MicroRNA-30c suppressed giant-cell tumor of bone cell metastasis and growth via targeting HOXA1


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Abstract. - OBJECTIVE: To dissect the functioning mode of miR-30c on giant cell tumor of bone cell metastasis and growth and provide therapeutic targets for giant cell tumor of bone. PATIENTS AND METHODS: By quantitative Real-time polymerase chain reaction (qRT-PCR), miR-30c expression level in 62 pairs of giant cell tumor of bone cells tissue samples and five breast cancer-derived cell lines. Using miR-30c mimics and inhibitors, we analyzed the effects of miR-30c over-expression and knockdown on cell proliferation, invasion, and migration. Dual-luciferase activity assay was recruited to examine the potential target gene HOXA1, which predicted by several databases. Protein level was studied using Western blot. RESULTS: MiR-30c expressed significantly lower in giant cell tumor of bone tissue samples and cell lines. Over-expression miR-30c in giant cell tumor of bone cells decreased the cell proliferation, invasion, and migration abilities while down-regulation miR-30c in giant cell tumor of bone cells increased these abilities opposite-ly. Dual-luciferase and Western blot confirmed HOXA1 as a target gene of miR-30c. Furthermore, up-regulation of HOXA1 reserved the suppressive effect of miR-30c over-expression on cell growth and progression. CONCLUSIONS: miR-30c could suppress giant cell tumor of bone cell proliferation and progression via HOXA1, which might provide a new target for giant cell tumor of bone diagnosis and therapy.

Key Words: Giant cell tumor of bone, miR-30c, Proliferation and invasion, HOXA1.

Introduction

Giant cell tumor of bone (GCT) is one of the most common primary invasive bone tumors in China, accounting for about 6% of all bone tumors, and is common in people aged 20-40 years1,2. Giant cell tumors of the bone occur in the metaphysis of the long bones of the limbs, and are rare in the sacrum, pelvis, and spine3. Giant cell tumor of bone often shows strong local invasion, while osteolytic bone destruction is typical of its clinical features4. Osteolytic bone destruction can lead to severe bone pain and pathological fractures, and seriously affect the quality of life of patients5,6. Giant cell tumor of bone is insensitive to radiotherapy and chemotherapy, and surgical resection is the most important treatment7. With the rapid development of surgical techniques, the overall resection of giant cell tumor of bone is widely used in clinical practice, but the recurrence rate of giant cell tumor of bone has not been high, reaching 22.4-41.7%8. Giant cell tumor is defined as borderline or malignant primary bone tumor given its local invasive growth and postoperative recurrence rate9. Osteolytic bone destruction and abnormal proliferation are the most typical pathological features of giant cell tumor of bone, as well as the important reason for affecting the quality of life and the overall survival time of patients with giant cell tumor, but the specific pathogenesis is not clear.

MicroRNA (miRNA) is a class of non-coding RNA with an average length of 22 nucleotides. It is widespread in vivo and participates in a series of physiological and pathological processes in vivo10. MicroRNA works by binding to a non-coding region (3’-UTR) of a specific target gene mRNA and inhibiting or directly cutting off the target mRNA translation process10. Bioinformatics analysis of the human genome
library shows that more than 1/3 of human genes can be regulated by microRNA \textsuperscript{12}. At present, the role of microRNA in tumorigenesis has become the focus of basic research at home and abroad, but its specific role in giant cell tumor of bone has not been described yet\textsuperscript{13}. MiR-30c was showed to act as a tumor suppressor in several cancers \textit{via} targeting different genes; for instance, in prostate cancer, miR-30 c inhibited survival by regulating ASF/SF2\textsuperscript{14}. In gastric cancer, it decreased cell proliferation, metastasis and epithelial to mesenchymal transition (EMT) targeting MTA1\textsuperscript{15}, and miR-30c decreased non-small cell cancer cell proliferation \textit{via} targeting Rab18\textsuperscript{16}. However, whether miR-30c was abnormally expressed in GCT and how it affects GCT still remains unclear.

In our study, to reveal the relationship between miR-30c and GCT, we first evaluated the miR-30c expression level of 62 pairs of GCT and adjacent normal tissues as well as in 5 cell lines. We found miR-30c expressed lower in GCT tissue samples and cells. Then, by up-regulation and down-regulation of miR-30c, we observed that miR-30c could decrease cell proliferation, invasion and migration in GCT. Furthermore, HOXA1 was identified to be a potential target for miR-30c using dual-luciferase assay and Western blot. The restoration of HOXA1 could impair tumor suppression role of miR-30c. Taken all together, our study could provide miR-30c as a novel target for GCT therapy in the future.

\section*{Patients and Methods}

\subsection*{Tissue Samples and Cell Lines}
All 62 pairs of GCT and adjacent normal tissue samples were collected from the First Hospital of Harbin City and all the patients had not received any chemotherapy before surgery. All recruited patients signed the informed consents and the study was approved by the medical Ethics Committee of the First Hospital of Harbin City. The tissue samples were conserved in liquid nitrogen before next experiments. The clinical features were collected according to the standard of the American Joint Committee on Cancer (AJCC). Human giant-cell tumor of bone cell lines (GCT cells) was purchased from the Cell Bank of Chinese Academy of Sciences. GCT cells were cultured in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Gibco) (Carlsbad, CA, USA), and were maintained at temperature of 37°C with 5% CO\textsubscript{2} in the humid air.

\subsection*{Cells Transfection}
The miR-30c mimics and inhibitors were provided by Genepharm (Shanghai, China). GCT cells were planted into six-well plate and culture to density of 60-70%, then, incubated with miR-30c mimics, miR-30c inhibitors or relative negative control in serum-free DMEM containing lipofectamine 3000 for 48 h according to the specifications. The transfection efficiency was measured by qRT-PCR.

The plasmid pcDNA3.1-HOXA1 was synthesized by Genepharm (Shanghai, China). Cells were cultured for 24 h in six-well plate, and then incubated with pcDNA3.1-HOXA1 mixed with lipofectamine 3000 in serum-free DMEM. After maintained 48 h in normal culture atmosphere, the expression of HOXA1 was confirmed.

\subsection*{RNA Extraction and Quantitative Real-time PCR}
Total RNA of tissue samples and cells were isolated by using TRIZol reagent (Invitrogen, Carlsbad, CA, USA) and reversed using a miRNA Reverse Kit (TaKaRa, Dalian, China). The miR-30c expression level was detected using SYBR Premix kits (TaKaRa, Dalian, China) with ABI Step One (ABI, Vernon, CA, USA) and U6 was used as internal control. Each experiment was confirmed three times. All the relative expression levels were measured by using the 2\textsuperscript{-ΔΔCT} method.

\subsection*{CCK8 Assay}
CCK8 assay kit (Dojindo, Kumamoto, Japan) was applied to measure the proliferation of cells. The cells seeded in a density of 1x10\textsuperscript{3} cells with 100 uL medium were cultured for 24, 48, 72, 96 h after transfection. A total of 10 uL CCK8 reagent was added into the wells and absorbance of 450 nm was measured.

\subsection*{Colony Formation Assay}
To further investigated cell growth of GCT cells, cells were plated in 6-well plates at a density of 600 per well and maintained in normal medium for 10 days. The colonies were fixed in 70% methanol for 20 min and then stained with 0.5% crystal violet for 10 min on ice, washing each well 3 times with phosphate-buffered saline (PBS).

\subsection*{Wound-Healing Assay}
Wound-healing assay was applied to detect the ability of cell migration. Cells were put into
six-well plates and cultured to a density of 100%. After washing with PBS for 3 times, 3 scratches per well were created using 200 μL tip on the surface of cells. Then, the cells were incubated in serum-free DMEM for 48 h and the wound healing condition was measured using microscope at 0 h and 48 h.

**Transwell Assay**

Transwell inserts (Millipore, Billerica, MA, USA) were used to measure the invasion ability. A total of 1×10⁵ treated cells in 10% FBS medium were seeded into the top chamber of the insert, which had plated Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and the lower chamber was immersed in FBS-free medium. After 24 h incubation, the membrane containing cells on its lower surface was fixed with precooled methanol and stained with 0.5% crystal violet. Next, the cells stained were calculated after pictures taken using a microscope in five random visions.

**Dual-Luciferase Assay**

The Dual-Luciferase reporter system (Promega, Madison, WI, USA) was employed to test the activity of luciferase. The HOXA1 3’-UTR cDNA fragment containing the wild type or mutant miR-30c binding site was amplified and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). GCT cells were transfected with miR-30c mimics and the conducted PGL3 vector using lipofectamine 3000. The activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured as the fold-change to the basic pGL3 vector relatively.

**Western Blot**

Reagent RIPA (Beyotime, Shanghai, China) was utilized to extract protein from cells. BCA protein assay kit (TaKaRa, Otsu, Shiga, Japan) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then they were transformed onto the polyvinylidene fluoride (PVDF) membrane and then incubated with antibodies. Cell Signaling Technology (Danvers, MA, USA) provided us with rabbit anti-HOXA1 and rabbit anti-GAPDH, as well as goat anti-rabbit secondary antibody. Chemiluminescent film was applied for assessment of protein expression with ImageJ software.

**Statistical Analysis**

Statistical analysis was done using SPSS 19.0 software (IBM, Armonk, NY, USA) and all quantitative data were showed as mean ± SD. One-way ANOVA test was used for comparison between groups followed by post hoc test (Least Significant Difference). p values < 0.05 were considered having obvious significant.

**Results**

**MiR-30c Expressed Lower in GCT Tissues and Cell Lines**

To detect the relationship between the miR-30c expression and giant cell tumor of bone, we examined miR-30c levels in 62 pairs of GCT tissues and adjacent normal tissues as well as GCT cel-
ls. Clearly shown in the Figure 1A, GCT tissues expressed significantly lower miR-30c level than adjacent normal tissues. This result indicated that miR-30c could suppress GCT tumorigenesis.

To further study the effect of miR-30c in GCT, we next over-expressed or knockdown miR-30c expression by transfected with miR-30c mimics or inhibitor (Figure 1B and C).

**MiR-30c Over-Expression Inhibited the Proliferation of GCT Cells**

CCK8 and colony formation assay were used to explore the miR-30c effects in cell proliferation. GCT cells treated with miR-30c mimics performed reduced proliferation activity than negative control group (Figure 2A), while GCT cells transfected with miR-30c inhibitors displayed increased growth ability than its control group (Figure 2B). Also, GCT cells formed lesser colonies after miR-30c mimics treatment (Figure 2C) and GCT cells formed more colonies after miR-30c inhibitors transfection compared to control group, relatively (Figure 2D). All these data suggested that miR-30c could inhibit GCT cells proliferation.

**Up-Regulation of miR-30c Inhibited the GCT Cell Migration and Invasion**

We measured the miR-30c influence in GCT cells migration abilities using wound-healing assay. Over-expressed miR-30c in GCT cells significantly decreased the migration ability (Figure 3A); in contrast, knockdown of miR-30c in GCT cells increased the wound-healing ability compa-
red with control cells (Figure 3B). In addition, to evaluate the influence of miR-30c in cell invasion, we carried out transwell invasion assay and found sharp decline after miR-30c overexpression (Figure 3C) but markedly enhancement after miR-30c inhibition in cell invasion ability (Figure 3D). These results illustrated miR-30c suppressed GCT cell migration and invasion.

Figure 3. MiR-30c effected the migration and invasion of GCT cells. A and B, Wound-healing assay was performed to determine proliferation of GCT cells treating with miR-30c mimics or inhibitors compared to each negative control. E and F, Transwell invasion assay was used to detect the invasion ability of miR-30c mimics treated GCT cells (C) or miR-30c inhibitors treated GCT cells (E). Data are presented as the mean ± SD of three independent experiments. **p< 0.01.
HOXA1 was a Target Gene of miR-30c

To further explore the molecular mechanism of miR-30c involved in GCT, we next searched three databases: Targetscan, miRwalk, and PicTar. After comprehensive analysis, we found HOXA1

Figure 4. HOXA1 was a direct target of miR-30c. A, The predicted binding sites of miR-30c in the 3'-UTR of HOXA1. B, Dual-luciferase reporter assay was used to determine the binding site. GCT cells treated by mimics or NC were transfected with pGL3 construct containing the WT or mutant HOXA1 3'-UTR site. C and D, Levels of HOXA1 protein measured by Western-blot in miR-30c overexpression GCT cells (C) and miR-30c knockdown GCT cells (D). The protein levels were normalized to that of GAPDH. Data are presented as the mean ± SD of three independent experiments. **p < 0.01.

HOXA1 was a Target Gene of miR-30c

To further explore the molecular mechanism of miR-30c involved in GCT, we next searched three databases: Targetscan, miRwalk, and PicTar. After comprehensive analysis, we found HOXA1
as a candidate target gene of miR-30c. To confirm the prediction, we employed dual-luciferase assay using conducted wild-type or mutant HOXA1 3'-UTR vector (Figure 4A). The result of dual-luciferase assay displayed a significant activity decrease in the WT group, but no difference in mutant group (Figure 4B). And then, the protein expression of HOXA1 in miR-30c mimics or inhibitor treated cells was measured by Western blot analysis. Obviously, upregulation of miR-30c reduced the HOXA1 level in GCT cells (Figure 4C) while downregulation of miR-30c in GCT cells increased the HOXA1 expression (Figure 4D). The above results suggested that HOXA1 was a direct target of miR-30c.

**HOXA1 Over-Expression Counteracted the Effect of miR-30c up-Regulation**

As we speculated miR-30c suppressed GCT cells proliferation, migration and invasion via down-regulating HOXA1, we established plasmid pcDNA3.1-HOXA1 to reverse the effect of miR-30c mimics to further confirm these results. As shown in Figure 5A, CCK8 assay showed that HOXA1 over-expression in miR-30c mimics treated GCT cells significantly rescued the cell proliferation ability. Furthermore, the decrease of invasion activity caused by miR-30c mimics was reversed by HOXA1 up-regulation (Figure 5B). Then, Western blot assay showed HOXA1 level was rescued by HOXA1 up-regulation (Figure 5C). These data indicated that miR-30c suppressed GCT cells proliferation and progression via targeting HOXA1.

**Discussion**

In recent years, miRNA has been found to be closely related to the pathogenesis of cancer. The
majority of the miRNAs associated with cancer are located in chromosome site.17-19. And their abnormal expression can regulate tumor related gene, cell proliferation, apoptosis, and invasion of tumor cells or blood vessel formation process20-22. However, the role of miRNA is extensive and complicated. Elucidating the regulation of gene expression and its mechanism in the occurrence and development of tumors will play a crucial role in clinical diagnosis and treatment.

In our work, we analyzed the expression level of miR-30c in GCT tissues for the first time and found lower miR-30c expression in GCT tissue samples compared to the normal group. These findings emphasized that miR-30c might function as a tumor inhibitor in GCT as well as in several other cancers including prostate cancer, non-small cell lung cancer, and gastric cancer.

Next to confirm the function of miR-30c in GCT, we conducted loss- and gain- of function experiments using miR-30c mimics and inhibitors. By CCK8 assay and colony formation assay, we studied the proliferation activity of established cell lines. GCT cells performed, significantly reduced the ability of cell growth after miR-30c up-regulation, while GCT cells treated with miR-30c inhibitors showed increased cell proliferation ability compared to relative control group. These results indicated miR-30c could inhibit GCT cell proliferation, which was presented in breast cancer and non-small cell lung cancer. Furthermore, cell migration and invasion abilities changes were visualized. As reported in prostate cancer cells, miR-30c inhibits cell migration and invasion; miR-30c over-expression also reduced GCT cell migration and invasion while down-regulation of miR-30c promoted these activities in GCT cells. These findings clearly confirmed that miR-30c inhibited GCT progression and tumorigenesis. As far as we know, this is the first report explaining miR-30c function in GCT.

In order to detect the molecular mechanism of miR-30c, we speculated HOXA1 as a potential target gene due to several databases. Homeobox protein Hox-A1 (HOXA1), is a protein that encoded by the HOXA1 gene in humans. The gene is part of the chromosome 7 cluster and encodes DNA binding transcription factors that regulate gene expression, morphogenesis, and cell differentiation. More and more studies have found that HOXA1 can regulate tumour development; for example, HOXA1 can promote the proliferation of non-small cell lung cancer21, malignant progression inhibition of breast cancer22, prolife-
MicroRNA of SLE


