Hsa_circ_0005909 promotes cell proliferation of osteosarcoma cells by targeting miR-338-3p/HMGA1 axis

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Research

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Abstract

Objective: Osteosarcoma (OS) is the most common malignant bone tumor in pediatric population. The main goal of this study is to investigate the role of has_circ_0005909 and the underlying signaling pathway involved in OS.

Methods: Cell proliferation was measured using CCK-8 assay kit and clone formation assay. Change of RNA and protein expression was determined using RNA extract and quantitative real time PCR (RT-qPCR) assay and Western blotting, respectively. CircInteractome was used to predict the target of circRNA and starBase v2.0 was used to predict the target of miRNAs. Luciferase assay was used to confirm the predicted results from CircInteractome and starBase v2.0.

Results: Expression of circ_0005909 was upregulated in both OS tissues and cell lines. The predicted results from CircInteractome and starBase v2.0 demonstrated that circ_0005909 could sponge miR-338-3p and that HGMA1 was the direct target of miR-338-3p. Cell viability and cell clones was inhibited by knockdown of circ_0005909 but increased by dual inhibition of circ_0005909 and miR-338-3p. Phosphorylation of ERK, Akt, PI3K was inhibited by sh-circ_0005909 while this inhibition was repressed by co-transfection of sh-circ_0005909 and HGMA1.

Conclusion: Expression of circ_0005909 was upregulated in both OS tissues and cell lines which upregulated expression of HGMA1 through sponging miR-338-3p, resulting in the activation of MAPK-ERK and PI3K-Akt signaling pathways to promote the development of OS.

Introduction

Osteosarcoma (OS) is a class of primary malignant bone tumor, which was most common in children and adolescence [1]. The 5-year survival was approximately 65–70% in OS patients with localized disease while it was only 19–30% for metastatic disease [2]. The standard systematic chemotherapeutic drug for OS are: high-dose methotrexate with leucovorin rescue (HDMTX), doxorubicin, and cisplatin (MAP) [3]. With the complete surgical resection, this adjuvant chemotherapy achieved 60–70% of 5-year event-free survival in patients with non-metastatic disease [3]. However, all these 3 drugs are cytotoxic agents with many side effects, including cardiotoxicity. Therefore, new mechanism and treatment regimens should be explored to improve the outcomes and life quality of OS patients.

Circular RNAs (circRNAs) are a type of long, non-coding RNAs which is resistant to endonuclease due to its a closed loop structure [4, 5]. circRNAs contains conserved miRNA binding site, which resulted in repression of target miRNA activity [6]. Unlike circRNAs, microRNAs (miRNAs) is a class of small, linear RNAs with only 18–25 nucleotides (nt) in length [7]. miRNAs are also non-coding RNAs which repress translational process via binding to 3'-untranslated region (3'-UTR) of target mRNAs [8]. Acting as a competing endogenous RNA (ceRNA), circRNAs can protect the target mRNA from degradation to prevent translational repression [9].

Recent studies have reported that both circRNAs and miRNAs participate the regulation of tumor growth and metastasis [10]. As reported by Li *et al.*, upregulation of circ-0001785 could sponge miR-1200 to induce the overexpression of HOXB2, resulting in the progression of OS [11]. Acting as a ceRNA, circ_0102049 could upregulate MDM2 expression through targeting miR-1304-5p, promoting cell growth in OS [12]. On the contrary, circ_HIPK3 was downregulated in OS and overexpression of circ_HIPK3 prevented cell proliferation, migration and invasion in OS, demonstrating an inhibitory effects on the pathogenesis of OS [13]. These data suggest that circRNAs play important roles in cancer diagnosis and therapy which is mediated by target miRNAs. The main goal of this study is to investigate the role of has_circ_0005909 and the underlying signaling pathway involved in OS.

Methods

Human osteosarcoma tissue samples

Human OS tissue samples and their adjacent tissues (n = 15) were collected at ZiBo Central Hospital and written informed consent was obtained from all patients. The protocol of this research project has been approved by the Ethics Committee of ZiBo Central Hospital and was in accordance with the Declaration of Helsinki [14].

Cell Culture

Human OS cell lines (MG63, HOS, 143B and U2OS cells) and human osteoprogenitor cell line hFOB1.19 cells were obtained from American Type Culture Collection (ATCC, USA). All cells were incubated in Dulbecco's modified Eagles' medium (DMEM, HyClone, USA) supplemented with 1% penicillin/streptomycin (Invitrogen, USA) and 10% fetal bovine serum (FBS, Invitrogen, USA) under 37°C with 95% air and 5% CO₂ in a humidified incubator.

For transfection experiments, cells were cultured in sterile 12-well plates at the density of 1×10^6 cells/well. miR-338-3p mimics, miR-338-3p inhibitors small hairpin RNA of circ_0005909 (sh-circ_0005909) and their negative controls were purchased from Guangzhou Bersin Biotechnology Co. Ltd (BersinBio, China) and transfected into the cultured cells with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturers' protocols. The transfected cells were cultured under 37°C with 95% air and 5% CO₂ for 24 hours (h) for further experiments and analysis.

RNA extract and quantitative real time PCR (RT-qPCR) assay

After treatment, total RNA was extracted with Trizol reagent (TaKaRa, China) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed to cDNA with the PrimeScript RT Master Mix (TaKaRa, China). The relative RNA expression was examined using SYBR[®] Green Quantitative RT-qPCR Kit (Sigma-Aldrich, USA) and calculated by means of the $2^{-\Delta\Delta Ct}$ method. The primer sequences (Sigma-Aldrich, USA) were shown in table 1.

Table 1 Primer sequences for RT-qPCR

Gene	Primer sequences
GAPDH	Forward: CCACATCGCTCAGACACCAT Reverse: CCAGGCGCCCAATACG
circ_0005909	Forward: GTATCCACTTGCCCTTTA Reverse: TTACTCCAGCCTGTCTC
U6	Forward: CGCTTCGGCAGCACATATAC Reverse: TTCACGAATTTGCGTGTCAT
miR-338-3p	Forward: ATCCAGTGCGTGTCGTGG Reverse: TGCTTCCAGC ATCAGTGAT
HMGA1	Forward: GGTCGGGAGTCAGAAAGAGC Reverse: ATTCTTGCTTCCCTTTGGTCG

RNase R treatment and colony formation

5 µg of total RNA was incubated with or without 3 U/µg of RNase R (Epicentre Biotechnologies, USA) 37 °C for 15 min. Expression of RNA was subsequently purified and extracted by phenol-chloroform and then subjected to qRT-PCR.

CCK-8 assay and clone formation assay

After treatment, cell proliferation was examined at 0, 24, 48 and 72 hours (h) by Cell Counting Kit-8 (CCK-8) assay (Dojido, Japan) in a 96-well plate according to the manufacturer’s instructions. Briefly, 10 µl of CCK-8 solution was added to each well. The solution was then measured spectrophotometrically at 450 nm after 2-h incubation at 37°C and the optical density (OD) value was recorded.

For clone formation assay, MG63 and 143B cells were mixed with 0.4 % of agarose and seeded into 12-well plates. 2 ml of culture medium was added to each well and incubated at 37 °C with 5 % CO₂ for 7 days. MG63 and 143B cells were fixed with 10 % methanol and stained with 0.1 % crystal violet. The cell colonies were counted and photographed under a microscope (Olympus, Japan).

Construct of plasmid and Luciferase assay

This protocol was followed the published paper [15]. Briefly, the full-length of *HMGA1* 3'-UTR or circ_0005909 containing wild type (wt) and scrambled (mut) miR-338-3p binding sequence were inserted downstream of the firefly luciferase gene in psiCHECK2 to generate the psiCHECK2-*HMGA1* 3'UTR-wt or

circ_0005909-mut plasmid and psiCHECK2-*HMGA1* 3'UTR-mut plasmid or circ_0005909-mut, respectively. The wt and mut plasmids were co-transfected into H293T cells with negative control, miR-338-3p mimics or their negative control (miR-NC) with control Renilla luciferase expression plasmid using Lipofectamine 2000. After 24h, relative luciferase activity was measured using the Dual-luciferase reporter Assay System (Promega, USA) according to the manufacturer's instructions.

Bioinformatics analysis

CircInteractome (<https://circinteractome.nia.nih.gov/>) was used to predict miR-338-3p binding site in the circ_0005909 and starBase v2.0 (<http://starbase.sysu.edu.cn/>) was used to predict the potential miR-338-3p binding sites to corresponding gene *HMGA1* 3'UTR to study the possible crossing network among circRNA, miRNA and target gene.

Statistical Analysis

GraphPad 8.1.0 was used to perform the statistical analysis in this study. Comparisons between two groups was analyzed using Student's *t*-test or one-way ANOVA. When comparing more than two groups, one-factor analysis of variance was performed. All data was presented as mean $\pm$ standard error of means (S.E.M.). $p < 0.05$ represents statistical significance.

Results

hsa_circ_0005909 was upregulated in OS.

Expression of circ_0005909 was upregulated in OS tissues compared with their adjacent normal tissues (Fig. 1A). Upregulation of circ_0005909 was also observed in human OS cell lines, MG63, HOS, 143B and U2OS cells, compared with human osteoprogenitor cell line hFOB1.19 cells (Fig. 1B). RNase R treatment was used to confirm the circular characteristics of circ_0005909. The results demonstrated that circ_0005909 did not show any significant change in RNase R treatment group while the expression of the linear control RNA, GAPDH, was significantly reduced after RNase R treatment compared with mock group in both MG63 and 143B cells (Fig. 1C). The expression of circ_0005909 was higher in plasma than that in nucleus in both MG63 and 143B cells (Fig. 1D).

circ_0005909 acted as a molecular sponge of miR-338-3p in OS cells.

CircInteractome was used to predict the target miRNA of circ_0005909. The results manifested that there was a complementary sequence between circ_0005909 and miR-338-3p (Fig. 2A). Luciferase activity was reduced in cell co-transfected with circ_0005909-wt plasmid and miR-338-3p mimics compared with negative control group and circ_0005909-mut group (Fig. 2B). Expression of miR-338 was decreased in OS tissues compared with their adjacent normal tissue, which is negatively correlated with the expression of circ_0005909 (Fig. 2C). RNA pull-down assay showed that higher expression of miR-338-3p enriched more circ_0005909 compared with negative control group in both MG63 and 143B cells (Fig. 2D).

Knockdown of circ_0005909 repressed proliferation in OS cells via regulating miR-338-3p.

Sh-circ_0005909 was used to knockdown circ_0005909 in MG63 and 143B cells. The expression of circ_0005909 was significantly reduced in cell transfected with sh-circ_0005909 compared with cell transfected with scramble RNAs in both MG63 and 143B cells (Fig. 3A). Results from CCK-8 assay demonstrated that cell viability was significantly reduced by sh-circ_0005909 while this reduction of cell viability was reversed by dual inhibition of circ_0005909 and miR-338-3p (Fig. 3B). The cell clone number was significantly decreased by sh-circ_0005909 while this decrease of cell clones was attenuated by dual inhibition of circ_0005909 and miR-338-3p (Fig. 3C).

HMGA1 was the direct target of miR-338-3p.

The prediction results from starBase v2.0 indicated that miR-338-3p could bind to the 3'-UTR of HMGA1 (Fig. 4A). Luciferase activity was reduced in cell co-transfected with HMGA1-wt plasmid and miR-338-3p mimics compared with negative control group and HMGA1-mut group (Fig. 4A). Both mRNA and protein expression of HMGA1 was repressed in MG63 and 143B cells transfected with miR-338-3p mimics compared with those transfected with negative control (Fig. 4B). The protein expression of HMGA1 was downregulated by sh-circ_0005909 while this downregulation was prevented by dual inhibition of circ_0005909 and miR-338-3p in both MG63 and 143B cells (Fig. 4C). Phosphorylation of ERK, Akt, PI3K was inhibited by sh-circ_0005909 while this inhibition was repressed by co-transfection of sh-circ_0005909 and HGMA1 in both MG63 and 143B cells (Fig. 4D).

Discussion

As mentioned, OS is an aggressive bone tumor in pediatric population with a poor prognostic outcome in patients with metastatic disease [2]. The pathogenesis and new mechanisms were undergone the deep investigation to improve the prognosis and prolonged the patients' life. In this study, overexpressed circ_0005909 was observed in both OS tissues and cell lines. Further experiments have found that circ_0005909 upregulated expression of HGMA1 through sponging miR-338-3p, which activated MAPK-ERK and PI3K-Akt signaling pathways and promoted the development of OS, providing a new understanding and therapeutic target for treatment of OS in future.

Many circRNAs have involved into the pathogenesis of OS and became an important diagnostic and prognostic indicator in OS [16]. In the present study, overexpression of circ_0005909 was observed in both OS tissues and cell lines, which is consistent with previous studies [17, 18]. Data of this study have shown that circ_0005909 was overexpressed in OS and that knockdown of circ_0005909 reduced cell proliferation in OS cell lines, indicating that upregulation of circ_0005909 contributed to the development and progression of OS. The previous study has revealed that circ_0005909 could upregulate HMGB1 via targeting miR-936 in OS [17]. However, results from this study manifested that circ_0005909 could also sponge miR-338-3p to increase cell proliferation in OS. This could be explained by "off-target" effects of miRNAs, which means each miRNA could bind to hundreds of target mRNAs or circRNAs with partial sequence match to regulate multiple biological processes [19, 20].

Results from the starBase v2.0 and luciferase assay have confirmed that HMGA1 was the direct target of miR-338-3p. HMGA1 is a family member of the high mobility group A(HMGA) proteins [21]. HMGA1 is upregulated in highly proliferative cells, and is closely associated with aggressiveness and poor prognosis of cancer [21]. In this study, expression of miR-338-3p was negatively associated with expression of circ_0005909 in OS, and overexpression of miR-338-3p inhibited the expression of HMGA1. Thus, there would be a positive correlation between the expression of HMGA1 and circ_0005909. These evidences further confirmed that circ_0005909 upregulated expression of HMGA1 via targeting miR-338-3p, leading to the development and progression of OS.

HMGA1 has been reported to activate MAPK-ERK1/2 signaling pathway and PI3K-Akt signaling pathway [22, 23]. In the present study, phosphorylation of ERK, PI3K and Akt was inhibited by knockdown of circ_0005909, suggesting that circ_0005909 involved in the activation of MAPK-ERK and PI3K-Akt signaling pathways. However, the downregulation of phosphorylated ERK, PI3K and Akt was reversed by overexpression of HMGA1. Taken together, these results demonstrated that circ_0005909 could promote the activation of MAPK-ERK and PI3K-Akt signaling pathways, which was mediated by upregulation of HMGA1, thus contributing to the pathogenesis of OS.

In summary, data of the present study have manifested that the expression of circ_0005909 was upregulated in both OS tissues and cell lines. Acting as ceRNA, circ_0005909 upregulated expression of HMGA1 through sponging miR-338-3p, which enhanced the activation of MAPK-ERK and PI3K-Akt signaling pathways to promote the development of OS, indicating a new diagnostic biomarker and future therapeutic target for OS.

Declarations

Conflict of interests

The authors declare that they have no conflict of interests.

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Figures

Fig.1

Figure 1

circ_0005909 was overexpressed in OS. (A) Expression of circ_0005909 was upregulated in OS tissues; (B) Expression of circ_0005909 was upregulated in OS cell lines; (C) circ_0005909 was a circular RNA; (D) circ_0005909 was mainly located in cytoplasm. ** $p < 0.01$; *** $p < 0.005$; ns: no statistical significance. OS: osteosarcoma tissues; Normal: normal tissues

Fig.2

Figure 2

circ_0005909 acted as a molecular sponge of miR-338-3p in OS cells. (A) The putative binding sites between circRNA_0005909 and miR-338-3p, and luciferase activity was reduced in cell co-transfected with circ_0005909-wt and miR-338-3p mimics; (B) Expression of miR-338-3p was downregulated in OS tissues; (C) Expression of miR-338-3p was negatively correlated with expression of circ_0005909; (D) circ_0005909 was enriched in cell transfected with miR-338-3p mimics. **p < 0.01; ns: no statistical significance. circ_0005909-wt: psiCHECK2 plasmid contained the full sequence of circ_0005909; circ_0005909-mut: psiCHECK2 plasmid contained the mutant sequence of circ_0005909; miR-NC: negative controls of miR-338-3p; miR-338-3p: miR-338-3p mimics.

Fig.3

Figure 3

Knockdown of circ_0005909 repressed proliferation in OS cells via regulating miR-338-3p. (A) Expression of circ_0005909 was reduced in cell transfected in sh-circ_0005909; (B) Cell viability was significantly reduced by sh-circ_0005909 while this reduction of cell viability was reversed by dual inhibition of circ_0005909 and miR-338-3p; (C) The cell clone number was significantly decreased by sh-circ_0005909 while this decrease of cell clones was attenuated by dual inhibition of circ_0005909 and miR-338-3p. **p < 0.01 versus (vs) scramble; #p < 0.05 vs sh-circ_0005909. Sh-circ_0005909: small hairpin RNA of circ_0005909; scramble: scramble RNA of sh-circ_0005909;

Figure 4

HMGA1 was the direct target of miR-338-3p. (A) The putative binding sites between 3'-UTR of HMGA1 and miR-338-3p and luciferase activity was reduced in cell co-transfected with HGMA1-WT and miR-338-3p mimics; (B) Both mRNA and protein was reduced in cell transfected with miR-338-3p mimics; (C) The protein expression of HMGA1 was downregulated by sh-circ_0005909 while this downregulation was prevented by dual inhibition of circ_0005909 and miR-338-3p in both MG63 and 143B cells; (D)

Dysregulation of phosphorylated Akt, ERK and PI3K by sh-circ_0005909 and HMGA1. **p < 0.01. HGMA1-WT: psiCHECK2 plasmid contained the full sequence of HMGA1 3'-UTR; HGMA1-MUT: psiCHECK2 plasmid contained the mutant sequence of HMGA1 3'-UTR.