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EXPERIMENTAL STUDY

Blockade of miR-663b inhibits cell proliferation and induces apoptosis in osteosarcoma via regulating TP73 expression

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ABSTRACT

OBJECTIVE: This study aimed to investigate the exact role of miR-663b in osteosarcoma (OS) progression and further explore the underlying molecular mechanisms.

MATERIALS AND METHODS: The miR-663b expression in human OS cell lines was determined by qRT-PCR, and the results suggested that miR-663b was highly expressed in human OS cells. TargetScan was used to predict the potential targets of miR-663b, and the prediction was confirmed by dual-luciferase reporter assay. To investigate the role of miR-663b in OS, miR-663b was down-regulated in U2OS cells using miR-663b inhibitor. CCK8 and flow cytometry were preformed to investigate the proliferation and apoptosis of U2OS cells. Moreover, qRT-PCR and western blot analysis were performed to measure the mRNA and protein expression.

RESULTS: We found that miR-663b directly targets TP73 and negatively regulates TP73 expression. MiR-663b inhibitor significantly decreased the proliferation ability of U2OS cells, while the percentage of apoptotic cells was markedly increased. The level of Bcl-2 was notably inhibited by miR-663b inhibitor, while Bax expression was significantly enhanced. Moreover, miR-663b down-regulation promoted p53 and p21 expression in U2OS cells.

CONCLUSIONS: MiR-663b down-regulation represses proliferation and induces apoptosis in OS by targeting TP73. Therefore, we provide a potential therapeutic target for OS treatment *(Fig. 6, Ref. 27)*. Text in PDF www.elis.sk.

KEY WORDS: osteosarcoma, miR-663b, TP73, proliferation, apoptosis.

Introduction

Osteosarcoma (OS), the primary malignant bone tumor in young adults and children, is frequently derived from long bones and characterized by osteoid production produced by malignant cells (1–5). OS has high mobility and aggressivity, mainly to the lung. At present, the main therapeutic mean for OS is the combination treatment with chemotherapy and surgery, however, the five-year survival of OS patients remains relatively low (6, 7). Therefore, it is urgent for researchers to explore new therapeutic targets for OS treatment.

MicroRNAs (miRNAs), a class of short non-coding RNAs, 18-22 nucleotides in length, can negatively regulate the expression of the target genes via binding to the 3'-untranslated regions (UTR) of the specific gene mRNAs (8). MiRNAs play an important role in regulating a variety of biological processes, including cell proliferation, cell cycle, apoptosis and differentiation (9–11). Evidences have indicated that miRNAs play critical roles in managing many normal biological processes, and abnormal expression of miRNAs is associated with various cancers occurrence, such as gastric cancer, lung cancer, OS, etc. (12–14), and miRNAs serve as oncogenes or cancer suppressors during the progression of OS (15, 16). For example, Bi et al (17) suggested that miR-100 serves as a cancer inhibitor in OS development via suppressing OS growth by regulating FGFR3 expression. MicroRNA-133a can prevent the proliferation and invasion of osteosarcoma cells by targeting IGF-1R (18). MiR-542-5p has been found to serve as an oncogene in OS though promoting OS proliferation by targeting HUWE1 (19).

MiR-663b has been found to be up-regulated in OS (14), however, the exact roles of miR-663b in OS and the underlying molecular mechanisms are still unclear. Thus, in this study, we will investigate the role of miR-663b in OS and further explore the underlying molecular mechanisms.

Materials and methods

Materials

The normal osteoblast hFOB1.19 cell line and human osteosarcoma (HOS) cell lines MG63 and U2OS were obtained from American type culture collection (ATCC); the RPMI 1640 me-

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dium, DMEM medium and fetal bovine serum (FBS) were obtained from Invitrogen company (Waltham, MA, USA); the primary antibodies and the second antibody were supplied by Cell Signaling Technology company (Danvers, MA, USA); the CCK8 kit was purchased from Eli Lilly and Company (USA); and the lipofectamine 2000 cell transfection reagent was purchased from Invitrogen company (USA).

Cell culture

The U2OS cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Invitrogen), and incubated in humidified atmosphere of 5 % CO_2 at 37 °C. The MG63 cells were cultured in DMEM medium. The normal osteoblast hFOB1.19 cell line was grown in F12/DMEM (v/v: 1: 1, Hyclone) medium containing 10 % FBS, 2.5mM/L glutamine (without phenol red), and 0.3 mg/ml G418 (Sigma, St. Louis, MO, USA). Fresh cell culture medium was replaced every 2–3 days.

Dual luciferase reporter assay

TargetScan was used in the present study to predict the targets of miR-663b, and TP73 was found to be a potential target of miR-663b. To reveal the prediction, dual luciferase reporter assay was applied. Wild-type and mutant 3′-UTRs of TP73 were amplified and then cloned into the psiCHECK-2 reporter. MiR-663b and miR-663b-TP73-WT 3'UTR or miR-663b-TP73-MUT 3'UTR vector were co-transfected into U2OS cells using Lipofectamine 2000 regent following the instructions provided by the manufacturer. 48 h later, the luciferase activity was assessed by using the Dual-Luciferase Reporter Assay System (Promega, USA).

Cell transfection

To investigate the role of miR-663b in OS, a stable miR-663b-low expression U2OS cell line was generated via performing cell transfection assay. In brief, U2OS cells were transfected with miR-663b inhibitor, its negative control, or miR-663b inhibitor + siTP73 RNA by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. 48 h after the cell transfection, the U2OS cells were collected and then used for subsequent analysis.

Cell proliferation assay

48 h after transfection, the U2OS cells were seeded into a 96-well culture plate (Corning Costar, Corning, NY, USA) (2,500 cells per well) and then incubated for 24 h at 37 °C under 5 % (v/v) CO_2. Subsequently, 10 μg/ml CCK8 solution was added to each well at certain time points, and then incubated at 37 °C for 2 h. At the end of the test, the OD value at 450 nm was detected by using a microplate reader (Thermo Labsystems, Waltham, MA, USA). Experiments were repeated at least 3 times.

Apoptosis analysis assay

48 h after transfection, cells were washed for at least 3 times using cold PBS solution. Then the U2OS cells were fixed with 70 % ethanol (30 min), rinsed with PBS solution, labeled with annexin V-FITC and propidium iodide (PI), and then incubated at room temperature for 30 min. Finally, cell apoptosis was analyzed using the flow cytometry (Becton Dickinson, New Jersey, USA) and cell apoptotic rate was calculated. Tests were repeated three times.

Western blot

48 h after cell transfection, log-phase U2OS cells were harvested, and total cellular protein was extracted by using RIPA Buffer (Auragene, Changsha, China). The BCA protein quantitative kit (Thermo, USA) was applied to determine protein concentration. Equal amount of protein samples were resolved by using SDS-PAGE analysis and then transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked with 5 % fat-free milk at room temperature for 1 h. Subsequently, the membrane was incubated with a primary antibody (anti-β-actin 1 : 1000; anti-p53 1 : 1000; anti-p21 1 : 1000; anti-TP73 1 : 1000; anti-Bcl-2 1 : 1000; anti-Bax 1 : 1000) at 4 °C overnight, then washed three times with TBST solution, followed by incubation with a HRP-conjugated secondary antibody (Anti-rabbit IgG, HRP-linked Antibody; 1 : 5,000) at room temperature for 1 h. To visualize the protein bands, an ECL kit (Applygen, Beijing, China) was applied following the manufacturer's protocol.

QRT-PCR

Total RNA of U2OS cells was extracted using TRIZOL reagent (Takara, Japan) according to the instructions supplied by the manufacturer. To evaluate the quality and integrity of the RNA, A260/A280 ratios were assessed. U6 and GAPDH served as internal controls for the miRNA and mRNA expression, respectively. The PrimeScript™ RT reagent kit (Takara, Japan) was used for cDNA generation. SYBR Premix Ex Taq (Takara, Japan) was carried out for RT-PCR analysis. The TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, USA) was used to reversely transcribe miRNA into cDNA, and RT-PCR was carried out by using the SYBR RT-PCR kit (Takara, Japan).

Statistical analysis

All quantitative data are shown as the mean ± SD. SPSS 17.0 statistical software (SPSS, Chicago, IL, United States) was applied for statistical analyses. Student's t-test or ANOVA was used to compare differences between groups. $p < 0.05$ was considered statistically significant.

Results

miR-663b is up-regulated in osteosarcoma cell lines

The expression level of miR-663b was determined by qRT-PCR in the human osteosarcoma MG-63, U2OS and osteoblast hFOB1.19 cell lines. The results suggested that compared with the control, the expression of miR-663b was significantly increased in MG-63 and U2OS cells, especially in U2OS cells, indicating that miR-663b was up-regulated in OS (Fig. 1). U2OS cells were used for further analysis.

P73 is a target of miR-663b

To investigate the role of miR-663b in the development of OS, TargetScan was performed to predict the target gene of miR-663b

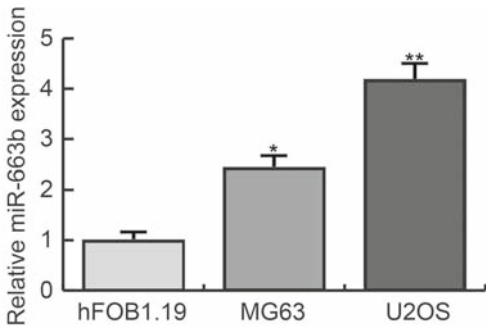

Fig. 1. miR-663b is up-regulated in osteosarcoma cell lines. We performed qRT-PCR to determine the miR-663b expression. hFOB1.19: normal human osteoblastic cell line; MG63 and U2OS: human osteosarcoma cell lines. *, **p < 0.05, 0.01 *vs* hFOB1.19. Experiments were repeated 3 times.

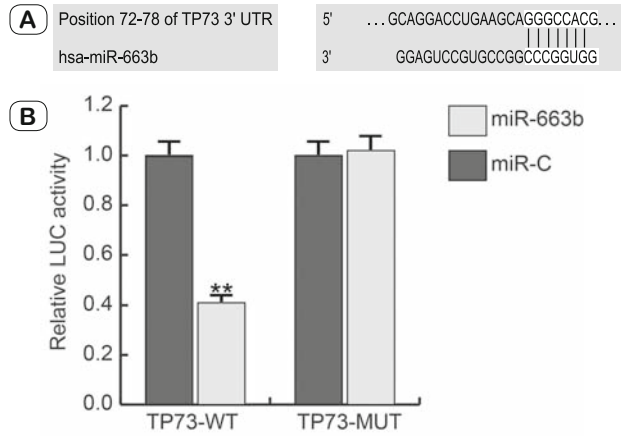

Fig. 2. TP73 is a direct target of miR-663b. A: Interaction between miR-663b and 3'UTR of TP73 was predicted using TargetScan; B: Luciferase activity of a reporter containing a wild-type TP73 3'UTR or a mutant TP73 3' UTR are presented. "TP73-3'UTR-MUT" indicates the TP73 3' UTR with a mutation in the miR-663b binding site. UTR, untranslated region. **p < 0.01 *vs* control. All data are presented as the mean ± SD of three independent experiments.

(Fig. 2A), and the luciferase reporter gene assay was carried out to confirm our prediction. The results showed that the luciferase activity was significantly reduced in miR-663b and miR-663b-TP73-WT co-transfected U2OS cells, but co-transfection of miR-663b with miR-663b-TP73-MUT did not reduce it (Fig. 2B). These results revealed miR-663b could inhibit expression of transcripts containing a miR-663b binding site, indicating that TP73 is a direct target gene of miR-663b.

Furthermore, we found that TP73 had low expression in U2OS cells (Figs 3A and B). Moreover, to determine the role of miR-663b in U2OS cells,U2OS cells were transfected with miR-663b inhibitor, its negative control, or miR-663b inhibitor + siTP73

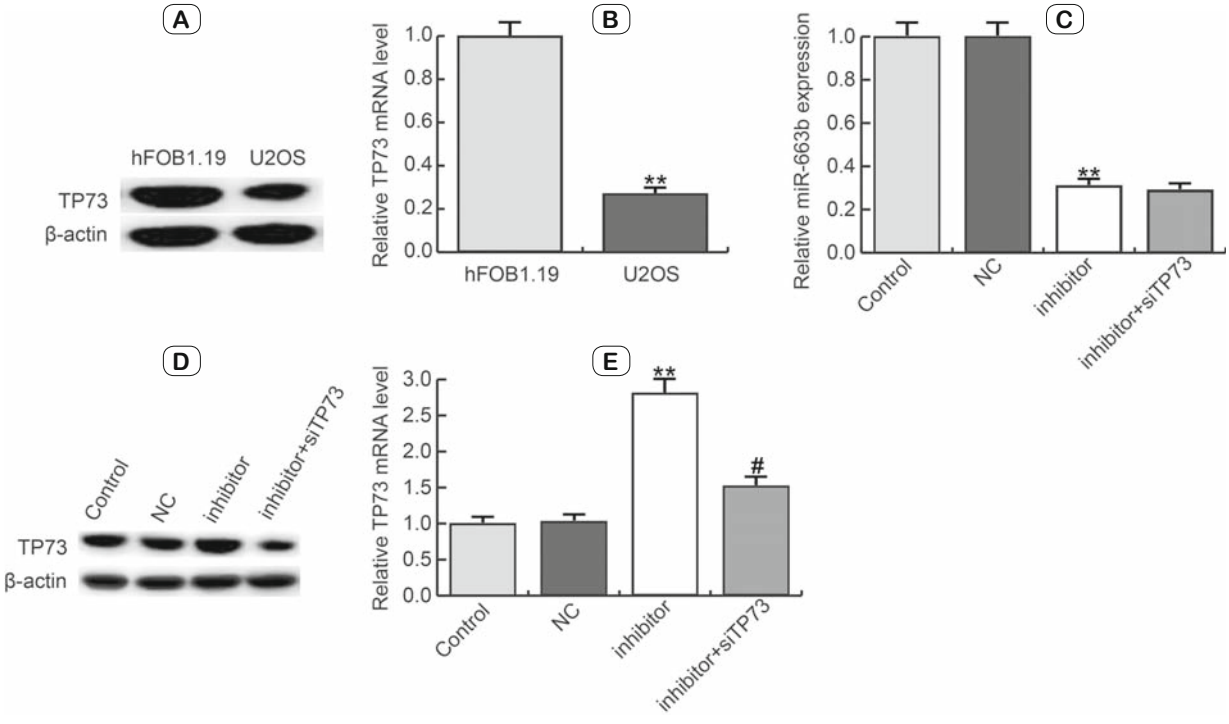

Fig. 3. Expression of TP73 in different groups. A: protein level of TP73 in hFOB1.19 and U2OS cell line was measured by western blotting; B: mRNA level of TP73 in hFOB1.19 and U2OS cell lines were measured by qRT-PCR; C: relative miR-663b expression in different groups was detected by qRT-PCR; D: protein level of TP73 in different groups was detected by western blotting; E: mRNA level of TP73 in different groups was detected by qRT-PCR. Control: cells without any treatment; NC: cells transfected with the negative control of miR-663b inhibitor; inhibitor: cells transfected with miR-663b inhibitor; inhibitor+siTP73: cells co-transfected with miR-663b inhibitor and siTP73 RNA. All data are presented as the mean ± SD of three independent experiments. ** p < 0.01 *vs* control; # p < 0.05 *vs* inhibitor.

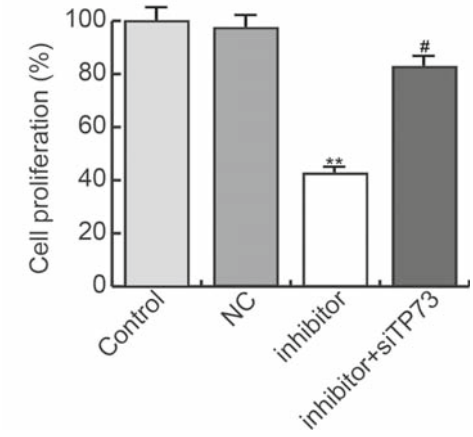

Fig. 4. miR-663b down-regulation inhibits the proliferation of U2OS cells. Cell proliferation was analyzed by CCK8 assay in the U2OS cells. Control: cells without any treatment; NC: cells transfected with the negative control of miR-663b inhibitor; inhibitor: cells transfected with miR-663b inhibitor; inhibitor+siTP73: cells co-transfected with miR-663b inhibitor and siTP73 RNA. All data are presented as the mean ± SD of three independent experiments. **p < 0.01 *vs* control; # p < 0.05 *vs* inhibitor.

RNA, and the transfection efficiency was assessed by qRT-PCR (Figure 3C). And we found that miR-663b negatively regulated TP73 expression in U2OS cells. miR-663b inhibitor significantly increased TP73 expression, and this increase could be eliminated by siTP73 RNA (Figs 3D and E).

miR-663b down-regulation suppresses the proliferation of U2OS cells

To explore the effect of miR-663b on OS cell proliferation, cell proliferation was measured by using CCK-8 assay. As shown in Figure 3, miR-663b inhibitor markedly decreased the proliferation ability of U2OS cells, and this decrease could be eliminated by siTP73 RNA (Fig. 4). The data indicated that miR-663b down-regulation inhibited the proliferation of osteosarcoma cells.

miR-663b down-regulation induces the apoptosis of osteosarcoma cells

To investigate whether cell apoptosis participated in the proliferation repression caused by miR-663b inhibitor apoptosis of the U2OS cells was analyzed by using flow cytometry. As shown in Figures 5A and B, compared with the control, miR-663b down-regulation notably increased the apoptotic rate of U2OS cells, and this increase could be eliminated by siTP73 RNA .

In addition, the expression level of Bcl-2 and Bax was determined, and we found that miR-663b inhibitor significantly decreased Bcl-2 expression, and the expression level of Bax was markedly enhanced. These effects could be offset by siTP73 RNA (Fig. 5C).

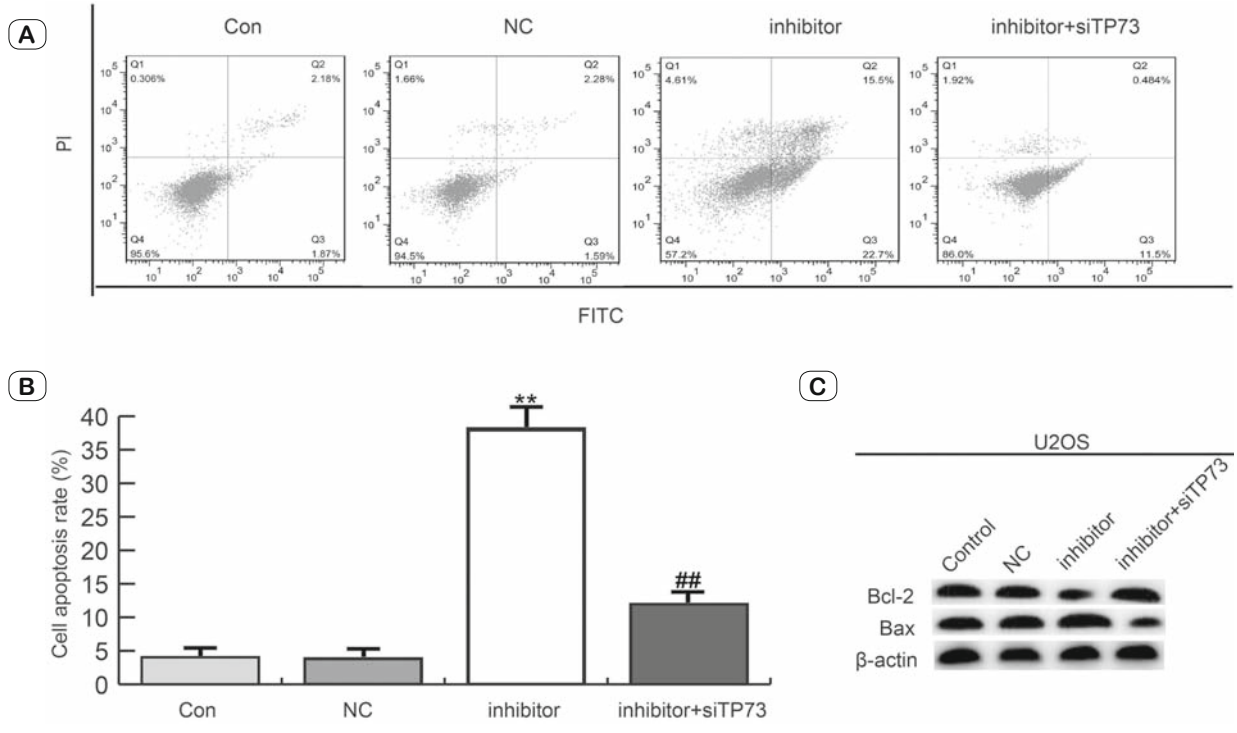

Fig. 5. miR-663b down-regulation induces the apoptosis of U2OS cells. Flow cytometry was performed to detect the cell apoptosis (A), and data was analyzed (B). C: Bax and Bcl-2 protein level in different groups were detected by western blotting. Control: cells without any treatment; NC: cells transfected with the negative control of miR-663b inhibitor; inhibitor: cells transfected with miR-663b inhibitor; inhibitor+siTP73: cells co-transfected with miR-663b inhibitor and siTP73 RNA. All data are presented as the mean ± SD of three independent experiments. ** p< 0.01 *vs* control; ## p < 0.01 *vs* inhibitor.

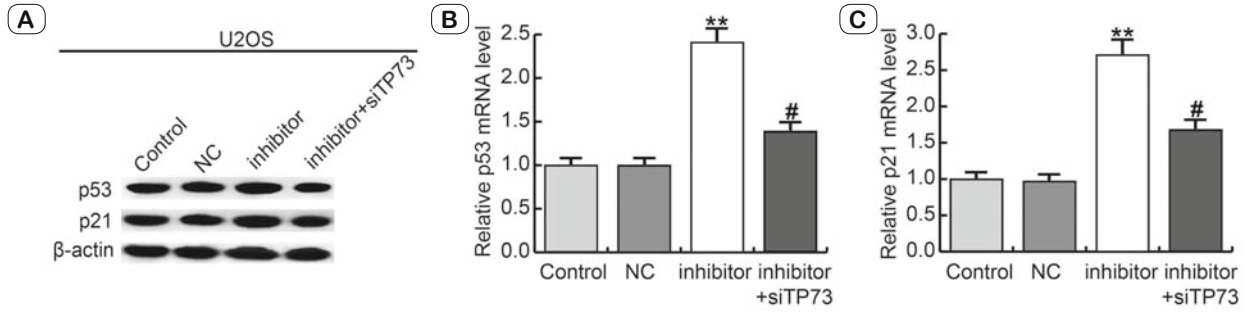

Fig. 6. p53 and p21 expression before and after miR-663b down-regulation. A: protein expression levels of p53 and p21 in U2OS cells; B and C: relative mRNA expression levels of p53 and p21 in U2OS cells. Control: cells without any treatment; NC: cells transfected with the negative control of miR-663b inhibitor; inhibitor: cells transfected with miR-663b inhibitor; inhibitor+siTP73: cells co-transfected with miR-663b inhibitor and siTP73 RNA. All data are presented as the mean ± SD of three independent experiments. ** $p < 0.01$ *vs* control; # $p < 0.05$ *vs* inhibitor.

miR-663b down-regulation promotes p53 and p21 expression

To explore the precise molecular mechanism of the effect of miR-663b on U2OS cell proliferation and apoptosis, the level of p53 and p21 was determined by using western blotting and qRT-PCR analysis. As shown in Figure 6, compared with the control, miR-663b inhibitor significantly enhanced the expression of p53 and p21 at both the mRNA and the protein level, and the changes could be offset by siTP73 RNA.

Discussion

In the present study, we revealed that miR-663b down-regulation plays critical roles in preventing OS cell growth and inducing OS cell apoptosis. miR-663b directly targets TP73 and negatively regulates TP73 expression. miR-663b low-expression thus indirectly enhanced p53 and p21 expression, which probably contributes to the inhibition of U2OS cell proliferation and the induction of U2OS cell apoptosis. First, we confirmed the high expression of miR-663b in osteosarcoma cell lines MG-63 and U2OS compared with the normal osteoblast hFOB1.19 cell line. And miR-663b expression is higher in U2OS cells than in MG-63 cells. Among the established human OS cell lines, U2OS cell line is widely used in scientific studies of OS (20). Therefore, in the present study, U2OS cell line was performed for the investigation of OS in vitro. We next found a novel functional link between miR-663b and TP73 in the progression of OS. The data also suggested miR-663b inhibitor significantly inhibited the proliferation and induced apoptosis of U2OS cells in vitro. p53 and p21 expression level was notably enhanced by miR-663b inhibition. Furthermore, we found that the effects of miR-663b inhibitor on U2OS cells could be reversed by siTP73 RNA. Our results revealed that miR-663b served as an oncogene in OS development, and miR-663b inhibitor plays important roles in tumor repression, thus, miR-663b may serve as a potential therapeutic target in the treatment of OS.

A growing number of studies suggest that miRNAs play an important role in cancer diagnosis, therapy and prognosis, and participate in tumorigenesis, tumor growth and tumor metastasis (10, 21). Thus, miRNAs may be potential targets for cancer therapy. A large number of studies reported that many miRNAs are involved in OS cell proliferation, migration and invasion (22–24). To date, the high expression of miR-663b in OS has been revealed however to the best of our knowledge the exact role of miR-663b in OS has not been investigated. Here, our results strongly proved that miR-663b expression was significantly up-regulated in OS cell lines, and miR-663b inhibition significantly prevented the proliferation and induced apoptosis of U2OS cells.

TP73 (p73) is a member of the transcription factor p53 family and was first discovered in 1997. Due to its homology with the structure and function of p53, the research of p73 instead of p53 has become a hot spot. P73 can induce apoptosis and cell cycle arrest by binding to p53 response gene or trans activating p53 response gene (25). p73 has low expression in OS, and the triggering of p73 dependent apoptosis in OS is controlled by the E2Fs-pRb2/p130 complex (26, 27). However, no interaction between miR-663b and TP73 in OS cells has been reported previously. In the present study, we revealed that TP73 was a direct target of miR-663b, thus, miR-663b indirectly regulated the expression of p53 and p21.

In summary, we found for the first time that miR-663b down-regulation inhibited the proliferation of OS cells and induced cell apoptosis via directly targeting TP73. Moreover, miR-663b inhibition could indirectly enhance the expression of p53 and p21. Therefore, miR-663b may serve as a potential therapeutic target for OS treatment.

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