATP6AP2) had

the potential as a candidate target. To confirm the direct binding sites of ATP6AP2 with miR-140-3p, we constructed Luciferase reporter gene with ATP6AP2 3'UTR and the mutant ATP6AP2 3'UTR, as shown in Figure 4B. Then we performed Luciferase reporter assay to detect the effects of miR-140-3p on luciferase intensity in A549 and H1299 cells transfected by ATP6AP2 3'UTR or mutant 3'UTR. We found that miR-140-3p reduced the luciferase intensity by about 50% in the FGF-3'UTR wild-type (WT) group compared to the ATP6AP2-3'UTR mutant (Mut) group (Figure 4C). Then, to determine ATP6AP2 expression was controlled by

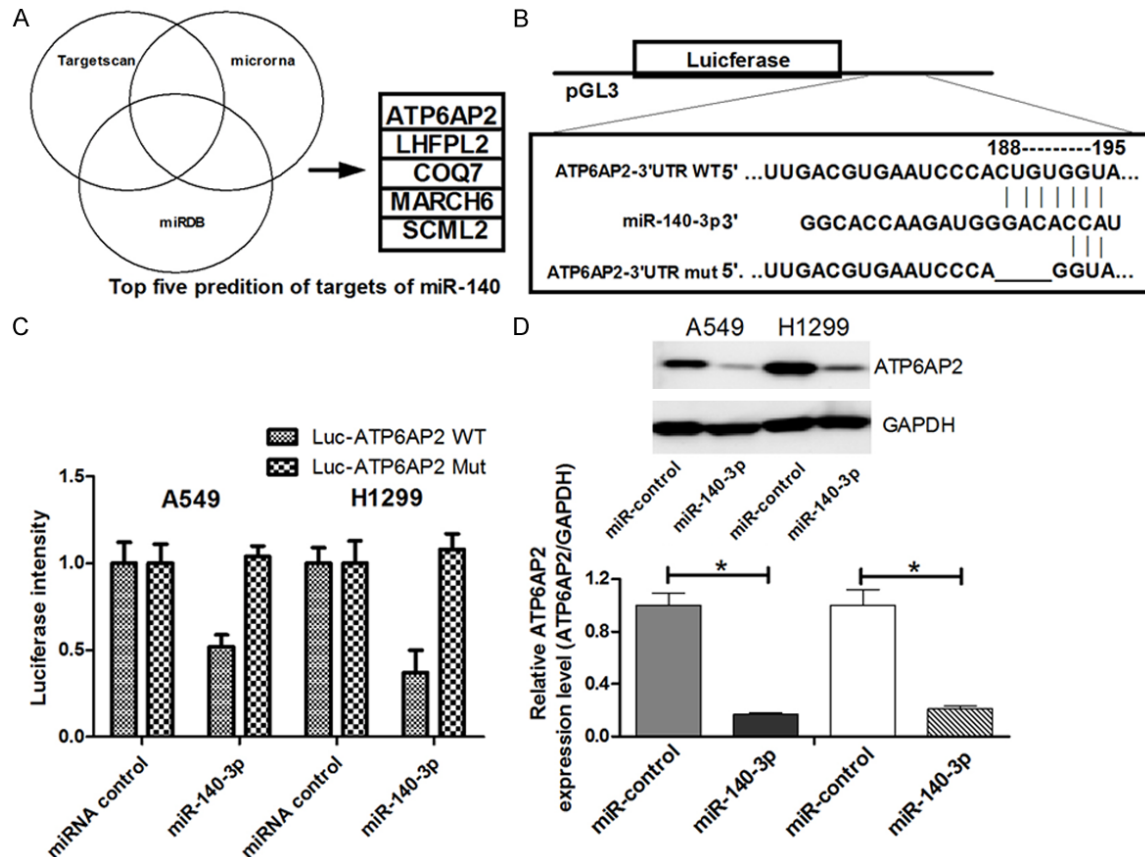


Figure 4. Screen the candidate target genes of miR-140-3p and its validation. A. A schematic representation showing the three miRNA target prediction algorithms screened five candidate target genes for miR-140-3p. B. Sequence alignment of miR-140-3p with the putative binding sites within the wild-type and mutant 3'-UTR regions of ATP6AP2. C. Luciferase fluorescence report assay demonstrated overexpression of miR-140-3p could reduce the intensity of fluorescence in A549 and H1299 cells transfected with the ATP6AP2 3'UTR vector, while had no effect on the ATP6AP2 3'UTR mutant vector. D. The protein level of ATP6AP2 was regulated by miR-140-3p in A549 and H1299 cell lines with GAPDH as a loading control. Three independent assays were performed and Data are represented as mean \pm SD. (* P <0.05).

miR-140-3p, we performed western blot in A549 and H1299 cells. As shown in **Figure 4D**, miR-140-3p suppressed ATP6AP2 expression by about 80% on both lung cancer cell lines. Overall, these results indicate that miR-140-3p suppresses ATP6AP2 expression by directly binding to its 3'UTR. These suggest that ATP6AP2 may act an important role in lung cancer proliferation and metastasis reduced by miR-140-3p mimics.

Discussion

To explore the molecular mechanism of lung cancer development is important for effective therapy methods. Aberrant miRNAs expression occurs frequently in human cancers. They have been reported to play important roles in carci-

nogenesis and tumor progression [12, 13]. Therefore, to determine the deregulated miRNAs in cancers is crucial. Previous studies discover miR-140 possible act as tumor suppressor in human malignances. Song et, al. showed that overexpression of miR-140 inhibited cell proliferation in both osteosarcoma and colon cancer cell lines [14]. More recently, Yang and colleagues reported that miR-140-5p is significantly decreased in HCC tissues and cell lines, and its overexpression suppresses tumor growth and metastasis by targeting transforming growth factor β receptor 1 and fibroblast growth factor 9 [15]. Yuan et, al. demonstrate miR-140 Suppresses tumor growth and metastasis of Non-Small Cell Lung Cancer (NSCLC) by targeting Insulin-Like Growth Factor 1 Receptor (IGF1R) [11]. Jude et, al. indicate that miR-140-

3p modulates CD38 expression in HASM cells through direct binding to the CD38 3'-UTR and indirect involving activation of p38 MAPK and NF- κ B [16]. And, miR-140-3p also found as one of the miRNA classifier that could distinguish SCC from normal lung tissues [17]. To date, however, the role of miR-140-3p in lung cancer carcinogenesis and the molecular mechanisms by which miR-140-3p exerts its functions remain unclear.

In the present study, we showed that miR-140-3p expression was significantly decreased in lung cancer tissues compared to adjacent normal tissues and six lung cancer cell lines. Overexpression of miR-140-3p could effectively inhibit lung cancer cell growth viability and reduce colony formation rate. Furthermore, miR-140-3p overexpression significantly repressed cell migration and invasion. These results suggest that miR-140-3p might be a novel tumor suppressor miRNA in lung cancer. Our results were all suggested the lung cancer suppressor role of miR-140-3p in vitro, so further study will explore the role of miR-140-3p in the lung cancer in vivo. Moreover, as for the silencing of miRNAs in cancers, hundreds of studies show that methylation may be one of the causes accounting for it. So, whether the down-regulation of miR-140-3p in lung cancer may be due to the hypermethylation of its promoter region or other mechanism are still need to be explored.

It has been demonstrate that miRNA plays tumor suppressive or oncogenic roles through binding to the the 3'UTR of target genes, so exploring the targets of miR-140-3p in lung cancer is crucial for understanding its regulation mechanism. In this study, we used three bioinformatics for target genes prediction. Finally, ATP6AP2 ATPase, H⁺ transporting, lysosomal accessory protein 2 came to our sight. We found that miR-140-3p decreased the ATP6AP2 expression on protein levels, suggesting that miR-140-3p negatively regulated ATP6AP2. Furthermore, Luciferase reporter assay directly confirmed the ATP6AP2 was the target of miR-140-3p in lung cancer cells.

ATP6AP2 gene encodes a protein that is associated with adenosine triphosphatases (ATPases). Proton-translocating ATPases have fundamental roles in energy conservation, secondary active transport, acidification of intracellular

compartments, and cellular pH homeostasis. There are three classes of ATPases-F, P, and V. The vacuolar (V-type) ATPases have a transmembrane proton-conducting sector and an extramembrane catalytic sector. ATP6AP2 has been found associated with the transmembrane sector of the V-type ATPases. A recent report found that the prorenin receptor (PRR) is a component of the Wnt receptor complex. And, PRR functions in a renin-independent manner as an adaptor between Wnt receptors and the vacuolar H⁺-adenosine triphosphatase (V-ATPase) (ATP6AP2) complex [18]. Our study demonstrated miR-140-3p inhibits lung cancer tumorigenesis and directly target ATP6AP2. According to these findings, we speculate the miR-140-3p/ATP6AP2 deregulation may stimulate the WNT signal in the lung cancer. But the detailed mechanisms should be explored.

In conclusion, the present study showed that miR-140-3p is significantly downregulated in lung cancer tissues and cell lines. Our study suggests that miR-140-3p regulates lung cancer cell proliferation, migration and invasion and directly suppressed the expression of ATP6AP2, functioning as a tumor suppressor. Enforce the expression of miR-140-3p may act as a novel therapeutic biomarker for lung cancer patients.

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Disclosure of conflict of interest

None.

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