Original Article
Expressions of miR-193b and miR-338-3p in renal clear-cell carcinoma cells and their effects on biological function

Guangbin Zhu, Changying Xie, Liliang Li

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Abstract: Objective: To evaluate the expressions of miR-193b and miR-338-3p in renal clear-cell carcinoma cells and their effects on biological function. Methods: Cancer tissues, adjacent normal tissues, and serum samples were obtained from 34 patients with renal clear-cell carcinoma. Peripheral blood samples from 30 healthy subjects were used as controls. The expression levels of miR-193b and miR-338-3p in renal clear-cell carcinoma cells and serum samples were compared with those in the control group. Results: The relative expression level of miR-193b was significantly higher in cancer tissues of patients with renal clear-cell carcinoma than in the adjacent noncancerous tissues (P<0.05). The relative expression of miR-338-3p was significantly lower in the cancer tissue than in the adjacent noncancerous tissues (P<0.05). The relative expression level of miR-193b in the serum of patients was significantly higher than that observed in the serum of healthy subjects (P<0.05). The relative expression level of miR-338-3p was significantly lower in patients than in healthy subjects (P<0.05). The results of MTT in vitro proliferation test showed statistical differences in the absorbance values of the three groups treated with miR-193b mimic at each time point (P<0.05). Transwell in vitro invasion test showed statistically significant differences in the number of invading cells in the three groups subjected to miR-193b mimic treatment (P<0.05). Transwell in vitro invasion test showed statistically significant differences in the number of invading cells in the three groups subjected to miR-193b mimic treatment (P<0.05). Significant difference was also observed in the number of invading cells between miR-193b mimic group (higher) and miR-control group (P<0.05). There were statistical differences in the number of transmembrane cells in the three groups of miR-338-3p cells (P<0.05). The number of transmembrane cells of miR-338-3p mimic and miR-control group was significantly lower than that of miR-338-3p inhibitor group (P<0.05), and the number of transmembrane cells of miR-338-3p mimic group significantly lower than the miR-control group (P<0.05). Conclusion: miR-193b and miR-338-3p was associated with renal clear-cell carcinoma. miR-193b has a function similar to that of a tumor inducer gene, while miR-338-3p functions as a tumor suppressor.

Keywords: miR-193b, miR-338-3p, renal clear cell carcinoma, proliferation, invasion

Introduction

About 70%-80% of renal parenchymal cancer belong to the renal clear-cell carcinoma type, accounting for about 3% of all malignant tumors. In recent years, the incidence of renal clear-cell carcinoma has increased, and about 250,000 new cases are reported every year, with more than 100,000 registered deaths iao [1, 2]. Surgical treatment is the most effective strategy for renal clear-cell carcinoma. The development of molecular targeted therapy has effectively improved the survival of patients with renal clear-cell carcinoma, but the overall therapeutic effect is still insufficient, and the mechanism underlying the development of renal clear-cell carcinoma is still unclear [3, 4]. Therefore, studies have been directed to evaluate changes on molecular levels in renal clear-cell carcinoma and discover novel therapeutic targets for the clinical treatment of renal clear-cell carcinoma.

MicroRNAs (miRNAs) are extensively expressed in eukaryotic organisms and are known to regulate cell proliferation, differentiation, and apoptosis. Abnormalities in miRNA biosynthesis are associated with a variety of pathophysiological
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processes [5, 6]. Many studies have reported that miRNAs are closely related to biological functions such as proliferation and invasion of tumor cells [7, 8]. miR-193b and miR-338-3p are two highly conserved miRNAs and studies have highlighted their regulatory roles in processes such as proliferation and invasion of ovarian, colon, gastric, and other cancers [9-14]. Furthermore, miR-193b and miR-338-3p are thought to alter the sensitivity of tumor cells to chemotherapeutic drugs [15, 16] and may play important roles in the development and progression of tumors, suggestive of their potential applications for the treatment of tumors. However, very few reports have explored the relationship between miR-193b as well as miR-338-3p and renal clear-cell carcinoma.

In this study, we investigated the potential therapeutic targets for renal clear-cell carcinoma by analyzing the expression levels of miR-193b and miR-338-3p in renal clear-cell carcinoma cells and evaluated their effects on biological behavior.

Methods and materials

Research subjects

Cancer tissues and the adjacent noncancerous tissues were obtained along with serum samples from 34 patients with renal clear-cell carcinoma who were admitted to our hospital from September 2013 to April 2017. In addition, peripheral blood samples from 30 healthy subjects were obtained during the same period. All patients with renal clear-cell carcinoma were diagnosed at the pathology department after tumor operation in our hospital. No distant metastasis was observed according to imaging diagnosis. The survival time was estimated to be ≥3 months. Pregnant and lactating women were excluded from the study. Patients included in the study had no diseases, neurological dysfunction, and abnormal vascular function. The study was approved by the hospital ethics community, and patients or their families signed an informed consent form.

Cell source

Caki-1 human renal clear-cell cancer cell line was purchased from Shanghai Yubo Biotechnology Co., Ltd., and the medium selection company provided McCoy’s 5A media (modified with tricine) containing 10% fetal bovine serum. The culture conditions were 37°C and 5% CO₂. The cell were divided into six groups: the miR-193b mimic, the miR-193b inhibitor, the miR-338-3p mimic, the miR-338-3p inhibitor, the miR-control and the blank control cell groups. The expression vectors for miR-193b mimic, miR-193b inhibitor, miR-338-3p mimic, miR-338-3p inhibitor, and miR-control were designed and synthesized by Herzen (Shanghai) Biotechnology Co., Ltd. miR-193b shared the control group with miR-338-3p.

Cell transfection

Lipofectamine™ 2000 transfection kit was purchased from Shanghai Yanjing Biotechnology Co., Ltd., and the digestive cells and trypsin were adopted for 24 h before transfection. The siRNA, mimic vectors or empty vectors were transfected when the cell reached about 80% confluency. The cells were cultured for 48 h at 37°C in 5% CO₂ incubator and the medium was changed after every 6 h. Transfection results were detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Every 100 mg of tissue sample was subjected to grinding and pulverization, followed by treatment with 1 mL of TRIzol to extract total RNA. Serum samples and cell specimens (cell density of 1 × 10⁷) were added to the serum and TRIzol lysate for total RNA extraction at a ratio of 3:1. After extraction, the integrity of the RNA was analyzed by 1.5% agarose gel electrophoresis, and the purity of the extracted RNA was detected by a micro nucleic acid analyzer. A260/A280 value of 1.45-1.60 was considered adequate to meet the experimental require-
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After the RNA extraction, qRT-PCR reaction was carried out. The reverse transcription reaction system included 1.0 μL of oligo-dT primer, 1.0 μL of dNTP mixture, 2 μg of total RNA, and 10 μL of RNase-free distilled water. The reaction was carried out at 65°C for 5 min. After the completion of the reverse transcription reaction, PCR amplification was performed with 2 μL of cDNA template, 32.5 μL of SYBR Green Mix, 0.5 μL of upstream and downstream primers, and 50 μL of double-distilled water, with an initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 60°C for 60 s. The final extension was performed at 72°C for 5 min. U6 snRNA was used as the reaction internal reference. All samples were repeated thrice and the results were analyzed by the 2^(-ΔΔCt) method. The primer sequence was designed and synthesized by Herzen (Shanghai) Biotechnology Co., Ltd. (Table 1).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay

Caki-1 cells were seeded at a density of 4 × 10^6 cells/mL in 96-well cell culture plates. Following treatment, 20 μL of MTT (5 mg/mL) solution was added to each well and cells were cultured for 4 h at 37°C. The supernatant containing the impurities was removed and each well was treated with dimethyl sulfoxide. The plate was placed on a horizontal vibration bench for 10 min. The absorbance of each well was measured at 570 nm wavelength using an enzyme-linked immunosorbent assay (ELISA) plate reader at 12, 24, 48, and 72 h. MTT test kit was purchased from Shanghai Lianmai Bioengineering Co., Ltd.

Transwell in vitro invasion test

Caki-1 cells were seeded at a density of 5 × 10^5 cells/mL in 100 μL volume in a transwell chamber. The number of cells that migrated was detected after 24 h. Three parallel tests were simultaneously performed. Transwell chamber was purchased from.

Statistical analysis

SPSS19.0 (Asia Analytica, Formerly SPSS, China) was used for statistical analysis. The data were analyzed with χ² test. The measurement data were expressed as mean ± standard deviation (SD). The comparison between groups was performed with the analysis of variance, while the comparison between two groups was performed with t-test. P<0.05 was considered statistically significant.

Results

General information

No significant difference was observed in the gender and age between patients with renal clear-cell carcinoma (P>0.05). The tumor diameter was ≤4 cm in 14 patients (41.18%), while 20 patients (58.82%) showed a tumor diameter of >4 cm. Thirteen cases of histological grade I-II (38.24%) and 21 cases of grade III-IV (61.76%) were observed. In addition, 24 and 10 patients had clinical stage I-II (70.59%) and III-IV (29.41%) cancer, respectively. Fuhrman classification of moderate and poor differentiation revealed 14 cases of (41.18%) poor differentiation and 20 cases of (58.82%) high differentiation. Lymph node metastasis occurred in 23 patients (67.65%) (Table 2).

Results of qRT-PCR for miR-193b

The relative expression level of miR-193b was 1.472 ± 0.023 in cancer tissues from patients with renal clear-cell carcinoma and 1.015 ± 0.014 in the adjacent noncancerous tissues. The relative expression level of miR-193b in cancer tissues was significantly higher than that in the adjacent tissues (P<0.05). On the other hand, the relative expression level of miR-193b in cancer tissues was significantly higher than that in the adjacent tissues (P<0.05). On the other hand, the relative expression level of miR-193b in the serum of patients with renal clear-cell carcinoma and healthy subjects was 1.289 ± 0.018 and 0.874 ± 0.012, respectively. The relative expression level of miR-193b in the serum was significantly higher in patients with renal clear-cell carcinoma than in healthy subjects (P<0.05). Statistical difference was

<table>
<thead>
<tr>
<th>Table 1. Primer sequence</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>miR-193b</td>
<td>5'-CTCGCTTCGGCAGCACA-3'</td>
<td>5'-AACGCTTCAGAATTGCAGT-3'</td>
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<tr>
<td>miR-338-3p</td>
<td>5'-ACATGATTTAACCTGGTGCCT-3'</td>
<td>5'-CTCAACTGTTGTCGGAGA-3'</td>
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<tr>
<td>U6</td>
<td>5'-ACTCTGGCCTAGCCAGCTGT-3'</td>
<td>5'-CTCCGGGAACCAGCATTGTA-3'</td>
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Expressions of miR-193b and miR-338-3p in renal clear-cell carcinoma cells

Table 2. General information

<table>
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<th>Statistic</th>
<th>p-valued</th>
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<td>Sex</td>
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<td>23 (67.65)</td>
<td>21 (70.00)</td>
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<td>Woman</td>
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<td>9 (30.00)</td>
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<tr>
<td>Age (year)</td>
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<td>51.82 ± 12.46</td>
<td>0.829</td>
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<td>Diameter of tumor</td>
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<td>≤4 cm</td>
<td>14 (41.18)</td>
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<tr>
<td>&gt;4 cm</td>
<td>20 (58.82)</td>
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<tr>
<td>I-II</td>
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<tr>
<td>III-IV</td>
<td>21 (61.76)</td>
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<tr>
<td>I-II</td>
<td>24 (70.59)</td>
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<tr>
<td>III-IV</td>
<td>10 (29.41)</td>
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<td>Fuhrman grade</td>
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<td>20 (58.82)</td>
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<td>10 (29.41)</td>
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<tr>
<td>No</td>
<td>11 (32.35)</td>
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Figure 1. A. qRT-PCR results of miR-193b in cancer tissues, adjacent noncancerous tissues, and serum samples of patients with renal clear-cell carcinoma. B. qRT-PCR results of miR-193b expression in the three groups of Caki-1 human renal clear-cell carcinoma cells. *P<0.05.

Results of qRT-PCR analysis of miR-338-3p

The relative expression level of miR-338-3p in cancer tissues of patients with renal clear-cell carcinoma was 0.654 ± 0.011, while that in the adjacent noncancerous tissues was 1.152 ± 0.018. Furthermore, the relative expression of miR-338-3p in cancer tissues was significantly lower than that observed in the adjacent noncancerous tissues (P<0.05). The relative expression level of miR-338-3p in serum of patients with renal clear-cell carcinoma and healthy subjects was 0.457 ± 0.013 and 1.038 ± 0.012, respectively. The relative expression level of miR-338-3p was significantly lower in serum of patients with renal clear-cell carcinoma than in the serum of healthy subjects (P<0.05). Statistical difference was observed in the relative expression levels of miR-193b in cells from the three
Expressions of miR-193b and miR-338-3p in renal clear-cell carcinoma cells

The occurrence and development of renal clear-cell carcinoma is a multistep multi-molecular pathological process. The near-infinite proliferation ability of malignant tumor cells and high invasion and metastasis abilities are the most important biological characteristics. The high recurrence and metastasis rates have posed great difficulties in the clinical treatment of
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The development of molecular biology and related therapeutic methods has attracted attention of several researchers to study the role of miRNAs in tumors. Studies have shown that the abnormal expression of miR-193b and miR-338-3p in various malignant tumors have affected the biological behavior of tumor cells [13, 14]. In this study, we examined the changes in the expression levels of miR-193b and miR-338-3p in tissues and serum samples from patients with renal clear-cell carcinoma and analyzed their effects on the proliferation and invasion ability of Caki-1 cells. As a result, we found that these miRNAs were key factors involved in the occurrence and development of renal clear-cell carcinoma and may serve as potential therapeutic targets for the treatment of renal clear-cell carcinoma.

We analyzed the relative expressions of miR-193b and miR-338-3p in tissues and serum samples of patients with renal clear-cell carcinoma and included cancerous tissues, adjacent noncancerous tissues, and serum samples from 34 patients with renal clear-cell carcinoma along with the blood samples from 30 healthy subjects. No significant difference in the gender and age between two groups was observed. qRT-PCR results showed that the relative expression of miR-193b in renal clear-cell carcinoma tissue was significantly higher than that observed in the adjacent noncancerous tissues, and the relative expression level in patients' serum was higher than that in the serum of healthy subjects. Furthermore, the relative expression of miR-338-3p in cancer tissues was significantly lower than that in the adjacent noncancerous tissues and serum samples of patients. In addition, the relative expression level miR-338-3p was lower in the serum samples from patients than in those from healthy subjects, suggesting that miR-193b and miR-338-3p may be involved in the development or progression of renal clear-cell carcinoma. Studies have reported that the expression of miR-193b is lower in ovarian cancer [9] but higher in colorectal and gastric cancers [10, 11]. Therefore, we speculate that miR-193b plays different roles in various parts of the tumor and may have several downstream target genes. Whether the target gene is activated or inhibited may depend on the tumor microenvironment in which the cancer cells are located. A study on miR-338-3p showed that the relative expression of miR-338-3p decreased in ovarian, colon, and gastric cancers [12, 13], consistent with the results of the present study. Hence, we speculate that miR-338-3p may play same functions during the development of different tumors. We also analyzed the effects of miR-193b and miR-338-3p on the proliferation and invasion of Caki-1 human renal clear-cell carcinoma cells. Caki-1 cells

![Figure 3. The effect of miR-193b on the proliferation of Caki-1 cells, as detected by MTT assay. *P<0.05 compared with miR-193b inhibitor group; #P<0.05 compared with miR-control group.](image1)

![Figure 4. The effect of miR-338-3p on the proliferation of Caki-1 cells, as detected by MTT assay. *P<0.05 compared with miR-193b inhibitor group; #P<0.05 compared with miR-control group.](image2)
Expressions of miR-193b and miR-338-3p in renal clear-cell carcinoma cells

We designed an overexpression vector and low-expression vector of miR-193b and miR-338-3p and used a blank vector as a control. We transfected these vectors into Caki-1 cells and analyzed the transfection results by qRT-PCR. The corresponding miRNA expression levels in miR-338-3p mimic and miR-193b mimic groups were higher than those observed in the miR-control group, while miRNA expression levels were lower in the miR-193b inhibitor and miR-338-3p inhibitor groups than in the miR-control group, suggestive of the successful transfection of cells. We used MTT in vitro proliferation test and transwell in vitro invasion test to analyze the proliferation and invasion ability of transfected Caki-1 cells. The results showed that the overexpression of miR-193b may result in the proliferation and invasion of Caki-1 cells. The proliferation and invasion abilities of Caki-1 cells showing low expression of miR-193b were significantly higher than those of cells with high expression of miR-193b, suggesting that miR-193b has similar tumor-promoting functions in renal clear-cell carcinoma cells. The proliferation and invasion abilities of Caki-1 cells with high expression of miR-338-3p were significantly lower than those of cells with low expression of miR-338-3p, indicating that miR-338-3p functions as a tumor suppressor in renal clear-cell carcinoma. It was shown that miR-193b promotes the proliferation and invasion of gastric and pancreatic cancer cells, while miR-338-3p regulates the proliferation, invasion, and other biological behaviors of gastric cancer cells, glioblastoma, non-small cell lung cancer, and other tumor cells [14, 19-22]. These reports have demonstrated that miR-193b and miR-338-3p have the ability to regulate tumor cell proliferation and invasion, as observed in the present study. However, the present study has a few shortcomings. The time span of this study was short, owing to the experimental conditions. Caki-1 human renal clear-cell carcinoma cells were used in the second part of the study. Given the differences between Caki-1 cells, tumor cells from patients, and tumor microenvironment, the results and conclusions of this study need to be further validated with more experiments and clinical data. This study will attract more researchers to evaluate molecular and therapeutic targets for renal clear-cell carcinoma.

In summary, miR-193b and miR-338-3p may play a very important role in the occurrence and development of renal clear-cell carcinoma. miR-193b performs the function of a tumor-promoting gene, while miR-338-3p acts as a tumor suppressor. miR-193b and miR-338-3p may be new potential therapeutic targets for renal clear-cell carcinoma treatment.

Disclosure of conflict of interest

None.
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