Original Article

microRNA-1908-5p inhibits proliferation and promotes apoptosis by targeting PP5 in NSCLC

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Abstract: MicroRNA-1908 is involved in the occurrence and development of various tumors. However, the mechanism of microRNA-1908-5p in the pathogenesis of non-small cell lung cancer (NSCLC) is not thoroughly studied. Protein phosphatase 5 catalytic subunit (PP5), a member of the protein phosphatase catalytic subunit family, may be a target of the microRNA-1908-5p. In order to further explore the mechanism of microRNA-1908-5p, real-time PCR was used to detect the expression of microRNA-1908-5p in non-small cell lung cancer tissues, and analyze the relationship between the expression of microRNA-1908-5p and clinical characteristics of lung cancer patients. The target of microRNA-1908-5p was predicted by bioinformatics and verified by Dual-luciferase assay. The effects of microRNA-1908-5p on the proliferation and apoptosis of lung cancer cells were examined at the cellular level. Nude mice tumorigenesis experiment was used to study the effect of microRNA-1908-5p on cancer cells. Western blot was used to detect the expression of related proteins. The results showed that the expression of microRNA-1908-5p in lung cancer tissues was significantly lower than that in adjacent tissues. The expression of microRNA-1908-5p in the non-metastatic lung cancer tissues was significantly higher than that in the metastatic lung cancer tissues, and the expression of microRNA-1908-5p was closely related to the survival rate of patients. Bioinformatics analysis combined with double luciferase assay showed that PP5 was a significant target of microRNA-1908-5p. Our results suggest that microRNA-1908-5p can regulate the pathogenesis of NSCLC by inhibiting PP5.

Keywords: MicroRNA-1908-5p, NSCLC, proliferation, prognosis, PP5

Introduction

There is an increasing need for effective treatment of cancer. Although in recent years, a variety of radiotherapy and chemotherapy methods targeting cancer cells have emerged on the basis of surgical treatment, which alleviate the disease to a certain extent, the prognosis of cancer patients is still unsatisfactory [1-3]. Lung cancer is one of the most common cancers with the highest morbidity and mortality in the world. More than 1 million new cases of lung cancer occur worldwide every year [4]. The pathologic types of lung cancer include non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), of which more than 80% are NSCLC, which is insensitive to radiotherapy and chemotherapy and has poor prognosis. Therefore, exploring the pathogenesis of NSCLC is very important for the diagnosis and prognosis of NSCLC [5-7]. Studies have shown that non-coding RNA is involved in the pathogenesis of various tumors. MicroRNA is a single-stranded non-coding RNA with length ranging from 10 to 23 nucleotides. It can bind to the 3’UTR of target gene, and then inhibit the translation of target gene, so as to achieve the goal of regulating gene expression [8]. MicroRNA-1908 is involved in the pathogenesis and metastasis of ovarian cancer, and the expression of microRNA-1908 is significantly down-regulated in ovarian cancer patients. The expression of microRNA-1908 in ovarian cancer tissues of patients with metastasis was significantly lower than that of patients without metastasis [9]. In addition, the expression of microRNA-1908 was significantly lower in osteosarcoma patients than in adjacent tissues [10]. However, the involvement of microRNA-1908-5p in the genesis and development of NSCLC has rarely been reported.
MicroRNA-1908-5p inhibits proliferation in NSCLC

Table 1. Primer sequences of product expression

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA-1908-5p</td>
<td>RT primer</td>
<td>5' CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGACCAATC 3'</td>
</tr>
<tr>
<td></td>
<td>Forward primer</td>
<td>5' ACACTCCAGCTGGGCGGCGGAGACG 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5' CTCAACTGGTGTCGTGGGA 3'</td>
</tr>
<tr>
<td>U6</td>
<td>RT primer</td>
<td>5' GTCTGATCCAGTGCAAGGTCCAGGTATTCGACTGATCGAGAAAAATATGAAC 3'</td>
</tr>
<tr>
<td></td>
<td>Forward primer</td>
<td>5' GCCGACGATGATGCGCATA 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5' CCTCACGAACGCACAGGCA 3'</td>
</tr>
<tr>
<td>PP5</td>
<td>RT primer</td>
<td>Radom Primer (TakaRa Random Primer DNA Labeling Kit)</td>
</tr>
<tr>
<td></td>
<td>Forward primer</td>
<td>5' GCGCAGACATGGAGAAA 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5' CTCGCTTCGGCAGCATA 3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>RT primer</td>
<td>Radom Primer (TakaRa Random Primer DNA Labeling Kit)</td>
</tr>
<tr>
<td></td>
<td>Forward primer</td>
<td>5' GCGCAGACATGGAGAAA 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5' CTCGCTTCGGCAGCATA 3'</td>
</tr>
<tr>
<td>PP5-3'UTR</td>
<td>RT primer</td>
<td>Radom Primer (TakaRa Random Primer DNA Labeling Kit)</td>
</tr>
<tr>
<td></td>
<td>Forward primer</td>
<td>5' GCGCAGACATGGAGAAA 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5' CTCGCTTCGGCAGCATA 3'</td>
</tr>
</tbody>
</table>

In order to further explore the mechanism of microRNA-1908-5p in the pathogenesis of lung cancer, it is necessary to analyze the potential targets of microRNA-1908-5p. PP5, a member of the protein phosphatase catalytic subunit family, had been predicted as a target of microRNA-1908-5p. Proteins in this family participate in pathways regulated by reversible phosphorylation at serine and threonine residues. Many of these pathways are involved in the regulation of cell growth and differentiation [11]. The product of this gene has been shown to participate in signaling pathways in response to hormones or cellular stress, and elevated levels of this protein may be associated with breast cancer development [12]. However, the relationship between PP5 and NSCLC is still unclear. In this study, we detected the expression of microRNA-1908-5p in clinical lung cancer samples, screened the target of microRNA-1908-5p by bioinformatics, and then explored the mechanism of microRNA-1908-5p and PP5 in the pathogenesis of NSCLC in vivo and in vitro.

Materials and methods

Clinical samples

Seventy-six samples from The Second Affiliated Hospital of Guangxi Medical University were diagnosed as NSCLC in 2010-2011. This research was approved by the Research Ethics Committee of Guangxi Medical University. All patients signed the informed consent.

Real-time PCR

According to the instructions of Takara's RNA extraction kit, total RNA was extracted from 76 samples and adjacent tissues. The total RNA reverse transcription products of cells were used as templates, and the sequences in Table 1 were used as primers. According to the instructions of Takara's kit SYBR PremixExTaq™ (Takara, Japan), the expression of related genes was detected by fluorescence quantitative method. The statistical results of Real-time PCR were expressed by relative CT (threshold cycle).

Dual-luciferase reporter gene assay

Referring to the early research [13], TargetScan, MicroWalk and Pictar online software were used to analyze the target of microRNA-1908-5p, and the predicted results of the three softwares were analyzed comprehensively. Finally, it was preliminarily concluded that PP5 might be a target of microRNA-1908-5p. The 3'UTR sequence of PP5 gene was obtained by bioinformatics analysis. The 3'UTR of PP5 gene was amplified by PCR using the primer sequence of Table 1. The amplified product was linked to psiCHECK-2 plasmid. The double Luciferase Report experiment was used to verify whether PP5 was the target of microRNA-1908-5p.

Gain-of-fuction and Loss-of-function

The SPC-A1 cells were purchased from the China Infrastructure of Cell Line Resources.
Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. 100 U/ml penicillin and 100 mg/ml streptomycin were added. We cultured cells in incubator at 37°C, 5% CO₂, and changed the medium every other day. When the cells were in good condition, they were transferred to 6-well cell culture plates and transfected with microRNA-1908-5p mimics and microRNA-1908-5p inhibitors according to the instructions provided by the supplier (Ribobio, China). The transfected cells were cultured overnight in the incubator and then used for subsequent experimental study.

**MTT assay**

After digestion with 0.25% trypsin, the transfected cells were shaken into cell suspension, and the cell concentration was adjusted to 10⁵ cells per well. The cells were inoculated on 48-well cell plate at 37°C and 5% CO₂ incubator for 24 hours. After that, the old culture medium was discarded and carefully rinsed with PBS twice. After that, serum-free RPMI-1640 culture medium 100 µL and 5 mg.mL⁻¹ MTT solution were added into each well for 20 µL. After 4 hours, the cells were cultured carefully with a micropipettor. The liquid in the pore was sucked out, 150 µL DMSO was added into each pore and incubated at 37°C for 10 minutes to promote the full dissolution of the crystals. The absorbance OD value of each hole was measured at 490 nm of the spectrophotometer every 24 hours. The growth curve of each group was calculated and drawn three times in parallel.

**Apoptosis detection**

After 24 hours of cell culture, the transfected cells were digested with 0.25% trypsin, shaken into cell suspension and transferred into 5 ml EP tube. The cell concentration was regulated to 10⁵ cells per pore. After FITC and PI staining, the apoptotic rate of transfected cells was analyzed by flow cytometry.

**Athymic mice assay**

Athymic mice (25 g ± 5 g, 6 weeks old) were purchased from Animal Center of Southern Medical University. A total of 30 mice were randomly divided into three groups: control group, mimic group and inhibitor group. Each group of 10 animals were fed in cages. The mice were cultured for one week before opera-

**Western blotting**

After 24 hours of cell treatment, the total protein of cells was extracted by using the instructions of protein extraction kit provided by Beyotime (China). After purification, SDS-PAGE electrophoresis was carried out and PAGE gel was transmembrane with nitric acid fibre membrane. Rabbit anti-human PP5 and GAPDH antibodies were incubated respectively. HRP-labeled antibodies were incubated overnight. After fixing and developing, the optical densities of PP5 and GAPDH protein bands were analyzed by Quantity One v4.4.0 software.

**Statistical analysis**

SPSS 19.0 was used for data analysis, and the differences among the groups were compared with one-way analysis of variance. All measurement data was expressed in mean ± standard deviation; P value less than 0.05 was significant.

**Results**

*The expression of microRNA-1908-5p was inhibited in NSCLC*

Real-time PCR was used to detect the expression of microRNA-1908-5p in lung cancer tissues. The results showed that the expression of microRNA-1908-5p in lung cancer was significantly lower than that in adjacent tissues (P<0.01) (Figure 1A). The low expression of microRNA-1908-5p was positively correlated with metastasis of lung cancer (P<0.01) (Figure 1B, Table 2). The results of Kaplan-Meier survival analysis indicated that high microRNA-1908-5p expression level had a longer survival time than those of low microRNA-1908-5p expression level (P<0.01) (Figure 1C).
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Flow cytometry was used to detect the apoptosis rate of SPC-A1 cells after treatment, and the results indicate that the apoptosis rate of the cells transfected with microRNA-1908-5p mimics is significantly higher than that of the control group, but the apoptosis rate of the cells transfected with microRNA-1908-5p inhibitors is significantly lower than the control group (P<0.01) (Figure 2A, 2B). In addition, we used CCK8 assay to detect the proliferation activity of SPC-A1 cells after treatment (Figure 2C). The results suggest that microRNA-1908-5p can significantly decrease the proliferation activity of SPC-A1 cells (P<0.01).

PP5 is a target of microRNA-1908-5p

PP5 belongs to a family of protein kinases whose members are presumed to be involved in cellular growth and development [25]. In this study, through bioinformatics prediction, it is concluded preliminarily that PP5 may be the target of microRNA-1908-5p (Figure 3A). The Dual-luciferase assay indicates that MicroRNA-1908-5p can significantly inhibit the ac-

Table 2. Correlation of the expression of microRNA-1908-5p with clinicopathologic features

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>n (% )</th>
<th>microRNA-1908-5p expression</th>
<th>P</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>58 (76.3)</td>
<td>8.36</td>
<td>0.754</td>
</tr>
<tr>
<td>Female</td>
<td>18 (23.7)</td>
<td>9.01</td>
<td></td>
</tr>
<tr>
<td>Site of tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left lung</td>
<td>46 (60.5)</td>
<td>10.12</td>
<td>0.881</td>
</tr>
<tr>
<td>Right lung</td>
<td>30 (39.5)</td>
<td>9.87</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>50 (65.8)</td>
<td>10.96</td>
<td>0.943</td>
</tr>
<tr>
<td>High/moderate</td>
<td>26 (34.2)</td>
<td>8.38</td>
<td></td>
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<tr>
<td>Lymph node Metastasis</td>
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</tr>
<tr>
<td>N0</td>
<td>24 (31.6)</td>
<td>2.46</td>
<td>0.008</td>
</tr>
<tr>
<td>N1</td>
<td>14 (18.4)</td>
<td>4.18</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>20 (26.3)</td>
<td>25.21</td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td>18 (23.7)</td>
<td>34.86</td>
<td></td>
</tr>
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</table>

MicroRNA-1908-5p suppresses SPC-A1 cell proliferation and induces SPC-A1 cells apoptosis

Flow cytometry was used to detect the apoptosis rate of SPC-A1 cells after treatment, and the
MicroRNA-1908-5p inhibits proliferation in NSCLC

Figure 2. miR-1908-5p affects the apoptosis and proliferation of SPC-A1 cells. A, B. Flow cytometry was used to test the apoptotic rate of SPC-A1 cells. NC, normal control; M, transfected with miR-1908-5p mimics; I, transfected with miR-1908-5p inhibitors. Compared with NC, *P<0.05. C. miR-1908-5p expression were regulated in SPC-A1 cells with mimics or inhibitors, and the proliferation of SPC-A1 cells were detected with MTT.

MicroRNA-1908-5p inhibits tumor growth in athymic mice

By subcutaneous injection, the SPC-A1 cells transfected with microRNA-1908-5p mimics or microRNA-1908-5p inhibitors were implanted into male nude mice. After 8 weeks, the nude mice were sacrificed, and the tumor was weighed and photographed. The results show that the weight of the tumor from the nude mice transfected with microRNA-1908-5p mimics is significantly less than that of the control group. In contrast, the results show that the weight of the tumor from the nude mice transfected with microRNA-1908-5p inhibitors is significantly higher than that of the control group (P<0.01) (Figure 4A, 4B). This result tells us that microRNA-1908-5p can inhibit the growth of tumor in vivo.

MicroRNA-1908-5p suppress expression of PP5 in vivo

PP5 is a potential target involved in tumor proliferation and apoptosis. After weighing, the tumor mass was used to detect the expression level of PP5. Western blot results indicated that microRNA-1908-5p mimics can significantly inhibit the expression of PP5. MicroRNA-1908-5p inhibitors could significantly promote protein expression of PP5 (P<0.01) (Figure 4C, 4D). These data indicated that microRNA-1908-5p may affect the proliferation and apoptosis potential of NSCLC cells by altering PP5 protein expression.

Discussion

Lung cancer is one of the malignant tumors that seriously endanger human health and has high mortality. The mechanism of its occurrence is complex and has not yet been fully...
clarified. As an important regulator in organisms, somemicroRNA is out of control in lung cancer. Abnormal expression of microRNA plays an important regulatory role in the occurrence and development of lung cancer, suggesting that it has great potential in clinical aspects of lung cancer [14-16]. The reasons for the low early diagnosis rate of lung cancer are the lack of popularization of high-quality CT and the misdiagnosis rate. The stable presence of microRNA in peripheral blood, sputum, and exhaled breath and the detection of high-quality microRNA make microRNA a promising early diagnostic indicator [17]. In the treatment of lung cancer, the regulation of microRNA is the key step in the occurrence and development of lung cancer [18]. It is not only the research direction of new drug targets in the future, but also the determination of drug resistance-related microRNA, that makes it possible to optimize existing drugs. In predicting prognosis, some studies have also identified prognostic-related microRNAs with certain specificity and sensitivity [19].

The mechanism of drug resistance in cancer patients is very complex, including drug transport, metabolism, DNA synthesis, and repair [20-22]. MicroRNAs with post-transcriptional regulatory mechanisms participate in the occurrence and treatment of lung cancer through a variety of mechanisms. Guo et al [23] compared the changes of microRNA and RNA expression profiles of chemosensitive NCI-H69 and insensitive NCI-H69AR cell lines (SCLC) under the action of adriamycin by microRNA chips and cDNA chips. Through the negative correlation between them, the microRNA groups related to drug resistance were identified. Functional analysis of miR-134 clusters (including mir-134/379/495, etc.) with significant differences showed that the target of miR-134 is a drug-resistant transmembrane glycoprotein MRP1/ABCC1, which has the effect of stagnating cell cycle in G1 phase. The down-regulation of miR-134 in lung cancer accurately explains the drug resistance of NCL-H69AR cell line. Ranade et al [24] observed the expression of microRNA in SCLC specimens and the relationship between drug resistance and prognosis. It was found that miR-92a-2 was closely related to chemotherapeutic resistance, only gender and high expression of miR-92a-2 were related to survival rate, which revealed the relationship between miR-92a-2 and drug resistance in lung cancer [25]. The expression of microRNA and its regulated target genes con-

Figure 3. The screening of microRNA-1908-5p direct target in SPC-A1 cells. A. The mutated PP5 3'-UTR sequence (Mut) was designed in accordance with wild type PP5 3'-UTR sequence (WT). B. The effect of PP5 WT/Mut 3'-UTR in HEK-293T cells after transfection with microRNA-1908-5p. The mutant showed higher luciferase activity than that of the wild type. C, D. SPC-A1 cells transfected with microRNA-1908-5p mimics or microRNA-1908-5p inhibitors, and the expression level of PP5 was detected by western blot. Compared with NC, *P<0.05.
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Figure 4. MicroRNA-1908-5p inhibits the tumorigenesis in vivo. A, B. Male athymic mice were injected subcutaneously with SPC-A1 cells in which the expression of microRNA-1908-5p was up-regulated or down-regulated. MicroRNA-1908-5p inhibits the growth of tumor significantly. C, D. The expression of PP5 was detected with western blot in tumor tissues. The experiment was repeated 3 times, *P<0.05.

...stitutes a regulatory network, which dynamically regulates the operation of various biological processes in organisms [26]. Because of the particularity of the mechanism of action, it is possible for a single microRNA to regulate multiple target genes or multiple microRNAs to co-regulate the same target gene [27, 28]. Therefore, confirmed microRNA-target gene pairing only reveals a part of the microRNA regulatory network. In this study, we investigated the role of microRNA-1908-5p in NSCLC carcinogenesis and metastasis. The results showed that the expression of microRNA-1908-5p was significantly downregulated in NSCLC compared with normal tissues, and microRNA-1908-5p participated in apoptosis and proliferation in NSCLC. Then, we investigated the microRNA-1908-5p's potential target in SPC-A1 cells. The results showed that PP5 is an important protein in apoptosis and proliferation. Bioinformatics analysis and luciferase activity analysis indicated that PP5 is a direct target gene of microRNA-1908-5p.
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In conclusion, our results suggest that microRNA-1908-5p can inhibit proliferation and apoptosis of NSCLC by partly regulating the expression of PP5, which enriches understanding of the mechanism of the occurrence and development of lung cancer, and provides a reliable theoretical basis for early diagnosis of lung cancer, research, and development of gene drugs and prognosis.

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Disclosure of conflict of interest
None.

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References
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