The miR-224 promotes non-small cell lung cancer cell proliferation by directly targeting RASSF8

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Abstract. – OBJECTIVE: The purpose of this study was to explore the mechanism of microRNA-224 (miR-224) in NSCLC.

PATIENTS AND METHODS: Quantitative RT-PCR (qRT-PCR) was used to evaluate expression levels of miR-224. The association of miR-224 with the clinicopathologic features of NSCLC was evaluated in 56 patients. The roles of miR-224 in cell proliferation were analyzed in vivo and in vitro with pre-miR-224 transfected cells. Also, the regulation of RASSF8 by miR-224 was evaluated by qRT-PCR, Western blotting and luciferase reporter assays.

RESULTS: In this study, we identified miR-224 to be significantly up-regulated in NSCLC tissues and associated with tumor size. Increased miR-224 expression promotes NSCLC cell proliferation by down-regulating RASSF8 at the mRNA and protein levels. The AKT pathway was found aberrantly activated after over-expression of miR-224. RASSF8 was identified as a direct target of miR-224 by bioinformatics analysis and luciferase reporter assay.

CONCLUSIONS: The miR-224 played an oncogenic role in the proliferation of NSCLC by direct targeting RASSF8, and it is suggested that miR-224 may be a potential therapeutic target for NSCLC.

Key Words: Nonsmall cell lung cancer, miRNA-224, RASSF8, Proliferation.

Instruction

Lung cancer is the most common cause of cancer-related death worldwide, and nonsmall cell lung cancer (NSCLC) accounts for 80% of all lung cancer. NSCLC is comprised of adenocarcinoma, squamous cell carcinoma and large cell undifferentiated carcinoma. Although advances have made in surgical and medical treatments, the 5-year survival rate of lung cancer patients remains frustratingly poor, and the molecular pathogenesis of NSCLC remains poorly understood. Understanding the molecular mechanisms of NSCLC progression would help to identify potential therapeutic targets and optimize already existing therapies.

MicroRNAs (miRNAs) are a class of small, non-protein-coding RNAs that can post-transcriptionally regulate protein expression by negatively regulating mRNA stability or inhibiting them from translating into proteins. It has been proven that miRNAs play essential role in various cellular progresses, such as growth, apoptosis, differentiation and development. Currently, miRNA expression profiling studies have shown that several miRNAs functions as protooncogenes or tumor suppressors in human cancers, such as liver cancer, lung cancer. MiR-224 has been reported to be over-expressed in breast cancer, hepatocellular carcinoma and NSCLC. It has been proven that miR-224 promotes cellular migratory, invasive, and proliferative capacity and tumor growth both in vitro and in vivo by targeting SMAD4 and TNFAIP1 in NSCLC.

RASSF8, a number of Ras-association domain family (RASSF), is a candidate tumor suppressor. It is not only found in the nucleus, but is also a membrane-associated at sites of cell-cell adhesion, co-localizing with the adherens junction (AJ) component b-catenin and binding to E-cadherin. It has proven that RASSF8 inhibits cell growth by regulating Wnt and NF-κB signaling pathway in lung cancer. Besides, it was reported that the over-expression of RASSF8 leads to inhibition of anchorage-independent growth in soft agar in lung cancer cells. In esophageal squamous cell carcinoma, RASSF8 downregulation promotes lymphangiogenesis and metastasis. The role of RASSF8 in tumor growth has attracted increasing interest.
In this study, we demonstrated that miR-224 was frequently upregulated in NSCLC, and the increased miR-224 can promote lung cancer cell proliferation in vitro and tumor growth in vivo. Furthermore, we identified RASSF8 as a functional target of miR-224. The over-expression of RASSF8 inhibited lung cancer cell proliferation. Our results suggested that miR-224/ RASSF8 axis partially elucidates the molecular mechanism of NSCLC growth and represents a new potential therapeutic target for NSCLC treatment.

**Patients and Methods**

**Cell Culture**

Two NSCLC cell lines (A549 and H460) and normal human bronchial epithelial cell line (16HBE) were purchased from Cell Bank, Chinese Academy of Sciences (Xuhui District, Shanghai, China). NSCLC cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) medium containing 10% FBS3 (HyClone, South Logan, UT, USA) and 100 U/mL penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). 16HBE cells were cultured in keratinocyte serum-free medium with 25 μg/mL bovine pituitary extract and 0.2 ng/mL recombinant epidermal growth factor (Invitrogen Life Technologies, Carlsbad, CA, USA). Cell cultures were incubated in a humidified atmosphere of 5 % CO₂ at 37°C.

**Tissue Samples**

A total of 56 pairs of human NSCLC tissues and their corresponding normal adjacent tissues (NATs) were collected from People’s Hospital of Rizhao (Rizhao, Shandong Province, China) between 2005 and 2010. None of the patients had received radiotherapy or chemotherapy before surgery. Disease histology was determined in accordance to the criteria of the World Health Organization. The use of human tissues was approved by the People’s Hospital of Rizhao Ethics Committee.

**Plasmid Construction**

The human pre-miRNA expression construct Lenti-miR-224 vector and human miRZip-224 anti-miR-224 miRNA construct were obtained from System Biosciences (Palo Alto, CA, USA). To generate RASSF8 expression construct, the ORFs were amplified by PCR and cloned into pC-DH-RASSF8 vector. LightSwitch empty vector and RASSF8-3’-UTR were ordered from Active Motif (Carlsbad, CA, USA). Mutations were carried out using Stratagene QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA).

**Transfection**

The transfection of RASSF8 expression vector was performed using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA), and RNA-related vectors were transfected into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA).

**Quantitative Real-time PCR**

Total RNA was extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). For miR-224 detection, reverse-transcribed complementary DNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and quantitative RT-PCR (qRT-PCR) was performed with SYBR Premix ExTaQ (TaKaRa, Dalian, China) with the Roche LightCycler 480 system (Roche, Basel, Switzerland). The relative expression ratio of miR-224 was calculated by 2^(-ΔΔCT) method. TaqMan gene expression assays for RASSF8 were purchased from Applied Biosystems (Carlsbad, CA, USA) to determine its expression. GAPDH was used as internal control for normalization of mRNA expression. All reactions were conducted in triplicates. All primers used in the study were presented in Table I.

**Western Blot Analysis**

The cells were lysed with RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Danvers, MA, USA), and separated in 10% SDS-PAGE gels and blotted on Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA, USA). Then, Membranes were blocked with 5% BSA in Tris-Buffered Saline with Tween 20 (TBST) buffer and incubated with primary antibody, followed by incubation with appropriate secondary antibody at room temperature. Specific proteins were detected using the enhanced chemiluminescence system (GE Healthcare, Chicago, IL, USA). Antibodies against p-AKT, AKT, RASSF8 and GAPDH were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). GAPDH was used as internal reference.
miR-224 and non-small cell lung cancer cell proliferation

**Table I.** Primer pairs used in the study.

<table>
<thead>
<tr>
<th>Primers for qRT-PCR</th>
<th>MiR-224 for qRT-PCR F: GAGCCAAGTCACTAGTGTT</th>
<th>MiR-224 for qRT-PCR R: GTGCAGGGTCCGAGGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RASSF8 for qRT-PCR F: AGCAGTTCATCCAGCACAGCA</td>
<td>RASSF8 for qRT-PCR R: GAGATGAACCAGGTCGCTTC</td>
<td></td>
</tr>
<tr>
<td>Primers for RASSF8 cloning</td>
<td>AGCAGTTCAATCCAGCACAGCA</td>
<td></td>
</tr>
<tr>
<td>RASSF8 for cloning F:</td>
<td>GAGATGAACCAGGTCGCTTC</td>
<td></td>
</tr>
<tr>
<td>RASSF8 for cloning R:</td>
<td>sequence for knockdown of RASSF8 siRNA: ATGACTCCATGGATGAAATGA</td>
<td></td>
</tr>
</tbody>
</table>

F indicates forward primer. R indicates reverse primer.

**Cell Proliferation Assays**

Cell proliferation was monitored using Cell Proliferation Reagent Kit I (MTT) (Roche, Basel, Switzerland). After transfection, cells were plated at 3000 cells/well in 96-well plates, and cell proliferation was measured every 24 hours following the manufacturer’s protocol. On the indicated days, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reagent (AMRESCO, Solon, OH, USA) was added, and the cells were incubated for 4 h at 37°C. The supernatants were then removed, and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) (150 μL/well). The absorbance at 490 nm for each sample was measured using a multilabel plate reader (PerkinElmer, Waltham, MA, USA).

**Luciferase Reporter Assay**

To determine if miR-224 directly targets the 3’-UTRs of RASSF8, 5 × 10^4 16HBE cells were seeded in 24-well plates overnight and then transfected with miR-224 mimic (Thermo Scientific, Waltham, MA, USA) plus empty 3’-UTR vector or 3’-UTR vectors containing WT or MUT-3’-UTR. After 48 h, the cells were harvested and assayed using Dual Luciferase Assay (Promega, Fitchburg, WI, USA) according to the manufacturer’s protocols. Transfections were performed in duplicate and repeated at least three times in independent experiments.

**In Vivo Tumor Growth Analysis**

Animal studies were performed according to Institutional Animal Care and Use Committee guidelines of China. The cells transfected with miR-224 were harvested by trypsin treatment, washed with PBS and resuspended in Matrigel/ PBS (1:1). 10^6 cells were injected into the lung of nude mice (3 mice per group), followed by 20 days growth.

**Statistical Analysis**

All statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences between variables were assessed using the χ² test. All data presented in this study have been repeated at least three times from three independent experiments and are presented as means ± SD. Measurement data were analyzed using the Student’s t-test. Differences were considered significant at p-values < 0.05.

**Results**

**The Expression of miR-224 is Up-regulated in NSCLC Tissues**

Previous studies suggested that miR-224 over-expression promotes tumor progression in NSCLC\(^1\). We performed qRT-PCR analysis of miR-224 expression in three NSCLC cancer cell line (A549 and H460) and a normal human bronchial epithelial cell line (16HBE). The level of miR-224 was markedly up-regulated in NSCLC cell lines compared with the normal cell lines (p < 0.01, Figure 1A).

Furthermore, clinicopathological analysis of 56 patients showed that an increase in miR-224 was significantly correlated with larger tumor size (p = 0.003) (Table II).

We further investigated the expression of miR-224 in 30 pairs of NSCLC tissues and the corresponding NATs. qRT-PCR analysis showed that the level of miR-224 expression was significantly higher in NSCLC tissues compared with the NATs (p < 0.01, Figure 1B).

**Over-expression of miR-224 Promotes Proliferation of Lung Cancer Cells in vitro**

To examine the effect of miR-224 on the proliferation of lung cells, a vector containing miR-
224 precursor was constructed and transduced into NSCLC cell lines (H460). To knockdown miR-224 in cells, a vector containing miRZip-224 anti-miR-224 miR was constructed and transfected into A549. The successful over-expression or knockdown of miR-224 was confirmed by qRT-PCR (Figure 2A). The proliferative abilities were significantly increased in miR-224-transfected cell lines H460 when compared with miR-NC transfected cell lines. In A549 cells, the knockdown of miR-224 significantly decreased proliferative abilities (Figure 2B).

**Over-expression of miR-224 Induces Expression of AKT Signaling Pathway**

AKT mediates some of cellular processes, including cell survival, migration, metabolism and autophagy\(^{15}\). It is aberrantly expressed in many cancers\(^{16}\). In this study, we found that the expression of p-AKT was dramatically increased when miR-224 was over-expressed in H460 cells compared with control cells. While in A549 containing miR-224 inhibitor, the expression of p-AKT was reduced when compared with control cells (Figure 2C).

**MiR-224 Targets the 3'-UTR of RASSF8**

To explore downstream targets of miR-224, we performed bioinformatics analyses, including TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do). RASSF8 was predicted a theoretical target gene. Therefore we performed further analysis to confirm the target.

**Table II.** MiR-224 expression and clinicopathological features in non-small cell lung cancer (NSCLC) patients.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Low expression</th>
<th>miR-224 expression</th>
<th>(p)-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 (27)</td>
<td>13 (48.1%)</td>
<td>14 (51.9%)</td>
<td>0.607</td>
</tr>
<tr>
<td>≥50 (29)</td>
<td>14 (48.3%)</td>
<td>15 (51.7%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (23)</td>
<td>10 (43.5%)</td>
<td>13 (56.5%)</td>
<td>0.570</td>
</tr>
<tr>
<td>Female (33)</td>
<td>17 (51.5%)</td>
<td>16 (48.5%)</td>
<td></td>
</tr>
<tr>
<td>Tumour size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5 (18)</td>
<td>10 (55.6%)</td>
<td>8 (44.4%)</td>
<td>0.003</td>
</tr>
<tr>
<td>≥5 (38)</td>
<td>6 (21.1%)</td>
<td>32 (78.9%)</td>
<td></td>
</tr>
<tr>
<td>Site of tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left lung (21)</td>
<td>9 (42.9%)</td>
<td>12 (57.1%)</td>
<td>0.590</td>
</tr>
<tr>
<td>Right lung (35)</td>
<td>18 (51.4%)</td>
<td>17 (48.6%)</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II (37)</td>
<td>15 (40.5%)</td>
<td>22 (59.5%)</td>
<td>0.031</td>
</tr>
<tr>
<td>III + IV (19)</td>
<td>2 (10.5%)</td>
<td>17 (89.5%)</td>
<td></td>
</tr>
</tbody>
</table>

*\(\chi^2\) test.
A luciferase reporter assay was performed in 16HBE cells to verify that miR-224 directly targets RASSF8. A mutation of 3'-UTR of RASSF8 was constructed using the QuikChange Mutagenesis Kit at the binding site, named as MUT (Figure 3A). The luciferase reporter constructs harboring MUT or control were constructed and then transfected into 16HBE cells. Luciferase activities were then measured. The relative luciferase activity was significantly reduced in cells containing 3'-UTR-WT and miR-224 than in control cells. The reduction of luciferase activities was attenuated when cells were cotransfected with miR-224 and MUT 3'-UTR (Figure 3B).
Besides, the over-expression of miR-224 significantly reduced RASSF8 expression at mRNA and protein levels in H460 cells. The silence of miR-224 increased the expression of RASSF8 at the mRNA and protein levels in A549 (Figure 3C and D).

**The Over-expression of miR-224 Promotes NSCLC Proliferation in vivo**

Given that miR-224 remarkably promoted the proliferation of NSCLC cells *in vitro*, we further investigated whether miR-224 could affect tumor proliferation *in vivo*. H460 stably expressing miR-224...
and control cells were injected into the lung of nude mice. After 20 days of inoculation, over-expression of miR-224 in H460 cells markedly increased tumor growth compared with H460/vector group in vivo, as shown in Figure 4 A and B.

**Discussion**

Recently, many studies have been focused on studying aberrant expression of miRNAs in cancer, indicating that miRNAs contributes to the pathogenesis of most human malignancies. A number of miRNAs functions as oncogenes or tumor suppressors in the majority of cancers. Strategies that interfere with miRNA function are considered to offer novel target for cancer treatment. MiRNAs are important regulators of many biological processes. In NSCLC, miR-let-7c inhibits migration and invasion by targeting ITGB3 and MAP4K3, miR-21 promotes growth and metastasis by targeting PTEN, and MicroRNA-135b promotes lung cancer metastasis. Besides, miR-224 enhances invasion and growth in NSCLC by

![Figure 4. MiR-224 promotes NSCLC proliferation in vivo. (A) Tumor growth in nude mice (n = 5 per group). (B) Comparison of tumor size at day 20 after inoculation with H460 (miR-224) or H460 (vector) cells.](image)
shifting the equilibrium of the partial antagonist functions of SMAD4 and TNFAIP1. Furthermore, miR-224 expression is frequently upregulated in various human tumor types, including colorectal cancer, hepatocellular cancer (HCC), and renal cancer. These studies suggest that miR-224 is an onco-miR. In the current study, we demonstrated that miR-224 is commonly upregulated in NSCLC, and the re-expression of miR-224 can promote NSCLC cell growth in vitro and in vivo. These findings agree with the results of Cui et al. and Wang et al.

This study suggested that RASSF8 was a candidate miR-224 target. The luciferase reporter assay firstly demonstrated its downregulation was mediated by the direct binding of miR-224 to the RASSF8 3′-UTR. We found that over-expression of miR-224 promoted cell growth by silencing RASSF8. As over-expression of RASSF8 can often lead to toxicity, we aimed to investigate the effects of decreased levels of RASSF8 on cell growth by RASSF8 depletion. After decreasing expression of RASSF8, the promotion effects of miR-224 on growth of NSCLC cells are partially increased.

RASSF8 is ubiquitously expressed in all major organs and tissues, including the kidney, liver, brain, heart and lung. Endogenous RASSF8 is expressed in both the nucleus and cell membrane, and it promotes cell-cell adhesion by maintaining adherens junction (AJ) stability. In previous studies, RASSF8 has been proposed as a candidate tumor suppressor in cancer. Lock et al. showed that RASSF8 expression might control cellular proliferation of NSCLC cells by regulating β-catenin localization and the canonical Wnt signaling pathway and, conversely, p65 localization and the NF-κB signaling pathway. Falvella et al. proposed that Expression of RASSF8 protein by transfected lung cancer cells led to inhibition of anchorage-independent growth in soft agar in A549 cells and reduction of clonogenic activity in NCIH520 cells. Besides, Korkola et al. found that RASSF8 expression was downregulated in male germ cell tumors. These results provided further evidence for our work.

Besides the gene RASSF8, p-AKT signaling pathway was also mediated by miR-224. Over-expression of miR-224 promoted the expression of p-AKT, and deletion of miR-224 inhibited the expression of p-AKT. It has been demonstrated that in colorectal cancer miR-224 activates PI3K/Akt signaling and promotes cell survival through modulation of FOXO3a, p21Cip1, p27Kip1, and cyclin D1 expression. And the inhibition of AKT signaling pathway promotes apoptosis. This pathway represents a new mechanism which underlie the development of NSCLC, while the specific mechanism still needs further studies to verify.

Lastly, our in vivo research presented that mice with miR-224 over-expression showed significantly increasing in tumor size compared to control size. The results strongly support the results from in vitro analyses. Thus, our studies might provide a potential molecular mechanism of NSCLC proliferation.

Conclusions

The increased levels of miR-224 in NSCLC patients indicated that miR-224 was a potential biomarker in the proliferation. The results showed that miR-224 plays an oncogenic role in NSCLC as miR-224 promotes tumor cell proliferation by targeting RASSF8. It is suggested that miR-224 may be a potential therapeutic target for NSCLC.

Conflict of Interest

The authors declare no conflicts of interest.

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