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Original Research

MiR-33b inhibits osteosarcoma cell proliferation through suppression of glycolysis by targeting Lactate Dehydrogenase A (LDHA)

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Abstract: Osteosarcoma (OS) is one of the most common types of malignant bone tumor in adolescent. MicroRNAs (miRNAs) are widely studied regulatory molecules which play important roles in tumor development, differentiation, growth, invasion, chemosensitivity and cellular metabolism. Recently, miR-33b has been reported to act as a tumor suppressor in osteosarcoma. However, the detailed mechanism of miR-33b in regulating osteosarcoma cell proliferation remains unclear. In this study, we detected miR-33b was significantly downregulated in osteosarcoma tissues compared to their matched adjacent nontumor tissues. The decreased expressions of miR-33b were also found in multiple osteosarcoma cell lines, including MG63, Saos-2, U2OS and SOSP-9607 when compared to normal osteoblast cell line hFOB. Overexpression of miR-33b suppressed U2OS cell proliferation and anaerobic glycolysis. We identified Lactate dehydrogenase-A (LDHA) as a direct target of miR-33b in osteosarcoma tumors and cells by Western blot and luciferase assay. Moreover, inhibition of LDHA significantly suppressed glycolysis and cell proliferation of osteosarcoma cells. Restoration of LDHA in miR-33b-overexpressing osteosarcoma cells reversed the suppressive effect of miR-33b on cell proliferation. In addition, we report a significantly negative correlation between LDHA mRNA and miR-33b expression in osteosarcoma tumors: miR-33b is downregulated in OS tumors with high levels of LDHA (92.9%). Meanwhile, high miR-33b expressions were found majorly in OS tumors with low LDHA mRNA levels (82.4%). This study reveals that miR-33b plays a suppressive role in the regulation of osteosarcoma cell proliferation through direct targeting LDHA. The miR-33b/glycolysis/LDHA axis may contribute to development of therapeutic anti-tumor agents for osteosarcoma.

Key words: MiR-33b, osteosarcoma cell, glycolysis, Lactate Dehydrogenase A (LDHA).

Introduction

Osteosarcoma (OS) is one of the most common types of malignant bone tumor in adolescent (1). Although increasing attention has been paid to the diagnosis and therapy of osteosarcoma, OS is still featured by complexity and resistance to conventional treatments, mainly due to its metastasis and recurrence (2). Recent reports provided evidences to show that the abnormal genetic and epigenetic changes of osteosarcoma is highly correlated with abnormal expressions of oncogenes or methylation of tumor suppressor genes (3). Therefore, it is important to identify new biomarkers of osteosarcoma for the development of novel strategies for osteosarcoma patient treatments.

MicroRNAs (miRNAs) are a class of endogenous, short (18-25 nucleotides), noncoding RNAs, which repress their target genes translation through degradation of mRNA by binding to the 3'untranslated region (3'UTR) (4). miRNAs are widely studied regulatory molecules which play important roles in tumor development, differentiation, growth, invasion, chemosensitivity and cellular metabolism (5,6). Thus, dysregulations of miRNAs have emerged as an important mechanism for the development and progression of cancers (7). Specifically, miR-33b has been reported as a tumor suppressive miRNA in various cancers such as lung cancer (8), breast cancer (9), gastric cancer (10), colorectal cancer (11) and multiple myeloma (12), indicating that targeting miR-33b may have a therapeutic potential as an anti-cancer treatment.

The enzyme lactate dehydrogenase-A (LDHA) catalyzes the interconversion of pyruvate and reduced nicotinamide adenine dinucleotide (NADH) generated by glycolysis to lactate and NAD+ (13). It has been reported LDHA is overexpressed in most cancers (14). Moreover, attenuated expression of LDHA by siRNA or inhibition of LDHA activities by specific inhibitor has been demonstrated to limit cell proliferation, migration and invasion, while promoting apoptosis of cancer cells (15), thereby presenting LDHA as a promising target for anticancer therapy. In this study, a role of miR-33b in glycolysis in osteosarcoma tumor and cell lines was characterized. The regulatory mechanisms of the miR-33b-modulated osteosarcoma proliferation were investigated. We will identify novel targets of miR-33b in osteosarcoma. This study contributes to the development of the miRNA-based therapeutic approaches against osteosarcoma.

Materials and Methods

Cell lines and cell culture

Human osteoblast cell line hFOB and four osteosar-

coma cell lines, namely MG-63, U2OS, SOSP-9607, and SAOS-2, were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were propagated in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum and streptomycin (100 mg/mL), penicillin (100 U/mL). hFOB cells were cultured in osteoblast growth medium (Promo Cell, Heidelberg, Germany). All cells were incubated in an incubator providing a humidified 5% CO_2 containing atmosphere at 37ºC.

Tumors and adjacent tissues

The OS tumors and their matched adjacent non-tumor samples were obtained from same patients who had undergone routine surgery at Department of Orthopedics, China-Japan Union Hospital Jilin University, Jilin, China between 2012 and 2016. Patients were not pretreated with radiotherapy nor chemotherapy prior to surgery. Written informed consent was obtained from the Ethics Committees from the China-Japan Union Hospital Jilin University, Jilin, China. Tissues were immediately frozen and stored in liquid nitrogen for total RNA extraction.

Cell transfection

Cells were plated into 6-well plate in growth medium 12-16 hours before transfection. After refreshment of cell culture medium, cells were transfected with 25 nM miR-33b mimic or control mimic using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The control mimic and miR-33b mimic were purchased from GenePharma (Shanghai, China).

RNA extraction and qRT-PCR analysis

Total RNA was extracted from cells or tissues with Trizol reagent (Invitrogen, Calsbad, CA, USA). Poly(A) tailing reaction was performed using the qScript™ microRNA cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA). cDNA synthesis was carried out using the qScript™ microRNA cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA). Real-time PCR was performed using the standard TaqMan PCR protocol. All RT reactions including non-template controls were run in triplicate. All miRNA quantification data were normalized to U6. The relative amount of transcript was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

Cells were plated in 96-well flat-bottomed microplate at 3,000/well. The cell proliferation was measured by MTT assay according to the manufacturer's protocol. Proliferation rates were determined at 0, 2, 4 and 6 days after transfection. Viable cells were counted by absorbance measurements at 570 nm using auto microplate reader Spectramax 250 (Molecular Devices, Sunnyvale, CA, USA). Experiments were repeated three times.

Luciferase reporter assay

The wild-type and mutant LDHA 3'UTR fragments were cloned into the downstream of the firefly luciferase coding region of pMIR-GLOTM Luciferase vector (Promega, Madison, WI, USA). Cells were co-transfected with 500 ng of the reporter construct or control vector and 20 nM miR-33b mimic or negative controls. Cells were harvested 24 hrs post-transfection and assayed with Dual Luciferase Assay (Promega, WI, USA) according to manufacturer's instructions. Firefly luciferase values were normalized to Renilla, and the ratio of Firefly/Renilla values was calculated as relative luciferase activity. All transfection assays were performed in triplicate.

Western blotting analysis

Proteins were extracted from cells using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA). The concentrations of proteins were determined by Bradford assay. Equal amount of proteins of each sample was separated on 10% SDS-PAGE gel, and then transferred to a PVDF membrane (Bio-rad, Hercules, CA, USA). After blocked with 5% non-fat dried milk, membrane was incubated with rabbit monoclonal anti-LDHA antibody (#3582, Cellsignaling, Beverly, Massachusetts, USA) at 1:1000 dilution or rabbit monoclonal anti-β-actin antibody (#4970, Cellsignaling, Beverly, Massachusetts, USA) at 1: 5,000 dilution at 4 ºC for overnight. After washing with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween20), the membranes were incubated for 2 h with goat anti-rabbit secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) at 1:5000 dilution. Proteins bands were visualized using ECL reagents (Pierce, Rockford, IL, USA).

Statistical analysis

Results are presented as the mean ± SD of three independent experiments. Prism 5.0 software was used to perform statistical analysis. Student's t-test was used to analyze the difference between two groups. One-way analysis of variance (ANOVA) was used to analyze the differences among more than two groups. Values of $p < 0.05$ were considered statistically significant.

Results

miR-33b is downregulated in osteosarcoma

Previous studies reported miR-33b is a tumor suppressive miRNA in multiple cancers (8-12). To assess the roles of miR-33b in osteosarcoma, we compared the expression of miR-33b in human osteosarcoma and their matched adjacent normal bone tissues by qRT-PCR. As

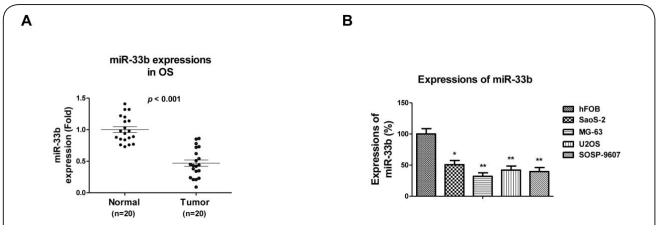

Figure 1. MiR-33b is downregulated in osteosarcoma. (A) The relative expressions of miR-33b in levels were determined by qRT-PCR PCR in 20 paired osteosarcoma tissues and their corresponding adjacent normal bone tissues. The expression of miR-33b was normalized to U6 snRNA. (B) The relative expressions of miR-33b were compared in human osteoblast cell line (hFOB) and osteosarcoma cells (SaoS-2, MG-63, U2OS and SOSP-9607) by qRT-PCR. The expression of miR-33b was normalized to U6 snRNA. *, $p < 0.05$; **, $p < 0.01$.

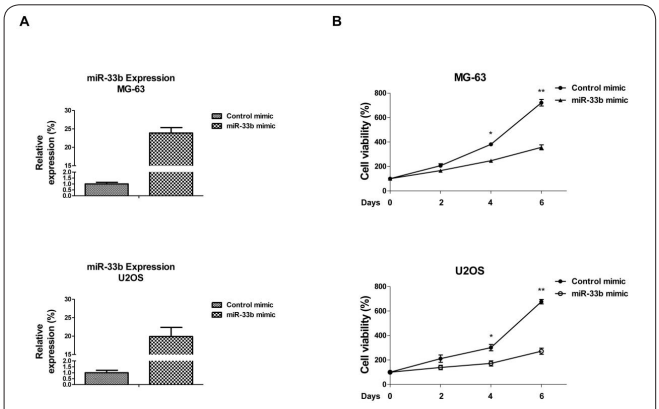

Figure 2. Overexpression of miR-33b inhibits proliferation of osteosarcoma cells. (A) qRT-PCR analysis of miR-33b expression after the transfection of control mimic or miR-33b mimic for 48 hours in MG-63 (upper) and U2OS (lower). (B) The MTT assay was performed to evaluate the proliferation rates of MG-63 (upper) and U2OS (lower) cells with or without transfection of miR-33b mimic. The expression of miR-33b was normalized to U6 snRNA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

shown in Fig. 1A, expressions of miR-33b was significantly downregulated in twenty osteosarcoma tissues compared with the corresponding adjacent normal bone tissues ($p<0.001$). Moreover, we found miR-33b was downregulated in four osteosarcoma cell lines, MG-63, U2OS, Saos-2 and SOSP-9607 compared with that in human normal osteoblast cell line hFOB ($p<0.05$, $p<0.01$) (Fig. 1B).

Overexpression of miR-33b represses tumor progression

To investigate the function of miR-33b in osteosarcoma, we transfected miR-33b mimic or control mimic into MG-63 and U2OS cells (Fig. 2A). As we expected, overexpression of miR-33b inhibited osteosarcoma cell proliferation rate at day 4 and day 6 in both cell lines compared with control mimic transfection ($p<0.05$, $p<0.01$) (Fig. 2B). Taken together, the above results revealed miR-33b is a tumor suppressive miRNA and suppresses osteosarcoma tumor progress.

Cellular glycolysis is suppressed by miR-33b

It has been widely known that tumor cells showed higher glycolysis rate than normal cells, a phenomenon called "Warburg effect" (16). To explore the mechanism of the miR-33b-modulated osteosarcoma growth inhibition, we measured the glycolysis rate of osteosarcoma cells with or without overexpression of miR-33b. The glycolytic flux was quantified by measuring glucose uptake and lactate excretion. We found overexpression of miR-33b significantly suppressed glucose uptake and lactate production of MG-63 and U2OS cells by 40%-60%, respectively (Fig. 3A-3B), indicating the limited cellular metabolic processes may be responsible for the suppressive roles of miR-33b in osteosarcoma cells.

miR-33b directly targets LDHA

We continued to explore the putative targets of miR-33b in glycolysis pathway. As predicted by Targetscan, there were complementary binding sites between hsa-miR-33b and the LDHA 3'-UTR (Fig. 4A). Overexpression of miR-33b reduced the protein levels of LDHA

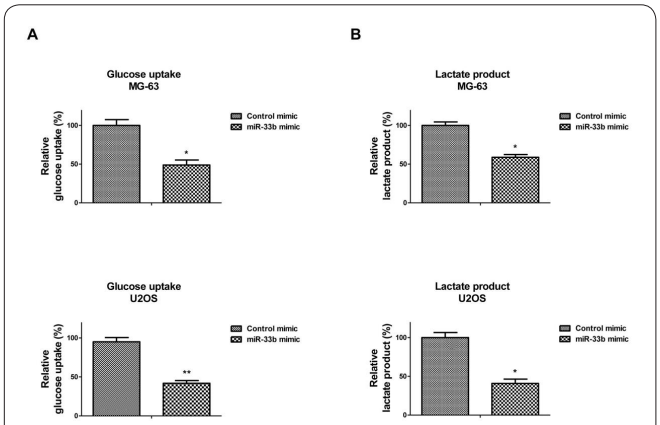

Figure 3. MiR-33b suppresses glycolysis of osteosarcoma cells. (A) MG-63 (upper) and U2OS (lower) cells were transfected with control mimic or miR-33b mimic for 48 hours, the glucose uptake and (B) lactate product were measured. *, $p < 0.05$; **, $p < 0.01$.

in osteosarcoma cells (Fig. 4B). To verify whether miR-33b could directly target 3'UTR region of LDHA, the potential binding sequence within the 3'-UTR of LDHA, as predicted by TargetScan, was mutated to generate a LDHA-mutant 3'UTR luciferase reporter. MG-63 and U2OS cells were co-transfected with control mimic or miR-33b with either original LDHA 3'UTR-luciferase reporter or LDHA mutant 3'UTR-luciferase reporter. The renilla luciferase activity was particularly suppressed by co-transfection of miR-33b with LDHA-3'UTR luciferase reporter ($p<0.01$). Morever, the reduction of renilla luciferase activity was pointedly abolished by co-transfection of miR-33b with LDHA mutant 3'UTR luciferase reporter (Fig. 4C), suggesting that miR-33b could specifically target the predicted 3'UTR region of LDHA in osteosarcoma cells. To test whether miR-33b could target LDHA in osteosarcoma tumors, we compared the expressions of miR-33b and LDHA from OS patients. Real-time RT-PCR results illustrated that the miR-33b is downregulated in OS tumors with high levels of LDHA mRNA (92.9%). Meanwhile, high

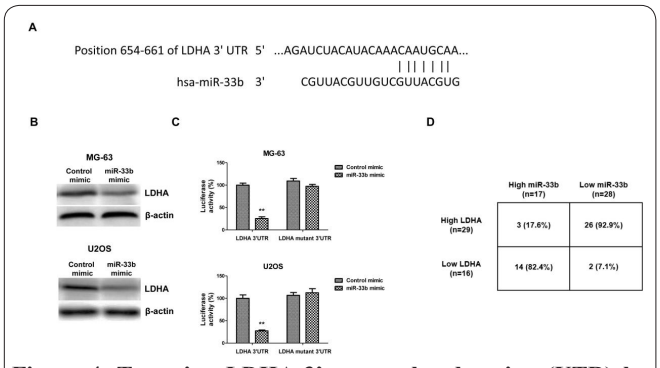

Figure 4. Targeting LDHA 3'-untranslated region (UTR) by miR-33b. (A) Prediction of miR-33b binding sites on 3'UTR region of LDHA mRNA by Targetscan. (B) MG-63 and U2OS cells were transfected with control mimic or miR-33b mimic for 48 hours, the expression of LDHA was detected by Western blot. β-actin is a loading control. (C) MG-63 and U2OS cells were co-transfected with control mimic or miR-33b mimic with LDHA-3'UTR-luciferase reporter and LDHA-mutant 3'UTR-luciferase reporter for 48 hours. The relative luciferase activity was measured by a dual-luciferase reporter assay. (D) Analysis of the correlation between expressions of LDHA mRNAs and miR-33b in osteosarcoma tumors. **, $p < 0.05$.

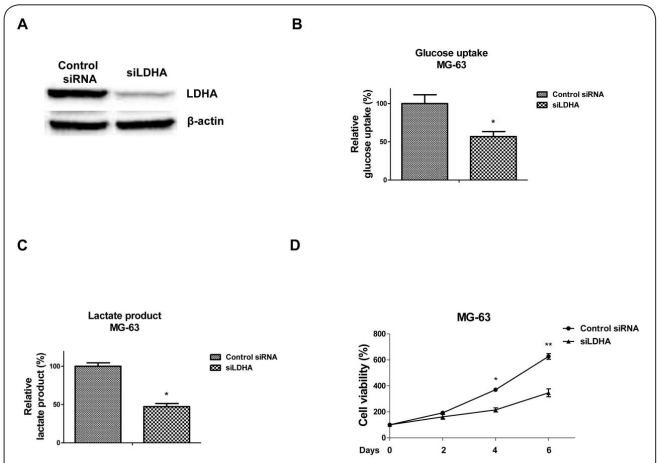

Figure 5. Knockdown LDHA suppresses proliferation rates of osteosarcoma cells. (A) MG-63 cells were transfected with control siRNA or siLDHA for 48 hours, the LDHA protein expression was determined by Western blot. β-actin is a loading control. (B) The glucose uptake and (C) lactate product of MG-63 cells with or without LDHA knockdown were measured. (D) The cell proliferation rates of MG-63 cells with or without LDHA knockdown were measured by MTT assay. *, $p < 0.05$; **, $p < 0.01$.

miR-33b expressions were found majorly in OS tumors with low LDHA mRNA levels (82.4%). These findings illustrated that miR-33b was most likely to target LDHA directly in osteosarcoma tumors, implying that overexpression of miR-33b might be an effective approach against osteosarcoma.

Knockdown of LDHA suppresses proliferation rates of osteosarcoma cells

The above results demonstrated that miR-33b targeted LDHA, thereby resulting in decreased cellular glycolysis rate. We therefore hypothesized that the inhibited cell proliferation may have been caused by the downregulation of LDHA. To test the hypothesis, we knocked down the LDHA expression by siRNA in osteosarcoma cells ($p<0.001$) (Fig. 5A). As expected, knockdown of LDHA limited glycolysis. The glucose uptake (Fig. 5B) and lactate product (Fig. 5C) were also decreased in LDHA knockdown cells compared with control cells ($p<0.05$). Moreover, we observed the cell proliferation rates of MG-63 were significantly suppressed by LDHA knockdown compared to the control siRNA ($p<0.05$, $p<0.01$) (Fig. 5B). Our data indicated that cell proliferation of osteosarcoma was inhibited by glycolysis attenuation.

Restoration of LDHA recovered cell proliferation of miR-33b-overexpressing osteosarcoma cells

We further studied whether the miR-33b-modulated cell proliferation inhibition was through mediation of LDHA expression. MG-63 cells were transfected with control mimic, miR-33b mimic alone or miR-33b mimic with pc-DNA3.1-LDHA overexpression plasmid. The protein levels of LDHA were successfully recovered in the co-transfection of miR-33b and LDHA cells compared to the control and miR-33b overexpression cells (Fig. 6A). Consistently, the glucose uptake (Fig. 6B) and lactate production (Fig. 6C) were increased in the co-transfected cells, clearly indicating that restoration of LDHA improved cellular glycolysis rate. MTT assay was then conducted to examine whether restoration of LDHA could recover cell proliferation. Results in Figure 6D demonstrated that cell proliferation was significantly increased in the miR-33b + LDHA cells compared to the control cells ($p<0.05$, $p<0.01$), indicating that overexpression of LDHA in osteosarcoma cells reversed the suppressive effects of overexpressing miR-33b on cell proliferation. Therefore, our findings suggest that miR-33b plays a suppressive role in the regulation of osteosarcoma cell proliferation by targeting LDHA.

Discussion

It has been previously shown that miRNAs play important roles during the tumorigenesis and malignant progression of osteosarcoma (5, 6). Among them, miR-145 inhibits invasion and metastasis of osteosarcoma cells via inhibiting the protein levels of VEGF (17) and miR-101 inhibits proliferation and induces apoptosis of osteosarcoma cells by targeting mTOR (18). Particularly, functions of miR-33b have been implicated in several human cancers (8-12). For instance, miR-33b is known to suppress the epithelial-to-mesenchymal transition (EMT) and migration of malignant melanoma cells by targeting HMGA2 (19). In addition, miR-33b could inhibit growth, invasion, and EMT of lung adenocarcinoma cells by suppressing Wnt/b-catenin/ZEB1 signaling (8). Recent studies demonstrated miR-33b was downregulated in osteosarcoma tissues compared with adjacent non-tumor bone tissues (20), indicating a tumor suppressive function of miR-33b. However, the detailed molecular mechanism of miR-33b in regulating progressions of osteosarcoma still remains unclear. In this study, we consistently found downregulation of miR-33b in osteosarcoma tumors and cell lines. In addition, we report overexpression of miR-33b significantly reduced osteosarcoma cell growth, suggesting inhibition of miR-33b might contribute to develop anti-osteosarcoma agents.

Altered cellular metabolism is one of the common features of cancer cells by the increased glucose uptake and fermentation of glucose to lactate (16). This phe-

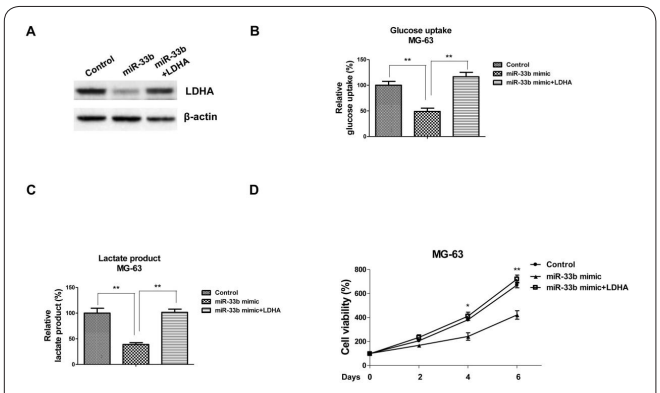

Figure 6. Restoration of LDHA recovers miR-33b-modulated glycolysis and proliferation of osteosarcoma cells. (A) MG-63 cells were transfected with control mimic, miR-33b mimic or miR-33b mimic plus LDHA overexpression plasmid for 48 hours. Western blot analysis showed LDHA protein expressions in these cells. β-actin is a loading control. (B) MG-63 cells were transfected with control mimic, miR-33b mimic or miR-33b mimic plus LDHA overexpression plasmid for 48 hours, then the glucose uptake, (C) lactate product and (D) cell proliferation rates were measured. *, $p < 0.05$; **, $p < 0.01$.

nomenon is observed even in the presence of sufficient oxygen supply and functional mitochondria, known as the 'Warburg Effect' (16). LDHA, a key glycolysis enzyme, is frequently upregulated in human malignancies and predicts a poor prognosis. A recent study reported pharmacological or genetic inhibition of LDHA inhibited osteosarcoma cell proliferation and invasive potential, suggesting an oncogenic role of LDHA in OS (21). We described overexpression of miR-33b significantly suppressed glucose uptake and lactate product of osteosarcoma cells, indicating key enzymes from glycolysis pathway might be the targets of miR-33b.

To identify molecular mechanisms for the miR-33b regulated glycolysis in OS, we performed bioinformatics analysis and found that the 3'UTR region of LDHA mRNA could be bound by seeding sequence of miR-33b. Western blot and luciferase assay confirmed that miR-33b could directly target LDHA in osteosarcoma. Furthermore, with restoration of LDHA in miR-33b overexpressing OS cells, the glycolysis rate and proliferation were recovered. This explains the miR-33b-modulated cell growth inhibition of OS cells by suppression of glycolysis. In summary, this study demonstrates a tumor suppressive role of miR-33b in the regulation of osteosarcoma cell proliferation

To our knowledge, this is the first study that reports LDHA is a direct target of miR-33b in osteosarcoma. Our findings will expand the understanding of the miRNA-based therapeutic strategies in limiting malignant progression of osteosarcoma.

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