

Original Research Article

Curcumin attenuates migration and invasion of osteosarcoma cells by regulating expression of miR-33b-5p/SIRT6 axis

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Abstract

Purpose: To investigate the potential role of curcumin in osteosarcoma (OS) cell migration and invasion, and also to explore the underlying mechanism of action.

Methods: OS cell lines, Saos-2 and U2OS, were treated with various concentrations of curcumin (0, 10, 20, 50 μ M) for 48 h. Transwell assay was used to determine the migratory and invasive activities of human OS cells while miR-33b-5p expression levels were measured using quantitative real time polymerase chain reaction (qRT-PCR). Expressions of metastasis-related genes, such as matrix metalloproteinases (MMPs), were quantified by qRT-PCR or western blot. The interaction between miR-33b-5p and Sirtuin 6 (SIRT6) was evaluated by luciferase assay.

Results: Curcumin inhibited the migratory and invasive activities of human OS cells, and downregulated MMP2 and MMP9 ($p < 0.05$). Moreover, in curcumin-treated OS cells, miR-33b-5p expression was upregulated while SIRT6 expression was significantly downregulated ($p < 0.05$). Overexpression of miR-33b-5p downregulated SIRT6 mRNA and protein expression, whereas transfection with miR-33b-5p inhibitor upregulated SIRT6 expression in OS cells ($p < 0.05$). Furthermore, curcumin suppressed SIRT6 expression by upregulating miR-33b-5p expression in OS cells. Finally, overexpression of SIRT6 or miR-33b-5p inhibitor reversed the suppression of OS cell metastasis induced by curcumin ($p < 0.05$).

Conclusion: Curcumin inhibits SIRT6 expression via upregulating miR-33b-5p expression, which leads to the inhibition of OS cell migration and invasion. Thus, curcumin is a potential therapeutic agent for the management of OS.

Keywords: Curcumin, MiR-33b-5p, SIRT6, Osteosarcoma

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INTRODUCTION

Osteosarcoma (OS) is common in children and adolescents, and the global incidence of OS is approximately 4 million per year [1]. Despite the benefits of surgery and chemotherapy, the

occurrence rate of metastasis is nearly 80 % [2], and the overall 5-year survival rate is lower than 20 % for patients with metastatic OS [3]. Therefore, it is necessary to develop anti-tumor drugs that inhibit OS metastasis.

Recently, active extracts from natural plants have been shown to have anti-tumor effects. Curcumin is a natural polyphenol *Curcuma longa* which is widely used in Asia as a traditional medicine and as a food additive for hundreds of years. Curcumin have therapeutic effects on various tumors, including inducing cell apoptosis, suppressing cell proliferation, sensitizing tumor cells to chemoradiotherapy, and inhibiting cell metastasis [4], [5]. However, the mechanisms underlying the anti-tumor effects of curcumin remain unknown.

By base-pairing with complementary sequences, microRNAs (miRNAs) regulate expression of many mRNAs and then indirectly control a series of biological processes like cell proliferation, apoptosis, differentiation, and metastasis [6-8]. MiRNAs also play important roles in tumorigenesis and cancer progression [8,9]. Among the microRNA-33 (miR-33), which includes miR-33a and miR-33b, is conserved in animals ranging from *Drosophila* fruit flies to humans [10]. In particular, miR-33b is located within the 17th intron of SREBP-1 which regulates lipid and cholesterol metabolism [11], and has been shown to suppress proliferation and metastasis of OS cells [12,13]. Recent studies have demonstrated a close association between miR-33b and the anti-tumor effect of curcumin. For example, curcumin induces gastric cancer cell apoptosis and inhibits melanoma metastasis by upregulating expression of miR-33b [14,15]. However, it is unknown whether curcumin directly regulates miR-33b to exert the therapeutic effects in OS.

Sirtuin 6 (SIRT6) is a mono-ADP ribosyl transferase and stress responsive protein deacetylase involved in multiple signaling pathways associated with DNA repair, aging, inflammation, and glycolysis [16]. It has been reported that SIRT6 promotes migration of pancreatic cancer cells and confers an inflammatory phenotype onto cancer cells, SIRT6 may be thus a promising anti-tumor drug target [17]. Here, the inhibitory effects of curcumin on the migratory and invasive activities of human OS cells were investigated. MiR-33b-5p (the main existing form of miR-33b)/SIRT6 axis was then uncovered as the mechanisms underlying the anti-tumor effect of curcumin.

EXPERIMENTAL

Cells and reagents

Saos-2 and U2OS osteosarcoma cell lines were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai,

China). The cells were cultured at 37 °C with 5 % CO₂ in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) supplemented with 10 % fetal bovine serum (FBS; Hyclone, Logan City, USA). Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was used for transfection of miR-33b-5p mimics, miR-33b-5p inhibitors (GenePharma, Shanghai, China), SIRT6 plasmids (Sino Biological, Beijing, China), and the corresponding controls. Curcumin (Sigma Aldrich, St. Louis, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, USA) at different concentrations (10, 20 and 50 μM).

Transwell migration and invasion assay

The transwell chambers were used (8-μm pore size; Corning, NY, USA). For the invasion assay, the chambers were pre-coated with Matrigel (BD Biosciences, New Jersey, USA). Cells treated with curcumin or transfected with miR-33b-5p mimics/inhibitors/SIRT6 plasmids were added to the upper chamber, and cells in the lower chamber were added with DMEM (10 % FBS). After incubated for 12 h, the non-migratory cells were removed. The cells in the lower chamber were fixed with paraformaldehyde (4 %, Sigma Aldrich, St. Louis, USA) and stained with crystal violet solution (Beyotime, Shanghai, China). The migrated or invaded cells were photographed and counted under a microscope (Leica, Solms, Germany).

Western blot

RIPA lysis buffer (Beyotime, Shanghai, China) was for extraction of total protein, and BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) was used to determine the protein concentrations. Protein extracts were subjected to SDS-PAGE electrophoresis and then transferred to PVDF membranes (Millipore, Billerica, USA), which were blocked in non-fat milk (5 %) and then incubated with monoclonal antibodies against MMP2, MMP9, SIRT6, and β-actin (1:1000, Abcam, Cambridge, UK). After washing, the membranes were incubated with HRP-labeled secondary antibodies (Abcam, Cambridge, UK), protein bands were visualized and band intensities were analyzed using Image J software.

Quantitative real-time PCR

To extract total RNA from OS cells, TRIzol reagent (Invitrogen, Carlsbad, USA) was used, and was reverse transcribed using the PrimeScript RT reagent Kit (Takara, Shiga, Japan). The ABI 7500 Real-Time PCR system (Applied

Biosystems, Foster City, USA) was used to perform quantitative real-time PCR (qRT-PCR) and to analyze the data. Relative mRNA expression levels were normalized using the $2^{-\Delta\Delta C_t}$ cycle threshold method. The primers were listed in Table 1.

Table 1: Gene primers used in qRT-PCR

Genes		Primer sequence
miR-33b	Forward	TGCATTGCTGTTGCATTG
	Reverse	GAACATGTCTGCGTATCTC
SIRT6	Forward	TGGCAGTCTTCCAGTGTGGTGT
	Reverse	CGCTCTCAAAGGTGGTGTGCGAA
SIRT-3'UTR-WT	Forward	GGCCACTAGTCCAGGGTGCTTGGGGAG
	Reverse	GGCCACTAGTGAGGACTTCTCCAGAAGCCT

Assay of luciferase activity

3' untranslated regions (UTR) wild-type (WT) of SIRT6 was PCR amplified, cut with *SpeI*, and ligated to an *XbaI*-linearized pGL3Control vector (Promega, Wisconsin, USA). The 3'UTR mutant (MUT) of SIRT6 was constructed by mutating the predicted binding sequence for miR-33b (CAAUGCA) using a commercial kit (Agilent Technologies, California, USA).

Saos-2 cells were transfected with SIRT6 3'UTR WT or SIRT6 3'UTR MUT reporter vector together with miR-33b-5p mimics or scrambled control. Cells were lysed and measured with Luciferase Assay System (Promega, Wisconsin, USA) 48 hours later.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 software. Student's t-test was used to analyze differences between groups. A value of $p < 0.05$ was considered statistically significant. All the experiments were performed in triplicate and results were shown as mean \pm standard deviation (SD).

RESULTS

Curcumin inhibited human OS cell migration and invasion

Human OS cell lines, Saos-2 and U2OS, were treated with various concentrations of curcumin (0, 10, 20, or 50 μ M), and the cell migration and invasion assays were performed. Curcumin inhibited the migration and invasion of OS cells in a dose-dependent manner (Figure 1 A and B).

Since MMPs are essential in cell invasion, the expressions of MMP2 and MMP9 were then detected. Consistently, curcumin dose-dependently down-regulated MMP2 and MMP9 protein expression (Figure. 1C). These results demonstrated that curcumin had inhibitory effects on human OS cell migration and invasion.

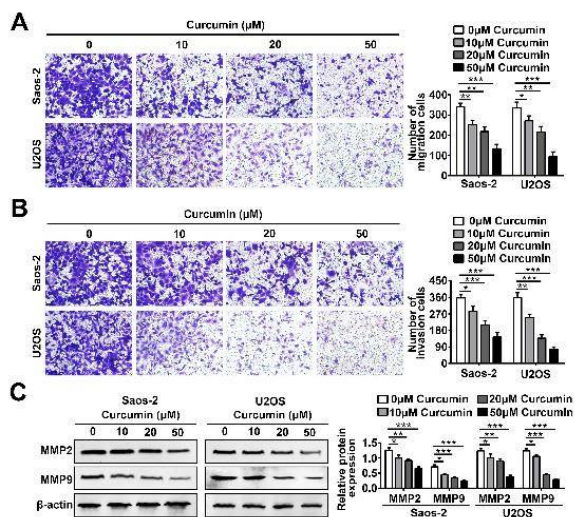


Figure 1: Effect of curcumin on human OS cell migration and invasion. Saos-2 and U2OS cells were treated with 0, 10, 20, or 50 μ M curcumin for 48 h. The transwell migration (A)/invasion (B) assay was performed, random fields were imaged, and the numbers of migrated/invaded cells in the fields were counted. (C) Western blotting of MMP2 and MMP9 protein expressions; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control group (0 μ M curcumin)

Curcumin regulated the expression of SIRT6 and miR-33b-5p in OS cells

To investigate the underlying mechanism, the expression levels of miR-33b-5p and SIRT6 were measured. Curcumin upregulated miR-33b-5p expression and downregulated SIRT6 expression in Saos-2 and U2OS cells in a concentration-dependent manner (Figure 2 A, B and C).

MiR-33b-5p directly inhibited SIRT6 expression

To confirm whether miR-33b-5p could regulate SIRT6 expression, miR-33b-5p mimics were transfected into Saos-2 cells. As expected, miR-33b-5p expression was up-regulated while SIRT6 expression was downregulated (Figure 3A and B). A putative miR-33b-5p binding site in the 3'-UTR of human SIRT6 was predicted by Targetscan 5.2 (www.targetscan.org), and SIRT6 3'-UTR was mutated (Figure 3C). In Saos-2 cells that transfected with SIRT6 3'UTR WT, overexpression of miR-33b-5p led to a significant reduction in luciferase activity, whereas in cells

transfected with SIRT6 3'UTR MUT, overexpression of miR-33b-5p did not affect luciferase activity (Figure 3C).

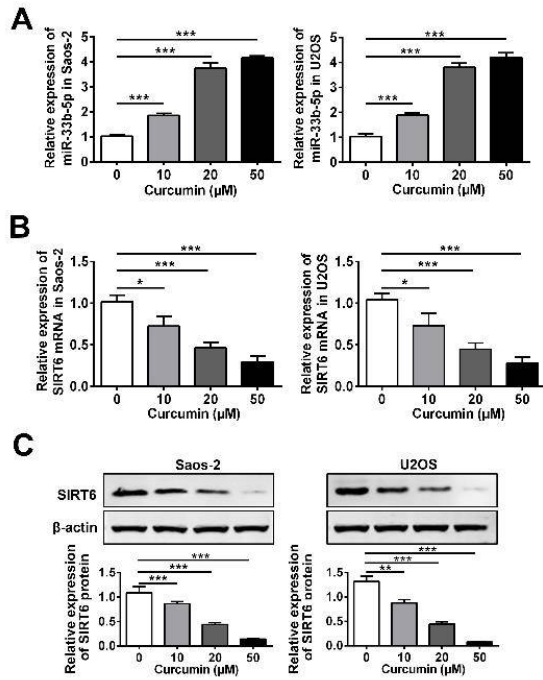


Figure 2: Effects of curcumin on the expression of SIRT6 and miR-33b-5p in OS cells. (A, B, C) Saos-2 and U2OS cells were treated with 0, 10, 20, or 50 μ M curcumin for 48 h, and then expression levels of miR-33b-5p and SIRT6 were measured; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control group (0 μ M curcumin)

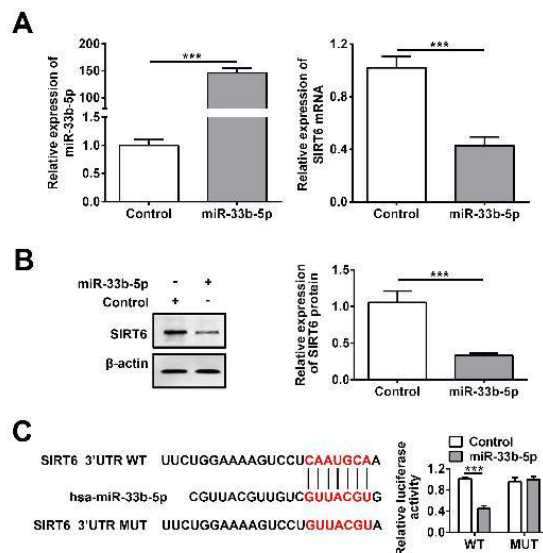


Figure 3: MiR-33b-5p directly inhibited SIRT6 expression. (A, B) MiR-33b-5p mimics or a scrambled control were transfected into Saos-2 cells, and the expression levels of miR-33b-5p and SIRT6 were measured. (C) Saos-2 cells were transfected with SIRT6 3'UTR WT or SIRT6 3'UTR MUT reporter

vectors along with miR-33b-5p mimics or a scrambled control, and then luciferase activity was measured; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control group

Curcumin reduced SIRT6 expression by upregulating miR-33b-5p expression in OS cells

To further demonstrate whether curcumin could inhibit SIRT6 expression by upregulating miR-33b-5p, miR-33b-5p inhibitor was transfected into Saos-2 and U2OS cells. As expected, miR-33b-5p expression was suppressed, whereas SIRT6 expression was upregulated (Figure 4A, B and C). Moreover, transfection with miR-33b-5p inhibitor partially abolished the inhibitory effect of curcumin on SIRT6 expression (Fig. 4B and C).

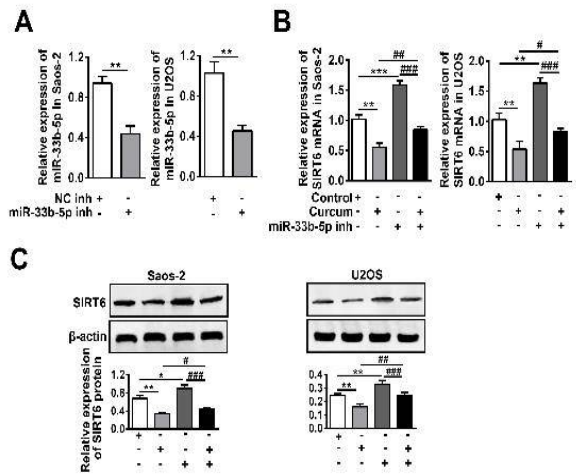


Figure 4: Curcumin suppressed SIRT6 expression by upregulating miR-33b-5p expression in OS cells. (A) miR-33b-5p inhibitor or control inhibitor was transfected into Saos-2 and U2OS cells, and then miR-33b-5p expression was measured. (B, C) miR-33b-5p inhibitor or control inhibitor was transfected into 50 μ M curcumin-treated Saos-2 and U2OS cells, and then SIRT6 expression was measured; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with the curcumin-treated group or the miR-33b-5p inhibitors-transfected group

MiR-33b-5p inhibited OS cell migration and invasion by downregulating SIRT6

SIRT6 was overexpressed in OS cells, and the mRNA and protein expression of SIRT6 were higher in SIRT6-transfected OS cells as compared with vector-transfected OS cells (Figure 5 A and B). Then, miR-33b-5p mimics or control mimics was also transfected into OS cells. SIRT6 overexpression attenuated miR-33b-5p-induced inhibition of OS cell migration and invasion (Figure 5 C and D) and attenuated miR-33b-5p-induced suppression of MMP2 and

MMP9 in OS cells (Figure 5E). These results showed that the miR-33b-5p-SIRT6 axis regulated OS cell migration and invasion.

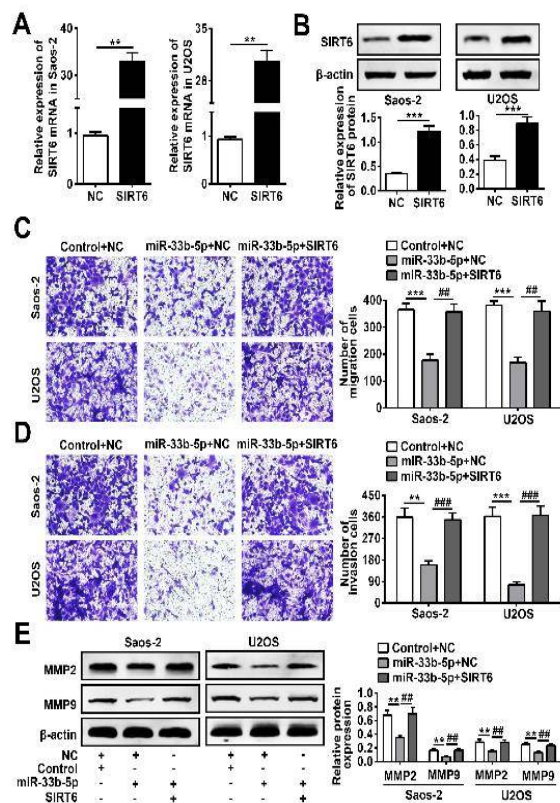


Figure 5: MiR-33b-5p inhibited OS cell migration and invasion by downregulating SIRT6. (A, B) SIRT6 or control plasmids were transfected into Saos-2 and U2OS cells, and SIRT6 expression was measured by qRT-PCR and western blot. (C, D, E) miR-33b-5p mimics/NC mimics and SIRT6 plasmid/control plasmid were co-transfected into Saos-2 and U2OS cells, and then transwell migration and invasion assays were carried out. MMP2 and MMP9 protein expression levels were measured using western blot; **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 compared with NC or Control+NC group; ##*p* < 0.01 and ###*p* < 0.001 compared with miR-33b-5p +NC group

Curcumin inhibited OS cell migration and invasion

To further confirm that curcumin suppressed OS cell migration and invasion by upregulating miR-33b-5p, miR-33b-5p inhibitor was transfected into Saos-2 and U2OS cells that treated with 50 μM curcumin. MiR-33b-5p inhibitor promoted OS cell migration and invasion. Moreover, transfection with miR-33b-5p inhibitor reversed the inhibitory effects of curcumin on OS cell migration and invasion (Figure 6A and B). Finally, western blot revealed that miR-33b-5p inhibitor attenuated the curcumin-induced suppression of MMP2 and MMP9 in Saos-2 and U2OS cells (Figure 6C).

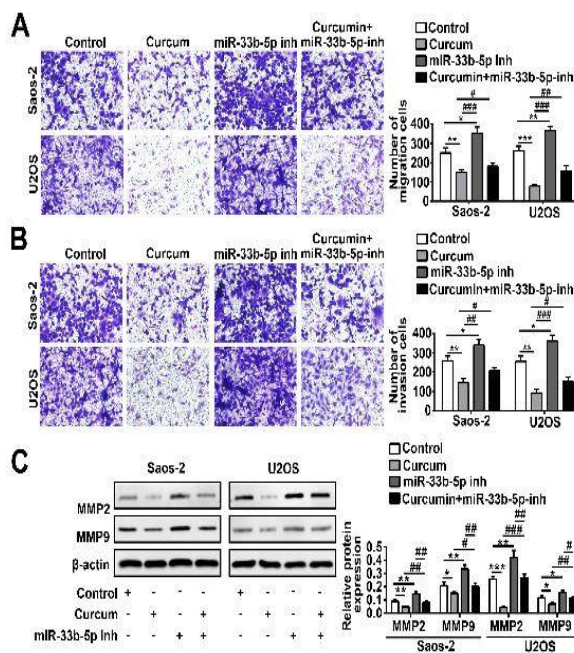


Figure 6: Curcumin inhibited OS cell migration and invasion by upregulating miR-33b-5p. A, B and C miR-33b-5p inhibitor was transfected into Saos-2 and U2OS cells that treated with 0 or 50 μM curcumin, and transwell migration and invasion assays were carried out and random fields were counted. MMP2 and MMP9 protein expression levels were measured by western blot; **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 compared with the control group; #*p* < 0.05, ##*p* < 0.01 and ###*p* < 0.001, compared with the curcumin-treated group or the miR-33b-5p inhibitors-transfected group

DISCUSSION

In this study, curcumin inhibited the migratory and invasive abilities of human OS cells (Saos-2 and U2OS) by downregulating SIRT6 expression. Indeed, SIRT6 functions as an oncogene in many types of tumors. In pancreatic cancer, SIRT6 contributes to cancer cell migration by upregulating Ca²⁺ responses [17]. In hepatocellular carcinoma, SIRT6 facilitates cancer initiation by repressing Survivin [18]. In breast cancer, SIRT6 induces resistance to paclitaxel and epirubicin by inhibiting FOXO proteins [19]. In OS, SIRT6 promotes cancer cell migratory and invasive abilities via the ERK1/2 pathway [20]. Therefore, the anti-tumor effect of curcumin on various tumors may be attribute to SIRT6 downregulation.

In the metabolism of fatty acid, miR-33a and miR-33b have been shown to suppress SIRT6 expression post-transcriptionally [21,22]. The present study indicated that curcumin reduced SIRT6 expression by upregulating miR-33b-5p in OS, but whether other miRNAs participate in the regulation of SIRT6 remains unknown. As a

result, it is difficult to determine the suppressive degree of SIRT6 expression caused by miR-33b-5p in curcumin-treated OS cells.

Typically, a miRNA directly targets various genes. MiR-33b has been shown to inhibit expressions of HMGA2, SALL4, and Twist1, which play vital roles in breast cancer metastasis [23]. In this study, miR-33b-5p was shown to suppress OS cell migration and invasion by downregulating SIRT6 expression. It remains to be determined whether miR-33b targets other genes involved in the progress and development of OS.

The natural agent curcumin has been shown to regulate expression of many miRNAs in tumors. In pancreatic cancer, curcumin inhibits cancer cell proliferative and invasive abilities by upregulating miR-7 expression [24]. In breast cancer, curcumin regulates miR-19 expression and the PTEN/AKT/p53 pathway, which inhibit cancer cell proliferation [25]. In non-small cell lung cancer, curcumin suppresses cancer cell growth and induces cancer cell apoptosis by upregulating miR-192-5p expression [26]. This study demonstrated that curcumin inhibited the migratory and invasive abilities of human OS cells by upregulating miR-33b-5p. It remains to be determined whether curcumin regulates expression of other miRNAs involved in OS cell migration and invasion.

Curcumin has been reported as a regulator of epigenetic alterations and the expression of miRNAs is closely related to epigenetic modification [27]. Thus, curcumin may alter miRNA expression by epigenetic modulation. In this study, miR-33b was found to be regulated by curcumin. Whether curcumin exerts its anti-tumor effects by epigenetic modification of miR-33b expression remains to be determined.

CONCLUSION

The findings of this study demonstrate that curcumin downregulates SIRT6 expression via upregulation of miR-33b-5p expression, resulting in the inhibition of OS cell migration and invasion. Therefore, curcumin is a promising natural agent for the management of OS.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. HL designed all the experiments and wrote the manuscript. ZZ, YH, JH, and YX performed the experiments.

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