MicroRNAs-491-5p suppresses cell proliferation and invasion by inhibiting IGF2BP1 in non-small cell lung cancer

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Abstract: MicroRNAs-491-5p (miR-491-5p) has been found to involve in tumor initiation and development in several tumors. However, the biological function and underlying molecular mechanism of miR-491-5p in non-small lung cancer (NSCLC) remain unclear. This study was therefore to investigate biological role of and underlying molecular mechanisms of in NSCLC. It was found that miR-491-5p expression was significantly downregulated in NSCLC tissues when compared with corresponding adjacent normal tissues (P<0.01), and the value was negatively related to advanced and tumor-node-metastasis (TNM) stage and lymph node metastasis (both P<0.01). We also demonstrate that restoration of miR-491-5p suppressed NSCLC cell proliferation by arresting NSCLC cells in the G1/G0 phase and accelerating apoptosis. miR-491-5p also inhibited cell migration and invasion in NSCLC cells. Mechanically, IGF2BP1 was identified as direct targets of miR-491-5p. And IGF2BP1 expression was significantly upregulated, and correlated negative with miR-491-5p expression in NSCLC tissues. In vivo assay showed that miR-491-5p suppressed tumor growth in nude model by repressing IGF2BP1 expression. Collectively, miR-491-5p functioned as a tumor suppressor in NSCLC by targeting IGF2BP1. Restoration of miR-491-5p expression may represent a promising therapeutic approach for targeting malignant NSCLC.

Keywords: Non-small lung cancer, miR-491-5p, proliferation, invasion, IGF2BP1

Introduction

Non-small cell lung cancer (NSCLC), including adenocarcinoma (ADC), squamous cell carcinoma (SCC), adenosquamous cell carcinoma (ASC) and large cell carcinoma (LCC), represents the most frequent type of lung cancer, and accounts for approximately 80-85% of all lung cancer cases [1, 2]. Despite radiotherapy, chemotherapy, and surgery have been recently used as standard treatment modalities for patients with NSCLC, the overall 5-year survival rate for NSCLC patients remains at approximately 15% since NSCLCs are often insensitive to chemotherapy and radiotherapy, and are fast-growing and highly invasive [3]. Therefore, it is urgent need to understand the molecular mechanisms underlying NSCLC development and progression for this disease diagnosis and treatment.

miR-491, has been found to be lost in several cancers, such as hepato-cellular carcinoma (HCC) [11], glioblastoma [12], colorectal cancer [13] and breast cancer [14]. miR-491-5p, a mature form of miR-491, has been showed to
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suppress the growth and metastasis of ovarian cancer, pancreatic cancer, breast cancer and cervical cancer by targeting Bcl-XL, TP53, JMJD2B and hTERT genes, respectively [15-18]. These results suggesting that miR-491-5p functions as tumor suppressor in these type cancers. However, the biological role and precise mechanisms of miR-491-5p in the progression of NSCLC have not been reported until now. Therefore, in the present study, we investigated the exact roles of the miR-491-5p and its underlying molecular mechanisms in NSCLC.

Materials and methods

Patients and tissue samples

36 paired NSCLC tissues and their corresponding adjacent normal lung tissues were obtained from patients who underwent NSCLC resection without preoperative treatment at Department of Thoracic Surgery, the First Hospital of Jilin University (Changchun, China), from April 2008 and June 2014, after receiving adequate informed consent. The corresponding adjacent normal tissues were obtained beyond 3 cm away from the boundary of NSCLC tissues. The tumor tissues and adjacent normal tissues were snap-frozen in liquid nitrogen immediately after resection and stored at -80°C until use. The study was approved by the Medical Ethics Committee of Jilin University.

Cell lines and culture

Four human lung cancer cell lines (A549, H1299, SPCA1 and H358) and normal lung cell (BEAS-2B) were brought from the Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., Grand Island, NY, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate at 37°C in a humidified air atmosphere containing 5% CO₂. Cells were used when they were in the logarithmic growth phase.

RNA extraction and quantitative PCR

Total RNA containing miRNA and mRNA from tissue samples and cells was extracted by using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instruction. For detection of miR-491-5p expression, complementary DNA (cDNA) was synthesized using the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), then were quantified using the miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) under an ABI 7900 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primers of miR-491-5p and U6 were used as previously [18]. For detection of IGF2BP1 expression, cDNA synthesis was performed using PrimeScript RT reagent Kit (Takara, Dalian, China) according to the manufacturer’s instructions, then was quantified using Real-time PCR Mixture Reagent (Takara) under ABI 7900 Fast system. The primers for IGF2BP1 and GAPDH were used as previous described [19]. The relative expression levels of miR-491-5p and IGF2BP1 were calculated by the 2^−ΔΔCt method. U6 and GAPDH were used as internal controls for miRNAs and mRNAs, respectively.

Cell transfection

miR-491-5pmimic (miR-491-5p) and corresponding negative control (miR-NC) (si-NC) were purchased from Ribobio (Guangzhou, Guangdong, China), and were transfected into A549 cells when cells were grown to 80-90% confluence, using the Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions at final concentration of 100 nM.

MTT assay

A total of 2×10⁴ cells were plated onto 96-well plates overnight and transfected with 100 nM miR-491-5p or miR-NC. 10 μl of sterile MTT dye (5 mg/ml) was added into each well containing 100 μl medium after cells were cultured for 24 h-72 h. The cells were then incubated at 37°C for 4 h in a 5% CO₂ incubator. Then MTT solution were removed and 150 μl dimethyl sulfoxide (DMSO, Sigma-Aldrich) were added to each well to dissolve the crystals for 10 min at 37°C. The absorbance in each well was measured using spectrophotometric analysis (BioTek, Grand Island, NY, USA) at 490 nm.

Cell cycle and cell apoptosis assay

The cells were seeded into 6-well plates and then transfected with the miR-491-5p mimic or miR-NC. After 48 h, the cells were harvested.
using trypsinization, washed in ice-cold PBS, and fixed in ice-cold ethanol in PBS. Then bovine pancreatic RNase (Sigma-Aldrich) was added to a final concentration of 2 mg/ml, and cultured at 37°C for 4 h in a 5% CO₂ incubator for 30 min. For the cell cycle distribution, the cells were stained with 20 mg/ml propidium iodide (PI; Sigma-Aldrich) for 20 min at room temperature. For the cell apoptosis assays, the cells apoptosis was determined using the Annexin V Apoptosis Detection Kit (Invitrogen) according to the manufacturer’s instructions. The cell cycle distribution and the cell apoptosis rates were quantified using an FACS Calibur flow cytometer (BD Biosciences, Mansfield, MA, USA).

Cell migration and invasion assay

To examine the migration ability of cells in vitro, a wound-healing assay was performed. In brief, the 2×10⁴ transfected cells were seeded into 24-well tissue culture plates for 24 h. Cells were then scratched using a sterile plastic micropipette tip to create an artificial wound. Migration of cells into the wound was observed and photographed under an inverted microscope at indicated time (0 h and 24 h).

Cell invasion was determined using 24-well Matrigel invasion chambers (Becton Dickinson) according to the manufacturer’s instructions. In briefly, transfected cells (5×10⁴) were seeded per well in the upper well of the Matrigel-coated invasion chamber in DMEM without serum. The lower chamber was filled with DMEM medium with 10% FBS to attract cells. After cells had been cultured at 37°C for 48 h, non-invading cells were removed from the top well with a cotton swab, while the bottom cells were fixed with 70% ethanol for 30 min and stained with 0.1% crystal violet for 10 min. The invaded cells were photographed and were counted in five randomly fields for each well under light microscope (Olympus, Tokyo, Japan).

Luciferase activity assay

The wild-type 3'-UTR segment of the IGF2BP1 mRNA (not the full length of IGF2BP1 3'-UTR) containing a putative miR-491-5p-binding site, was amplified using PCR and subcloned into pGL3-control vector (Ambion, Austin, TX, USA) at the Nhel and Xhol restriction sites, termed as: Wt-IGF2BP1-3'UTR. Amutant construct in miR-491-5p binding sites of IGF2BP1 3'UTR region also was generated using Quick Change Site-Directed Mutagenesis Kit (Agilent, Roseville City, CA), and subcloned into pGL3-control vector (Ambion, Austin, TX, USA) at the Nhel and Xhol restriction sites, and named as Mut-IGF2BP1-3'UTR. For reporter assay, A549 cells were plated onto 24-well plate (2×10⁴ cells/well) and transiently co-transfected with Wt/ Mut-IGF2BP1 reported plasmid, along with miR-491-5p/miR-NC using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured 48 h after transfection by the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase was used to normalize the Renilla luciferase.

Western blot

Cells or tissues were washed twice with ice-cold phosphate buffered saline, and lysed in RIPA buffer (Pierce, Waltham, MA, USA) on ice for 30 min, and then centrifuged at 13,000 g for 30 min. After centrifugation, proteins in the supernatants were quantified using a bicinchoninic acid (BCA) protein assay kit (Boster, China). Equal quantities (30 μg) of protein samples were loaded on 10% SDS-PAGE and transferred onto PVDF membrane (Mipore, Lake Placid, NY, USA). After blocked in 5% BSA, the membrane was incubated with antibody mouse anti-human IGF2BP1 (1:1000 dilution, Santa Cruz Biotechnology Inc., California, USA) and antibody mouse anti-human GAPDH overnight at 4°C, followed incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000 dilution, Santa Cruz, USA) for 1 h at room temperature. Protein band were determined with chemiluminescence detection system (Pierce) and visualized on Bio-Rad ChemiDocXRS (Bio-Rad Laboratories, Hercules, CA, USA).

Xenografted tumor model

Ten of five-week-old BALB/C nude male mice (20-25 g) were brought from the Experimental Animal Center of Changchun Institute for Biological Sciences (Changchun, China). All mice were maintained in the pathogen-free (SPF) conditions. All animal experiments were approved by National Institutes of Health Animal Care and the Use Committee guidelines of the Jilin University (Changchun, China).
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For the in vivo tumor assay, 2×10⁶ A549 cells stably expressing the miR-491-5p or miR-NC were collected and suspended in 0.2 ml PBS for each mouse (five in each group), and the cells were injected into left side of the posterior flank of nude mouse. Tumors growth were measured with calipers to estimate volume from day 7 to day 35 after injection according to the formula Volume (mm³) =1/2 width² × length. The animals were sacrificed after 35 days and the tumor tissue were removed for determination miR-491-5p and IGF2BP1 expression.

Table 1. Correlation between miR-491-5p status and clinical characteristics in patients with NSCLC

<table>
<thead>
<tr>
<th>Feature</th>
<th>N</th>
<th>miR-491-5p expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;60</td>
<td>16</td>
<td>0.35±0.08</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>≥60</td>
<td>20</td>
<td>0.37±0.09</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>0.38±0.11</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Man</td>
<td>19</td>
<td>0.34±0.07</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>T1/T2</td>
<td>23</td>
<td>0.41±0.11</td>
<td></td>
</tr>
<tr>
<td>T3/T4</td>
<td>13</td>
<td>0.27±0.05</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
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</tr>
<tr>
<td>No</td>
<td>26</td>
<td>0.46±0.13</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>0.11±0.02</td>
<td></td>
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<tr>
<td>TNM stage</td>
<td></td>
<td></td>
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<tr>
<td>I-II</td>
<td>22</td>
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<tr>
<td>III-IV</td>
<td>14</td>
<td>0.15±0.03</td>
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</table>

Figure 1. miR-491-5p expression is downregulated in non-small lung cancer (NSCLC) tissues and cell lines. A. Detection of miR-491-5p expression in 36 paired NSCLC tissues corresponding by quantitative RT-PCR (qRT-PCR) **P<0.01 compared to normal tissue. B. Detection of miR-491-5p expression in four human NSCLC cell lines (A549, H1299, SPCA1 and H358) and normal lung cell (BEAS-2B) by qRT-PCR. *P<0.05; **P<0.01 compared to BEAS-2B.

Statistical analysis

All data are presented as the means ± SD (standard deviation) from at least three independent experiments. Unpaired Student’s t test was used to determine the significance, using the GraphPadPrism version 6.0 software (GraphPad SoftwareInc., San Diego, CA, USA) and the SPSS 16.0 software (SPSS, Chicago, IL, USA). For all analyses, P<0.05 was considered statistically significant.

Results

MiR-491-5p expression was downregulated in NSCLC and associated with advanced clinical stage and NSCLC metastasis

To understand the potential biological significance of altered miR-491-5p expression in NSCLC progression, we evaluated miR-491-5p expression in 36 pairs of human NSCLC tissues and their corresponding normal tissues by quantitative RT–PCR (qRT-PCR). It was found that expression of miR-491-5p in NSCLC was significantly lower than that of corresponding adjacent normal tissue (Figure 1A). Then the relationship between the miR-491-5p expression levels and the clinicopathological characteristics of the NSCLC patients were investigated. As showed in Table 1, no significant correlations between miR-491-5p expression and age, gender, or tumor size were found, while we found that miR-491-5p expression was negatively associated with advanced TNM stage and lymph node metastasis. In addition, we also
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assessed the expression of miR-491-5p in four lung cancer line (A549, H1299, SPCA1 and H358) and normal lung cell (BEAS-2B), and found that the expression level of miR-491-5p was decreased in all four NSCLC cell lines compared with BEAS-2B (Figure 1B). The A549 cell line has lowest expression of miR-491-5p, thus it was selected for below study.

miR-491-5p inhibited cell proliferation in NSCLC cells

To elucidate potential effects of miR-491-5p in the progression of NSCLC, miR-491-5p was over-expressed in A549 cells (Figure 2A) by transfection of miR-491-5p. Alterations in cell proliferation, cell cycle distribution and apoptosis of miR-491-5p overexpression in A549 cells were determined. *P<0.05, **P<0.01 compared with miR-NC.
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sis induced by miR-491-5p were then examined. MTT assays revealed that restoration of miR-491-5p significantly decreased cell proliferation of A549 cells at the second and third days (Figure 2B, *P* < 0.05). To explore the possible mechanisms of miR-491-5p’s function in cell proliferation, we determined the cell cycle distribution of A549 cells transfected with miR-491-5p using flow cytometry, and found that overexpression of miR-491-5p in A549 cells induced a significant increase in the percentage of cells in the G1/G0 peak and a decrease in the percentage of cells in the S peaks (Figure 2C). In addition, we also measured cell apoptosis ratio in A549 cells after transfected with miR-491-5p. It was found that restoration of miR-491-5p in A549 cells obviously increased the rate of apoptosis (Figure 2D). These results suggested that miR-491-5p could inhibit cell proliferation by arresting the tumor cells at the G1/G0 phase and increasing tumor cell apoptosis.

miR-491-5p inhibited cell migration and invasion in NSCLC cells

We next assessed the effect of miR-491-5p on the migration and invasion of NSCLC cells by wound healing and invasion chamber assays, respectively. The results showed that overexpression of miR-491-5p in A549 cells markedly decreased the migratory and invasive capabilities (*P* < 0.05, Figure 3A and 3B).

IGF2BP1 is a direct target of miR-491-5p

To investigate the underlying molecular mechanism of growth inhibition by miR-491-5p in NSCLC cells, we identified the potential targets of miR-491-5p using two publicly available algo-
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IGFBP1 was identified as a potential target of miR-491-5p through Targetscan6.2 and miranda algorithms. The binding site for miR-491-5p was predicted to be conserved at position 763-769 (Figure 4A). To further determine whether miR-491-5p could directly associate with the 3'UTR of IGFBP1, a reporter plasmid harboring the wild-type or mutant binding site sequence in IGFBP1 3'UTR was constructed. Luciferase reporter assays were performed in A549 cells co-transfected with the wild-type or mutant 3'UTR IGFBP1 reporter plasmid and miR-491-5p mimic or miR-NC. Restoration of miR-491-5p significantly decreased luciferase activity of the wild-type IGFBP1 3'UTR-Wt in A549 cells (Figure 4B), while had no inhibition effect on the mutant IGFBP1 3'UTR reporter activity in A549 cells (Figure 4B). In addition, overexpression of miR-491-5p in A549 cells markedly inhibited the expression of IGFBP1 at both mRNA and protein level (Figure 4C and 4D). These results suggest that IGFBP1 is a direct target of miR-491-5p.

IGFBP1 expression was upregulated and inversely correlated with miR-491-5p expression in NSCLC tissues

Since the above results showed that the miR-491-5p could regulate IGFBP1 expression through targeting its 3'UTR in NSCLC cells, we investigated the IGFBP1 expression in NSCLC tissues and corresponding adjacent normal tissues. It was found that the expression of IGFBP1 on both mRNA and protein level was upregulated in NSCLC tissues compared with corresponding adjacent tissues (Figure 5A and 5B).
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Figure 5. IGFBP1 was up-regulated and inversely correlated with miR-491-5p expression in NSCLC tissues. A, B. IGFBP1 mRNA expression and protein expression in human NSCLC tissues and their corresponding normal tissues were determined by qRT-PCR and western blot, respectively. GAPDH was used as an internal control. **P<0.01 versus normal tissue. C. The reverse relationship between IGFBP1 mRNA expression and miR-491-5p expression was analyzed in NSCLC tissues by Spearman’s correlation.

Figure 6. miR-491-5p suppressed tumor growth in a xenograft model by repressing IGFBP1. A. Tumor growth curves for tumor volumes in xenografts of nude mice were established based on the tumor volume measured every week until five weeks. B. Tumor tissues weight was measured. C. Detection of miR-491-5p expression in tumor tissue by qRT-PCR. D. Detection of IGFBP1 protein expression in tumor tissues by Western blot. GAPDH was used as internal control. *P<0.05, **P<0.01 versus the miR-NC group.
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lation analysis, we found that IGFBP1 mRNA expression was inversely correlated with miR-491-5p expression in NSCLC tissues (Figure 5C; r=-0.522, P<0.05).

miR-491-5p suppressed tumor growth in a mouse xenograft model by repressing IGFBP1

Finally, an in vivo model was applied to evaluate the effect of miR-491-5p restoration on tumorigenicity. A549 cells stable expression miR-491-5p or miR-NC were subcutaneously inoculated in nude mice (n=5 for each group), respectively. It was found that the tumor growth was slower in A549/miR-491-5p group than that of A549/miR-NC group (Figure 6A). When mice were killed 35 days after injection, the tumor tissues were stripped and weighted. We found that the weight of tumor tissues in A549/miR-491-5p group was markedly reduced compared with A549/miR-NC group (Figure 6B). In addition, we also investigate the expression of miR-491-5p and IGFBP1 expression. It was found that miR-491-5p expression remarkably upregulated, while IGFBP1 expression obviously downregulated in A549/miR-491-5p group compared to A549/miR-NC group (Figure 6C and 6D). These results suggested that miR-491-5p suppresses tumor growth in a mouse xenograft model by repressing IGFBP1.

Discussion

Accumulating evidence showed that the aberrant expression of miRNAs play crucial roles in the occurrence and development of non-small lung cancer by regulating target genes [9, 10, 20]. For example, miR-30b/c could inhibit NSCLC cell proliferation by targeting Rab18 [21]; miR-98 suppress proliferation, migration, and invasion of lung cancer cells by directly binding to the 3’-UTR of ITGB3 Mrna [22]; miR-92a could promotes growth, metastasis, and chemo-resistance in non-small cell lung cancer cells at least partially by targeting PTEN [23], and so on. In this study, we found that miR-491-5p expression was decreased in NSCLC tissues and cell lines, and that miR-491-5p overexpression inhibited proliferation, migration, and invasion in NSCLC cells by targeting IGFBP1. These results might provide a new insight into the pathophysiological mechanism of NSCLC and a novel therapy target for NSCLC treatment.
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these results suggested that miR-491-5p exerts suppressive function partially by targeting IGFBP1.

In summary, the present study demonstrated that miR-491-5p was downregulated in NSCLC tissues and cell lines, and its expression level was significantly negative corrected with TNM stage and lymph node metastasis, and that restoration of miR-491-5p in NSCLC cells drastically decreased cell proliferation, migration, invasion, increased cell apoptosis and cell cycle arrest at G0/G1 stage in vitro, as well as suppressed tumor growth in nude mouse model by targeting IGFBP1. These findings suggested that miR-491-5p functions as a tumor suppressor in NSCLC by repressing IGFBP1 expression, and that miR-491-5p might serve as a promising therapeutic target in NSCLC.

Disclosure of conflict of interest

None.

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