Original Article

MicroRNA-194-3p inhibits the metastatic biological behaviors of spinal osteosarcoma cells by the repression of matrix metallopeptidase 9

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Abstract: Osteosarcoma is the most common primary malignant bone tumor, but only 3%-5% of cases occur in the spine. Spinal osteosarcoma presents a significant challenge, and most patients die in spite of aggressive surgery. MicroRNAs (miRNAs) are small noncoding RNAs that have a pivotal role in the post-transcriptional regulation of gene expression. The aim of this study was to investigate the role of miR-194-3p and to identify its potential mechanism in spinal osteosarcoma. Here, spinal osteosarcoma tissues showed down-regulated expression of miR-194-3p compared to adjacent non-tumorous tissues. The level of miR-194-3p was negatively correlated with metastasis in patients with spinal osteosarcoma. MiR-194-3p over-expression in spinal osteosarcoma cells significantly inhibited cell migration and invasion in vitro. Furthermore, mechanistic analyses showed that MMP-9 (matrix metallopeptidase 9) is a direct target of miR-194-3p, and the ectopic expression of miR-194-3p inhibits MMP-9 expression by directly binding to the 3'-untranslated region (3'-UTR) of the MMP-9 gene. In summary, our results demonstrate that miR-194-3p suppresses migration and invasion of spinal osteosarcoma cells by targeting MMP-9, indicating miR-194-3p may serve as a promising novel target for spinal osteosarcoma therapy.

Keywords: Spinal osteosarcoma, miR-194-3p, cell, gene, MMP-9

Introduction

Osteosarcoma, characterized by the production of osteoid or immature bone from malignant cells, is the most common malignant bone tumor with high morbidity in children and adolescents [1]. Although osteosarcoma is considered to be the most common primary bone tumor, it represents only 3%-5% of all spinal malignancies [2, 3]. Spinal osteosarcoma is a high-grade, malignant tumor with a poor prognosis. Various treatment methods have been advocated for spinal osteosarcoma, including chemotherapy, radiation therapy, and surgical resection; however, patients with metastatic or recurrent spinal osteosarcoma still have extremely poor survival outcomes [4]. Therefore, the identification and elucidation of the underlying molecular mechanisms that contribute to spinal osteosarcoma metastasis will be pivotal in the development of new therapeutic strategies for the cancer [5].

MicroRNAs (miRNAs) are a subset of endogenous, small, non-coding RNAs consisting of 18-24 nucleotides that inhibit target gene expression by directly binding with the 3'-untranslated regions (UTRs) of messenger RNAs (mRNAs) [6]. Accumulating studies suggest that miRNAs are involved in various cell processes, including proliferation, apoptosis, and metastasis [7]. MiRNAs have also been shown to be involved in the regulation of a variety of diseases in eukaryotes [8]. Recently, the dysregulation of miRNAs has been identified in osteosarcoma, which is closely associated with tumor initiation and progression. For instance, miR-494 inhibits the proliferation and metastasis of osteosarcoma cells by suppressing IRS1 (insulin receptor substrate 1) [9]. MiR-130a promotes the metastasis and epithelial-mesenchymal transition of osteosarcoma by targeting PTEN (phosphatase and tensin homolog) [10]. MiR-145 suppresses cellular metastasis in osteosarcoma via targeting MMP-16 (matrix
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Metallopeptidase 16) [11]. MiR-29b represses the proliferation and migration of osteosarcoma cells by targeting CDK6 (cyclin dependent kinase 6) [12].

MiR-194 has been identified as a tumor suppressive miRNA, and it down-regulates expression in several types of human cancers, including laryngeal squamous cell carcinoma [13], glioma [14], prostate cancer [15], and gastric cancer [16]. However, the biological function and molecular mechanism of miR-194-3p in spinal osteosarcoma remain to be elucidated. In this study, we showed that miR-194-3p was markedly down-regulated in spinal osteosarcoma tissues. We also demonstrated that miR-194-3p up-regulation inhibited the migration and invasion of spinal osteosarcoma cells. Notably, we identified that miR-194-3p negatively regulated MMP-9 (matrix metallopeptidase 9) by binding to its 3'-UTR of mRNA leading to the inhibition of MMP-9 translation.

Materials and methods

Ethics statement

This research involving human spinal osteosarcoma tissues has been approved by the Ethics Review Committee of the People’s Hospital of Xinjiang Uygur Autonomous Region. Consent forms were signed by all patients and the consent process was conducted in accordance with the regulations of Declaration of Helsinki.

Clinical samples

Pathology-confirmed spinal osteosarcoma tissues (n = 21) and adjacent non-tumorous tissues (n = 21) were obtained from the People’s Hospital of Xinjiang Uygur Autonomous Region between January 2001 and December 2014. Patients with any other malignancies were excluded. None of the patients had received preoperative treatment, such as radiotherapy or chemotherapy. All samples were flash-frozen in liquid nitrogen immediately and stored at -80°C until needed for further experiments.

Cell lines

Human osteosarcoma cell lines (U2OS and MG-63) were purchased and authenticated from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) containing streptomycin (100 mg/ml) and penicillin (100 U/ml), and maintained in an incubator at 37°C with 5% CO₂.

Cell transfection

Cells were seeded into 6-well plates and incubated at 37°C with 5% CO₂ overnight. miR-194-3p mimics and its mimics control (NC) were synthesized by GenePharma (Shanghai, China) in order to detect the function of miR-194-3p in spinal osteosarcoma. The sequences were as follows: mimics: 5'-GUCUAUUGUCGGGGUGACCC-3'; NC: 5'-CAGUAUAGUAUAGGACAA-3'. After 70-80% confluence, the cells were transfected with 100 nM mimics or NC by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocols.

RNA extraction and real-time PCR

Total RNA was isolated by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocols. Complementary DNA was synthesized with 1 µg of total RNA by using the One Step Prime script miRNA cDNA Synthesis Kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed using a SYBR Green Premix Ex TaqIIkit (Takara, Japan) on Light Cycler 480II (Roche, Rotkreuz, Switzerland). The U6 small nuclear RNA was used as an endogenous control. The primers used for the amplification were as follows: miR-194-3p: 5'-GTCTATTGTCGTCGGGGTGACC-3' (sense), miScript SYBR Green PCR kit Universal Primer (antisense); U6: 5'-CTCGCTTCGGGAGCACAGCACT-3' (sense), 5'-AAGCCTTCAGAAATTTGCGT-3' (antisense). The qPCR conditions consisted of 20 min of DNA polymerase activation at 95°C, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Data were analyzed using the comparative Ct method.

Cell migration and invasion assay

For the cell migration assay, the transfected cells were suspended in 200 µL of FBS-free DMEM medium and seeded on the upper compartment of a 24-well Transwell culture chamber, and 500 µL of complete medium was added to the lower compartment. After incubation at 37°C, the cells were fixed with methanol. The non-migratory cells were removed from the upper surface of the filter carefully with a cot-
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Figure 1. Real-time PCR analysis of miR-194-3p expression levels in spinal osteosarcoma tissues and adjacent non-tumorous tissues. The U6 was used as an endogenous control. The results showed that miR-194-3p is significantly down-regulated in spinal osteosarcoma tissues compared with adjacent non-tumorous tissues. *P < 0.05.

Ton swab. The migratory cells on the lower side of the filter were stained with crystal violet and counted.

For the invasion assay, the transfected cells were suspended in a 200 μL FBS-free DMEM medium and plated into the top chamber of BD Biocoat Matrigel-coated invasion chambers with 8.0-μm pore sizes (BD Biosciences, CA, USA), while a complete medium containing 10% FBS was added to the lower chamber. After processing the invasion chambers for 48 hours at 37°C, the non-invading cells were removed with a cotton swab, and the invading cells were fixed in 100% methanol and then stained with crystal violet solution, and then the cells in 5 random 100 × fields per well were counted.

Bioinformatics analysis of the miR-194-3p target gene

Three databases: miRanda (http://www.microrna.org), Pictar (http://pictar.mdc-berlin.de/) and Targetscan (http://www.targetscan.org/) were applied to identify miR-194-3p target gene.

Luciferase reporter assay

A 420-bp fragment of MMP-9 mRNA 3′-UTR containing the miR-194-3p binding side was amplified by PCR from MG-63 cells, and cloned into downstream of the renilla luciferase gene of psiCHECK-2 luciferase vector (Promega, WI, USA) to develop wild-type luciferase plasmid. The seed sequence of miR-194-3p binding site in MMP-9 mRNA 3′-UTR was mutated by using a QuikChange Mutagenesis Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocols. The mutant MMP-9 mRNA 3′-UTR fragment was also cloned into a psiCHECK-2 luciferase vector to develop the mutant luciferase plasmid. For dual the luciferase reporter assays, MG-63 cells at a density of 5 × 10^4 per well in 24-well plates were co-transfected with 0.5 μg of a wild-type luciferase plasmid or a mutant luciferase plasmid and 50 nM mimics or NC by using the lipofectamine 2000 reagent. After 48 h of transfection, the luciferase activity was measured using a dual luciferase reporter assay system (Promega, WI, USA).

Western blot

The MG-63 cells were lysed with a RIPA lysis buffer (Invitrogen, CA, USA). 12% sodium SDS-PAGE was used for separating the total proteins into equal amounts and transferred to polyvinylidene fluoride membranes (PVDF) (Millipore, MA, USA). After blocking with 5% non-fat milk for 2 h, the membranes were incubated with anti-MMP-9 and GAPDH antibodies (Millipore, MA, USA) overnight, followed by an anti-rabbit HRP-linked secondary antibody (Millipore, MA, USA). GAPDH was used as a loading control. Bands were obtained using Imaging System (BioRad, CA, USA), and quantified with Odyssey v1.2 software (LI-COR Biosciences, NE, USA).

Statistical analysis

The statistical analysis was conducted with SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). The data calculations were repeated at least three times and presented as the mean ± standard deviation (SD). The differences between two groups were evaluated using the Student’s t-test or one-way ANOVA. Values of P < 0.05 were considered to be statistically significant.

Results

MiR-194-3p is significantly down-regulated in spinal osteosarcoma tissues

We performed a real-time PCR assay to detect the miR-194-3p expression levels in both spinal osteosarcoma tissues and adjacent non-tumorous tissues. The results showed that miR-194-
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Table 1. The relationship between miR-194-3p expression and clinicopathologic characteristics in spinal osteosarcoma

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases (21)</th>
<th>miR-194-3p expression</th>
<th>p</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Low (11)</td>
<td>High (10)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>&lt; 20 years</td>
<td>6</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>≥ 20 years</td>
<td>15</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Sex</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>3</td>
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</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5 cm</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>≥ 5 cm</td>
<td>17</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Histological type</td>
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</tr>
<tr>
<td>Osteoblastic</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Others</td>
<td>9</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>13</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>7</td>
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<td>6</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

3p was significantly down-regulated in spinal osteosarcoma tissues compared to the adjacent non-tumorous tissues (Figure 1, P < 0.05).

The level of miR-194-3p is negatively correlated with metastasis in patients with spinal osteosarcoma

In order to further illuminate the possible relationship between miR-194-3p expression and the clinicopathologic features, 21 patients with spinal osteosarcoma was divided into low (n = 11) and high (n = 10) expression groups based on the median expression levels of miR-194-3p in spinal osteosarcoma tissues as the cut-off point (0.57; range from 0.17 to 0.94). As shown in Table 1, the expressions of miR-194-3p were significantly associated with metastasis (P < 0.05); however, no significant associations were observed between the miR-194-3p level and a patient’s age, gender, tumor size, histological type, or TNM stage. In addition, real-time PCR assay revealed that miR-194-3p expression was lower in the patients with metastasis than those patients with no metastasis (Figure 2, P < 0.05). These results indicate that miR-194-3p expression is negatively correlated with metastasis in patients with spinal osteosarcoma.

The restoration of miR-194-3p reduces the migration and invasion of spinal osteosarcoma cells

In order to identify the underlying roles of miR-194-3p in spinal osteosarcoma cells, U2OS and MG-63 cells were transfected with mimics or NC. Analysis of real-time PCR confirmed that U2OS and MG-63 cells transduced with mimics had significantly higher levels of miR-194-3p expression than the cells treated with NC (Figure 3, P < 0.05).

We further explored the cancer-related roles of miR-194-3p up-regulation in U2OS and MG-63...
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Restoration of miR-194-3p reduces the migration and invasion of spinal osteosarcoma cells. A. The number of migratory cells was remarkably decreased in U2OS and MG-63 cells after their transfection with mimics compared to the cells treated with NC. B. A transwell assay showed that the number of invasive cells was significantly reduced in U2OS and MG-63 cells after transfection with mimics compared to the cells treated with NC. *P < 0.05.

MMP-9 is a direct target gene of miR-194-3p

We then studied several online databases, including miRanda, Pictar and Targetscan to search the downstream target gene of miR-194-3p. Interestingly, we found that the fragment at 3'-UTR of MMP-9 mRNA could complementarily bind with miR-194-3p (Figure 5A). To identify whether miR-194-3p could directly regulate MMP-9, a dual-luciferase reporter assay was performed. We thus generated a wild-type luciferase plasmid and mutant luciferase plasmid and co-transfected them with mimics or NC into the MG-63 cells, and the luciferase activity was measured after 48 h of transfection. As shown in Figure 5B, when the MG-63 cells were transfected with mimics the relative luciferase activity significantly decreased in the wild-type luciferase plasmid (P < 0.05); however, the relative luciferase activity of the mutant luciferase plasmid remained unaffected. To further verify whether the over-expression of miR-194-3p down-regulated the endogenous MMP-9 expression, the mimics or the NC was transfected into the MG-63 cells. After 48 h transfection, the protein expression level of MMP-9 was detected by Western blot. Consistent with the results of the dual-luciferase reporter assay, we observed that the protein levels of MMP-9 were reduced in the MG-63 cells after they were treated with mimics (Figure 5C). These results indicate that MMP-9 is a direct target gene of miR-194-3p.

Discussion

Spinal osteosarcoma represents 3-15% of primary spinal tumors and 3%-5% of all spinal malignancies [17]. Despite the advances of modern medicine, the 5-year overall survival rate for patients with spinal osteosarcoma is less than 30% [18]. Metastasis is the major reason for the poor survival of spinal osteosarcoma patients. Therefore, the exploration of the molecular mechanisms involved in spinal osteosarcoma progression and potential novel therapeutic targets are very important.

Increasing evidence indicates that miRNAs may function as oncogenes or tumor suppressors in spinal osteosarcoma [19]. For example, Wang et al. showed that miR-103 promotes proliferation and inhibits apoptosis in spinal osteosarcoma cells by targeting p57 [20]. Wang et al. reported that miR-520b suppresses the proliferation, migration, and invasion of spinal osteosarcoma cells by downregulating Frizzled-8 (frizzled class receptor 8) [21]. Liu et al. demonstrated that miR-373 promotes growth and cellular invasion in spinal osteosarcoma cells by the activation of the PI3K/AKT-Rac1-JNK pathway [22]. MiR-194 is a vertebrate specific miRNA with a known role in mitochondrial energy production, the inhibition of inflammation, chondrogenesis and neuronal differentiation.
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Then, we performed functional experiments to assess the effects of miR-194-3p on the migration and invasion of spinal osteosarcoma cells. Consistent with previous studies [24-26], we found that the restoration of miR-194-3p reduced the migration and invasion of spinal osteosarcoma cells. These results suggest that miR-194-3p acts as a tumor suppressor gene in spinal osteosarcoma and is involved in the metastasis of spinal osteosarcoma.

It is clear that miRNAs execute their roles by regulating the expression of target genes. By performing a bioinformatics analysis, we found a potential target gene of miR-194-3p and selected MMP-9 as the potential candidate. MMP-9 was shown to be involved in the invasiveness and metastasis of endometrial cancer as well as other malignant tumors, such as osteosarcoma, renal cancer, and lung cancer [27-29]. Here, following the over-expression of miR-194-3p in MG-63 cells, MMP-9 expression was significantly decreased. Based on the above data, we performed a bioinformatics analysis using the TargetScan, miRanda, and PicTar online databases, identifying a potential miR-194-3p binding site in the 3'-UTR of MMP-9. Furthermore, a dual luciferase reporter assay also provided direct evidence that MMP-9 was a direct target gene of miR-194-3p.

In conclusion, our study is the first to demonstrate the decreased expression of miR-194-3p suppressed cell migration and invasion in spinal osteosarcoma. To the best of our knowledge, our research identified, for the first time, the miR-194-3p/MMP-9 axis in spinal osteosarcoma cells that is responsible for the metastasis. Thus, miR-194-3p may be a novel therapeutic target for the treatment of spinal osteosarcoma in the future.

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Disclosure of conflict of interest

None.

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