Oncology Research, Vol. 25, pp. 1161–1168
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0965-0407/17 $90.00 + .00
DOI: https://doi.org/10.3727/096504017X14841698396829
E-ISSN 1555-3906
www.cognizantcommunication.com

MicroRNA-21 Inhibits the Apoptosis of Osteosarcoma Cell Line SAOS-2 via Targeting Caspase 8

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Currently, multiple microRNAs (miRNAs) have been found to play vital roles in the pathogenesis of osteosarcoma. This study aimed to investigate the role of miR-21 in osteosarcoma. The level of miR-21 in 20 pairs of osteosarcoma and corresponding adjacent tissues was monitored by qPCR. Human osteosarcoma cell line SAOS-2 was transfected with either miR-21 mimic or miR-21 inhibitor, and then cell viability, survival, and apoptosis were measured by MTT, colony formation assay, and flow cytometry. A target of miR-21 was predicted by the microRNA.org database and verified in vitro by using luciferase reporter, qPCR, and Western blot analyses. Finally, cells were cotransfected with siRNA against caspase 8 and miR-21 inhibitor, and the apoptotic cell rate was determined again. Results showed that the mRNA level of miR-21 was highly expressed in osteosarcoma tissues compared with adjacent tissues. Overexpression of miR-21 improved cell viability and survival but suppressed apoptosis. Caspase 8 was a direct target of miR-21, and it was negatively regulated by miR-21. Moreover, miR-21 suppression attenuated caspase 8 silencing and induced the decrease in apoptosis. In conclusion, overexpression of miR-21 suppressed SAOS-2 cell apoptosis via directly targeting caspase 8.

Key words: MicroRNA-21; Osteosarcoma; Cell survival; Apoptosis; Caspase 8

INTRODUCTION

Osteosarcoma is a cancerous tumor of the bone characterized by the direct formation of immature bone or osteoid tissue by the tumor cells[1]. To date, the pathogenesis of osteosarcoma remains unclear, although the retinoblastoma gene, bone dysplasias, Li–Fraumeni syndrome, and Rothmund–Thomson syndrome have been identified as high risks for osteosarcoma[2–4]. Currently, surgical resection therapy and chemotherapy have been practiced in patients with osteosarcoma[5]. However, the outcome for those patients with metastatic or recurrent osteosarcoma remains dismally poor[6]. Therefore, a better understanding of the tumorigenesis and progression of osteosarcoma could lead to the development of novel therapeutic approaches for this tumor.

MicroRNAs (miRNAs) are a category of short, noncoding, single-stranded RNAs of about 22 nucleotides that are widely found in both animals and plants[7]. miRNAs regulate their targets by inhibiting their translation and/or by promoting degradation of their mRNA[8]. Currently, several miRNAs, such as miR-367, miR-224, and miR-184, have been suggested as candidates in the treatment of osteosarcoma by targeting their specific mRNAs[9–11]. The expression of miR-21 is remarkably increased in a number of tumors, such as breast, colon, gastric, and pancreatic cancers[12]. In osteosarcoma, miR-21 is highly expressed, and overexpression of miR-21 promotes proliferation and invasion and suppresses apoptosis in the human osteosarcoma line MG63[13]. Furthermore, miR-21 is capable of sensitizing human osteosarcoma cells to cisplatin, implying that miR-21 is implicated in the modulation of drug-induced resistance[14]. However, the detailed function of miR-21 in osteosarcoma and its underlying molecular mechanism remain elusive.

Therefore, this study aimed to investigate the impact of miR-21 on osteosarcoma cell apoptosis and its potential mechanism. The expression of miR-21 in osteosarcoma tissue and adjacent tissue samples was monitored. Human osteosarcoma cell line SAOS-2 was used and transfected with miR-21 mimic, miR-21 inhibitor, or control to assess the effects of miR-21 on cell survival and apoptosis. A target of miR-21 was predicted and verified in vitro, revealing a possible molecular mechanism of miR-21 in osteosarcoma. Our study showed the potential role of miR-21 in osteosarcoma and might provide possibilities for its use in treating this disease.

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MATERIALS AND METHODS

Human Tissue Samples and Cell Culture

Human osteosarcoma tissue and paired normal adjacent tissue samples were obtained during standard surgery from 20 osteosarcoma patients (12 men and 8 women; aged 19 to 46 years). These patients were admitted to the hospital between July 2012 and January 2015. None of the patients had received blood transfusion, radiotherapy, or chemotherapy before surgery. All tissues were frozen immediately in liquid nitrogen at the time of surgery and stored at −80°C for RNA extraction[15]. The study was approved by our local ethics committee, and written informed consent was obtained from all subjects for the use of their osteosarcoma and adjacent tissues for research.

Human osteosarcoma cell line SAOS-2 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA)[16]. Cells were incubated in a humidified 5% CO_2 incubator at 37°C.

RNA Extraction and Real-Time Quantitative PCR (qPCR)

Total RNA was extracted by TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). For cDNA synthesis, 2 μg of total RNA was reverse transcribed using Transcriptor First-Strand cDNA Synthesis Kit (Roche, USA) according to the manufacturer's instructions. The real-time qPCR was performed using FastSTART Universal SYBR Green Master (ROX; Roche) on the ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA)[15]. Data were analyzed using the $2^{-\Delta\Delta}$CT method. Levels of caspase 8 and miR-21 were normalized to those of GAPDH and U6, respectively. All primers were synthesized by GenePharma (Shanghai, P.R. China).

Cell Transfection

Cells were plated on 60-mm dishes and cultured for 24 h. Subsequently, miR-21 mimic, miR-21 inhibitor, or control, and/or small interfering RNA (siRNA) against caspase 8 (GenePharma) were transfected into cells. The transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions[17]. After 48 h, cells were collected for use in subsequent experiments.

Cell Viability Assay

Cell viability was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. After cells were transfected with miR-21 mimic, miR-21 inhibitor, or control, cells were planted into 96-well plates at a density of 2×10^3 cells/well and cultured for 1–4 days. Afterward, 20 μl of MTT (5 mg/ml; Sigma-Aldrich) was added into each well and incubated for another 4 h at 37°C. Then 150 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added, and the plates were shaken for 10 min. The absorbance was detected under a Multiskan EX (Thermo, Finland) at 450 nm[18].

Colony Formation Assay

After cells were transfected with miR-21 mimic, miR-21 inhibitor, or control, cells were seeded onto the cultured dishes (6 cm in diameter) at a density of 1×10^3 cells/well. Then the cells were incubated for 14 days at 37°C. Colonies were stained with Giemsa (Sigma-Aldrich) for 30 min, and colony numbers were counted under an optical microscope (Leica Microsystems, Wetzlar, Germany)[19].

Apoptosis Assay

Cell apoptosis was assessed using the Annexin-V/FITC and PI apoptosis detection Kit (Becton Dickinson, Franklin Lakes, NJ, USA). Briefly, 48 h after transfection, cells were collected and resuspended in 500 μl of annexin-binding buffer. After 30 min of incubation, 10 μl of annexin V–FITC and 5 μl of PI were added and the samples were incubated for another 30 min in the dark at room temperature. Apoptotic cells were immediately analyzed by flow cytometry (Becton Dickinson)[20].

Luciferase Reporter Assay

The 3′-UTR of caspase 8 was amplified by PCR and placed in the pmiR-Report vector (Ambion, Grand Island, NY, USA). These vectors were cotransfected with miR-21 mimic, miR-21 inhibitor, or control into cells using Lipofectamine 2000 (Invitrogen). After 48 h, luciferase assay was carried out using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) as previously described[21].

Western Blot

Cellular protein in the transfected cells was extracted by RIPA lysis buffer (Beyotime, Shanghai, P.R. China). Protein samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with 5% skim milk for 1 h at room temperature and were incubated with the special primary antibodies: caspase 8 (1C12), cleaved caspase 8 p18 (Asp387), or GAPDH (D4C6R) (all at a dilution of 1:1,000; Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. The membranes were

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then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were developed using enhanced chemiluminescence and analyzed by ImageJ 1.49 (National Institutes of Health, Bethesda, MD, USA)[19].

Statistical Analysis

All data were presented as the mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using the GraphPad Prism 5 software (GraphPad, San Diego, CA, USA), and data were analyzed by Student's *t*-test. A value of $p < 0.05$ was considered significant.

RESULTS

miR-21 Was Upregulated in Osteosarcoma

To investigate the role of miR-21 in osteosarcoma, the mRNA level expression of miR-21 in osteosarcoma and adjacent tissues was monitored by qPCR. A significant upregulation of miR-21 was found in osteosarcoma tissues when compared with adjacent tissues (Fig. 1). Thus, we suspected that miR-21 might be a pivotal regulator in the pathogenesis of osteosarcoma, and miR-21 might serve as a biomarker during osteosarcoma.

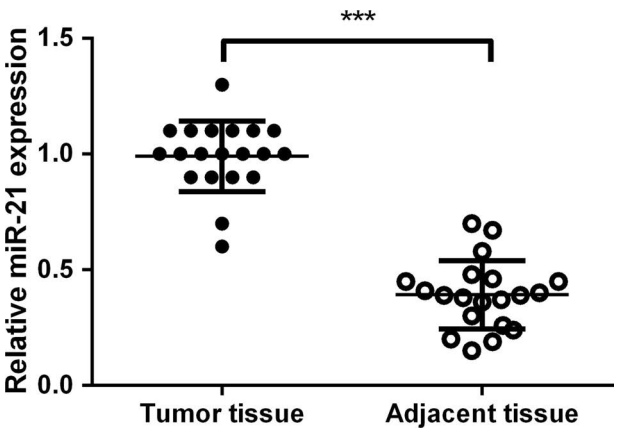

Figure 1. MicroRNA-21 (miR-21) was upregulated in osteosarcoma. mRNA level expression of miR-21 in osteosarcoma tissue and adjacent tissue samples ($n = 20$) was measured by quantitative PCR (qPCR). ***$p < 0.001$ when compared to the control.

Overexpression of miR-21 Increased SAOS-2 Cell Survival While Suppressing Apoptosis

In order to investigate the biological effects of miR-21 on osteosarcoma, SAOS-2 cells were transfected either with miR-21 mimic or miR-21 inhibitor. Subsequently, the viability, survival, and apoptosis of miR-transfected cells were determined by MTT, colony formation assay, and flow cytometry. As expected, the expression of miR-21 was significantly upregulated by transfection with miR-21 mimic and was downregulated by transfection with the miR-21 inhibitor (Fig. 2A). MTT results showed that overexpression of miR-21 significantly increased cell viability after cells were cultured for 2–4 days (Fig. 2B). However, suppression of miR-21 displayed the opposite results at the same time points. In accordance with the results from the MTT assay, colony formation assay showed that cell survival was significantly increased by miR-21 overexpression while being decreased by miR-21 suppression (Fig. 2C). miR-21 overexpression significantly decreased the apoptotic cell rate, while miR-21 suppression significantly induced cell apoptosis (Fig. 2D and E). Taken together, these results suggested that miR-21 was a key regulator in SAOS-2 cell survival and apoptosis.

Caspase 8 Was a Target Gene of miR-21

To explore the underlying molecular mechanism of miR-21 on SAOS-2 cell apoptosis, the microRNA.org (www.microrna.org) database was used to predict the target gene of miR-21. Results showed that caspase 8 might be a target gene of miR-21 (Fig. 3A). Furthermore, a dual-luciferase reporter assay showed that miR-21 reduced the activity of the luciferase reporter fused to the 3′-UTR-WT of caspase 8, but did not suppress that of the reporter fused to the Mut version, revealing that caspase 8 is a direct target of miR-21 (Fig. 3B and C). Next, the expression changes of caspase 8 were measured in the miR-21-overexpressing and -suppressing cells. Both caspase 8 and cleaved caspase 8 were downregulated by miR-21 overexpression and upregulated by miR-21 suppression (Fig. 4A–F). Furthermore, cells were transfected with caspase 8 siRNA alone or together with the miR-21 inhibitor. We then found that the levels of caspase 8 and cleaved caspase 8 were both downregulated by caspase 8 silencing, while this downregulation was partly abolished by miR-21 suppression (Fig. 5A and B). These results suggested that caspase 8 was a target gene of miR-21, and it was negatively regulated by miR-21.

Overexpression of miR-21 Suppressed SAOS-2 Cell Apoptosis via Targeting Caspase 8

To further explore the correlations between miR-21 and caspase 8 in SAOS-2 cell apoptosis, cell apoptosis was measured in caspase 8-silencing cells and in miR-21-suppressing and caspase 8-silencing cells. Caspase 8 silencing significantly inhibited cell apoptosis, and this inhibition was partly abolished by miR-21 suppression (Fig. 4A and B). Therefore, we inferred that the impact of miR-21 on SAOS-2 cell apoptosis might be via targeting caspase 8.

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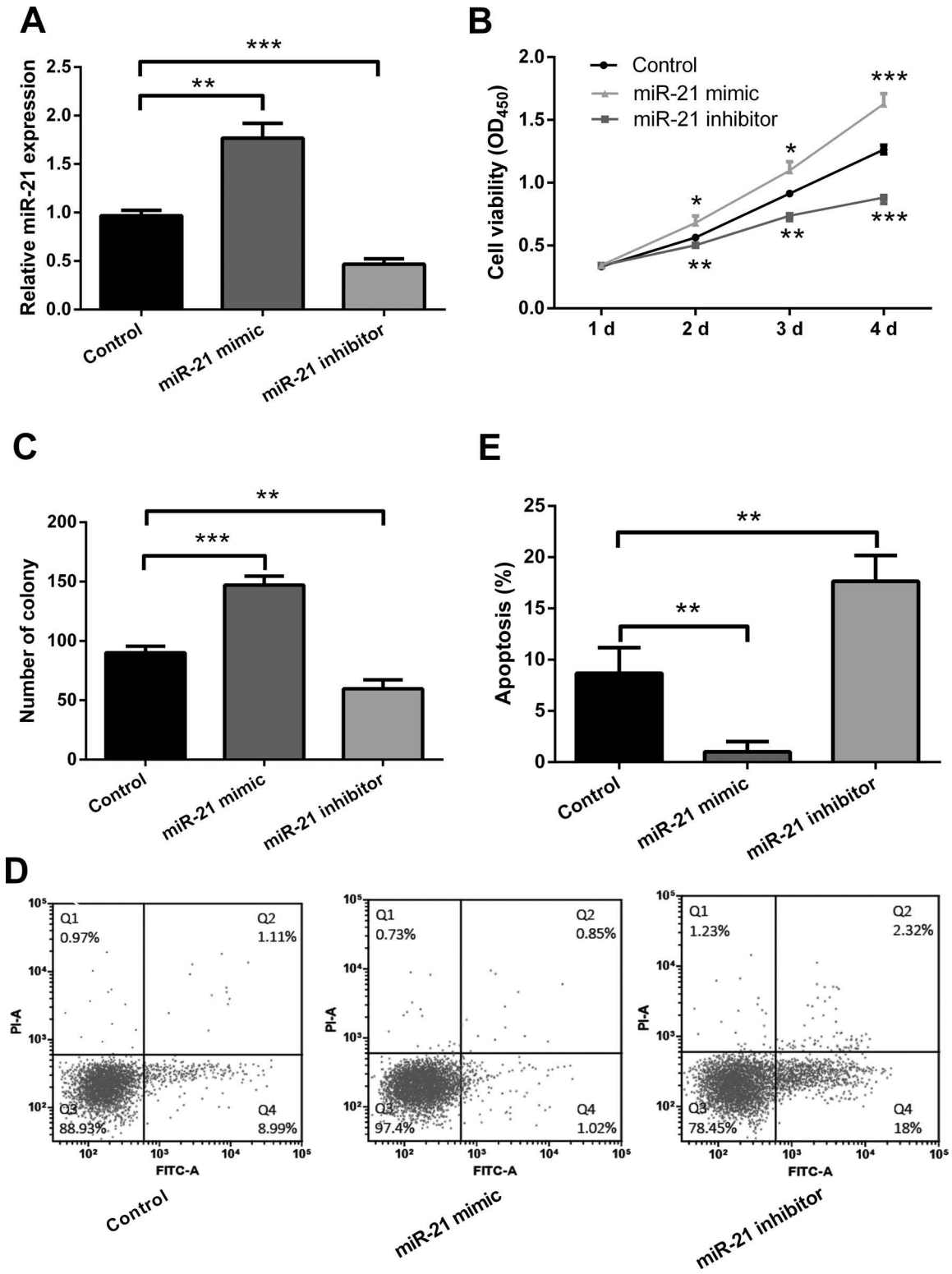

Figure 2. Overexpression of miR-21 increased SAOS-2 cell survival while suppressing apoptosis. miR-21 mimic, miR-21 inhibitor, or control was transfected into SAOS-2 cells. Subsequently, (A) the transfection efficiency was tested by qPCR, (B) cell viability was determined by MTT assay, (C) cell survival was measured by colony formation assay, and (D, E) the apoptotic cell rate was detected by flow cytometry. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the control.

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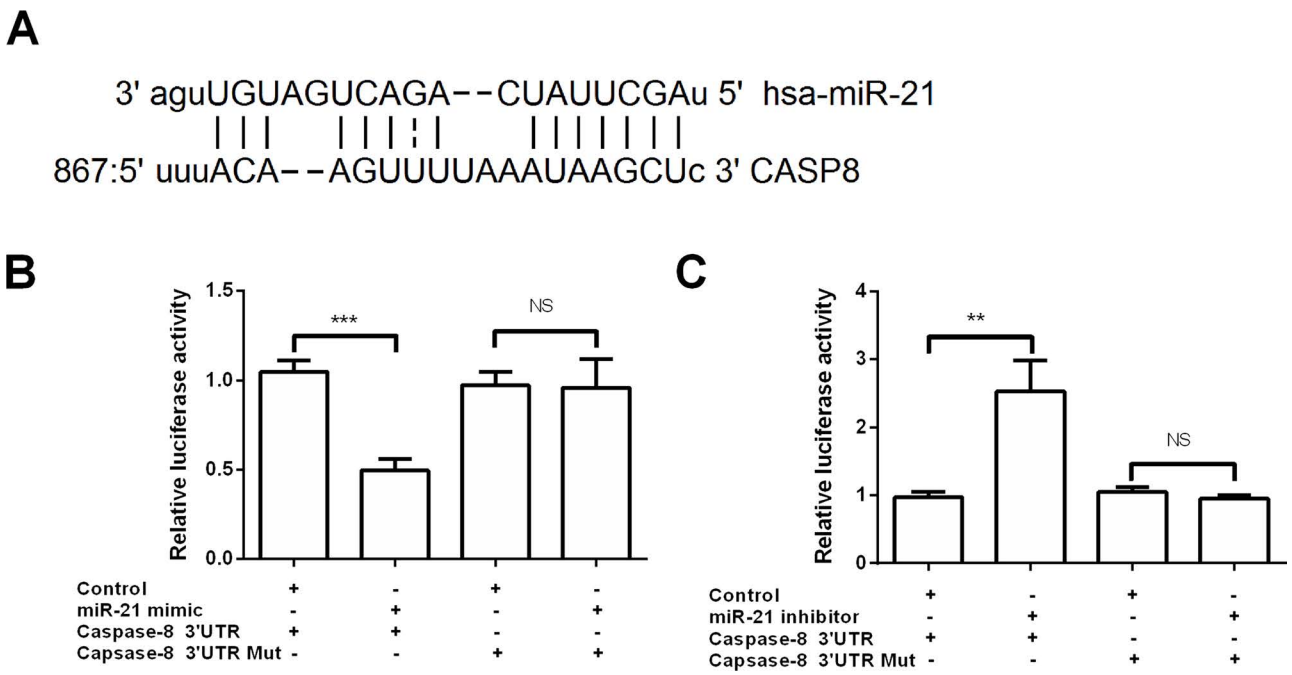

Figure 3. Caspase 8 was a target gene of miR-21. (A) The target of miR-21 was predicted using the microRNA.org database. (B) miR-21 mimic or (C) miR-21 inhibitor was cotransfected with the pmiR-Report vector containing full-length caspase 8 3′-UTR or a mutated seed site within pmiR-caspase 8 into SAOS-2 cells, and dual-luciferase reporter assay was then performed. NS, no significance. $^{**}p<0.01$, $^{***}p<0.001$ when compared to the control.

DISCUSSION

Currently, plenty of miRNAs have been found to serve as oncogenes or antioncogenes in osteosarcoma[9–11]. However, little is known about the potential role of miR-21 in the pathogenesis of osteosarcoma. In the current study, we found that the expression of miR-21 was notably elevated in osteosarcoma tissues. Overexpression of miR-21 significantly increased cell survival while suppressing apoptosis in SAOS-2 cells. Moreover, caspase 8 was verified as a target gene of miR-21, and in vitro investigations suggested that it was negatively regulated by miR-21. Of note, suppression of miR-21 attenuated caspase 8-silencing-induced decrease in apoptosis, implying that miR-21 inhibited SAOS-2 cell apoptosis via targeting caspase 8.

An increasing number of studies have demonstrated that miRNAs could modulate osteosarcoma cell apoptosis. Kawano et al. demonstrated that miR-93 decreased apoptosis in osteosarcoma cells[22]. Lin et al. found that miR-184 overexpression reduced doxorubicin-induced cell apoptosis in osteosarcoma cells[11]. In the current study, our findings suggested that miR-21 was upregulated in osteosarcoma tissues, and overexpression of miR-21 notably increased cell survival while suppressing apoptosis in the osteosarcoma cell line SAOS-2. Analogously, Lv et al. indicated that miR-21 promoted survival and suppressed apoptosis in the human osteosarcoma line MG63[13]. Our findings were partly consistent with the study of Lv et al., suggesting that miR-21 acted as an oncogene of osteosarcoma by controlling tumor cell survival and apoptosis.

Previous studies have demonstrated that multiple genes could serve as target genes of miR-21, including phosphatase and tensin homolog (PTEN), Ras homolog family member B (RhoB), and TIMP metallopeptidase inhibitor 3 (TIMP3)[23–25]. However, in this study we found that caspase 8 was a target of miR-21, and it was negatively regulated by miR-21. In fact, previous studies have demonstrated that caspase 8 was a downstream gene of miR-21. miR-21 has been found to be endowed with antiapoptosis properties by activating caspase 3 and caspase 8 fragments in the condition of renal ischemia reperfusion injury[26]. Moreover, the antiapoptotic function of miR-21 was sufficient for inhibition of caspase 8 activity in a transgenic mouse heart[27]. Our findings were consistent with these previous investigations and suggested that caspase 8 was a target of miR-21.

The caspase activation mechanism is a major signaling pathway leading to apoptosis[28]. Caspases are divided into effectors (such as caspases 3, 6, and 7) and initiators (such as caspases 8 and 9)[29]. The cleaved caspase 8 subsequently initiates apoptosis via proteolytic

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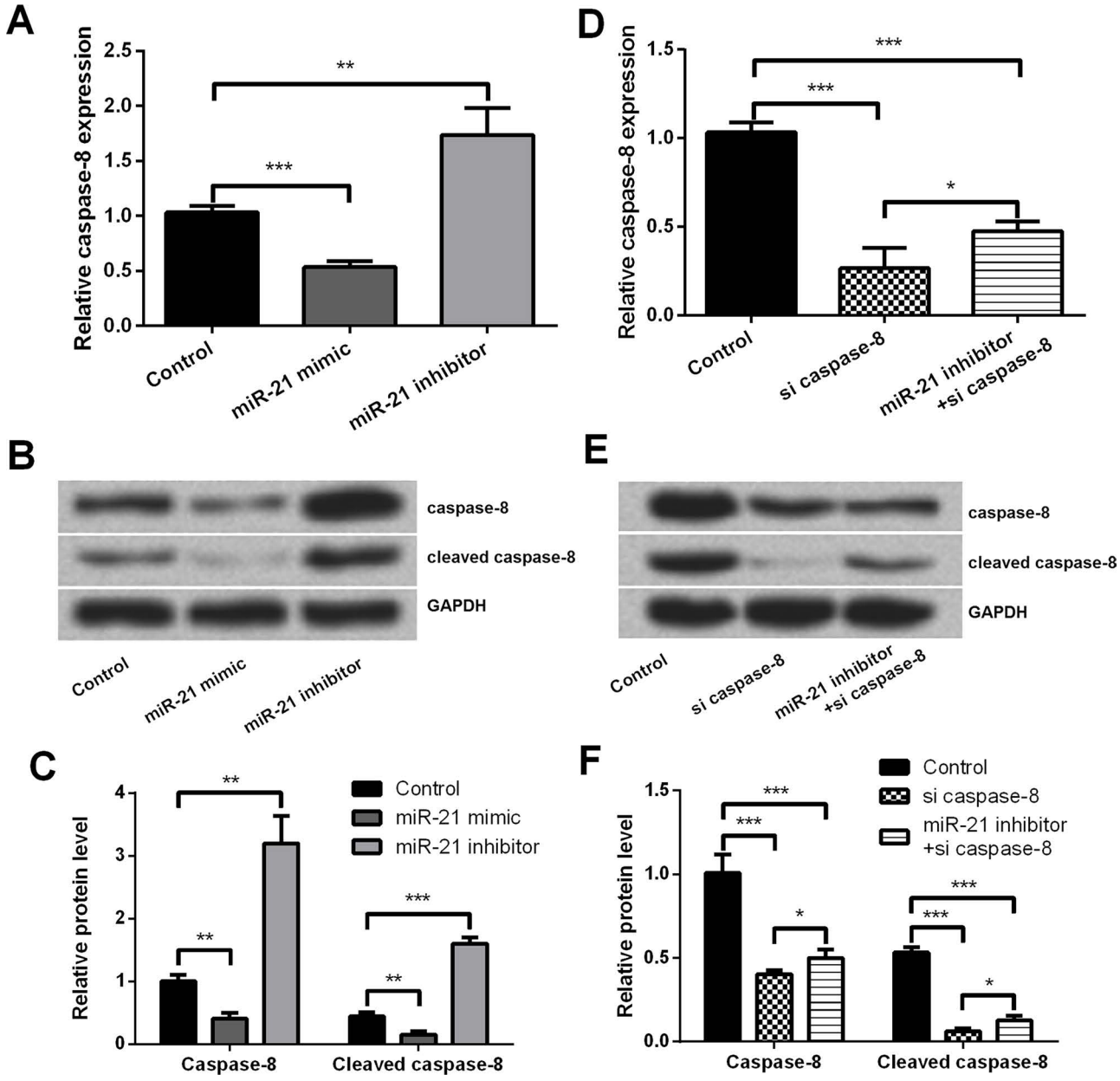

Figure 4. Capase-8 was negatively regulated by miR-21. Cells were transfected with miR-21 mimic, miR-21 inhibitor, or control. Then (A) the mRNA level expression of caspase 8 was measured by qPCR, and (B, C) the protein expressions of caspase 8 and cleaved caspase 8 were measured by Western blot. Cells were transfected with small interfering RNA (siRNA) against caspase 8 and/or the miR-21 inhibitor, and then (D) the mRNA level of caspase 8 and (E, F) the protein expressions of caspase 8 and cleaved caspase 8 were measured. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the control.

activation of downstream effector caspases[30]. Furthermore, it has been demonstrated that caspase 8 plays a vital role in osteosarcoma cell apoptosis[31–33]. In this study, caspase 8 silencing remarkably decreased the apoptotic cell rate, whereas miR-21 suppression partly recovered the decreased apoptosis. Taken together, we inferred that miR-21-inhibited apoptosis might be via targeting caspase 8.

In conclusion, miR-21 was elevated in osteosarcoma, and overexpression of miR-21 suppressed apoptosis via targeting caspase 8. These findings suggest that miR-21 might be a potential biomarker in the diagnosis of osteosarcoma. Besides, inhibition of miR-21 might be a therapeutic strategy for the treatment of this disease by controlling apoptosis. Nevertheless, further investigation is still needed to explore the deep mechanisms of miR-21 on osteosarcoma.

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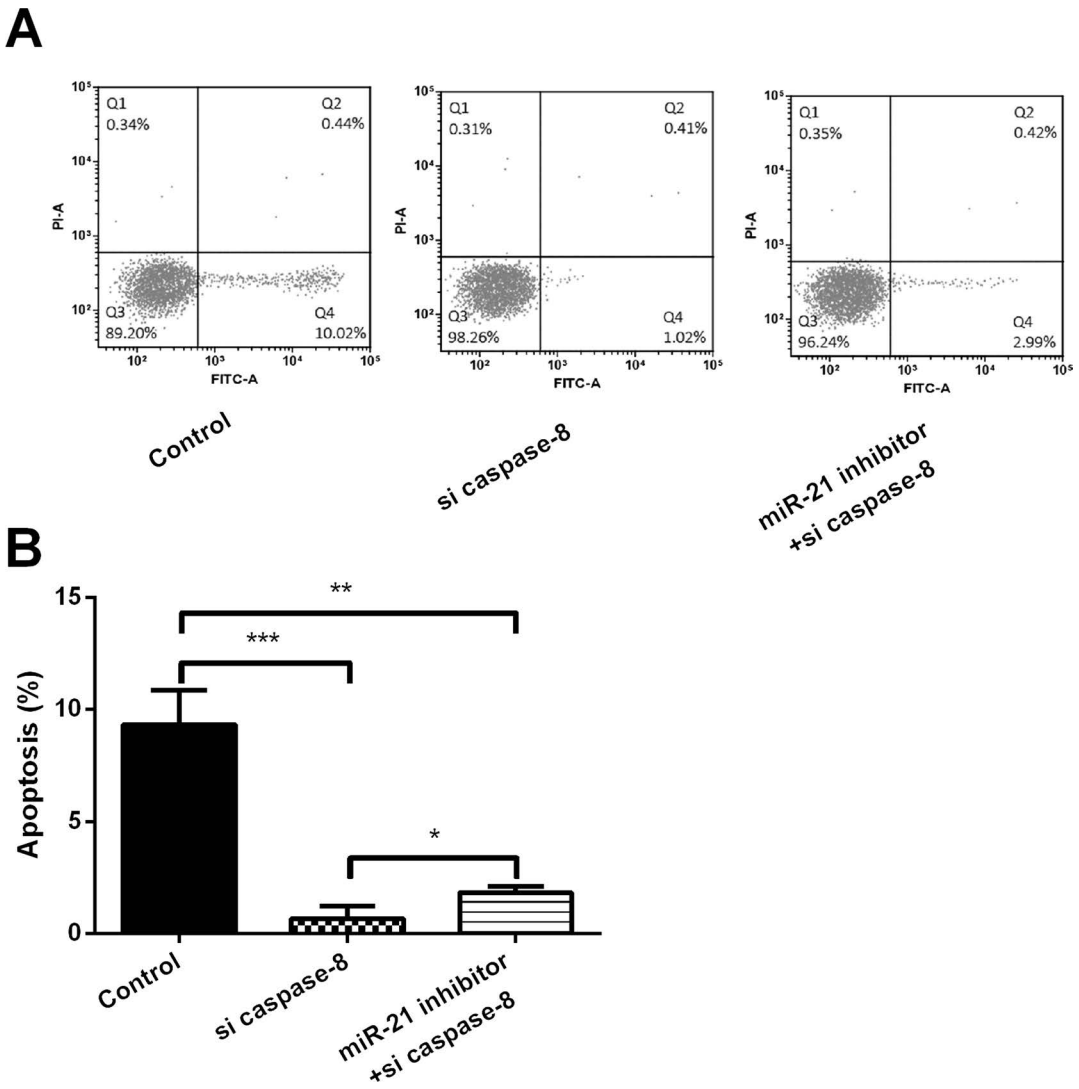

Figure 5. Overexpression of miR-21 suppressed SAOS-2 cell apoptosis via targeting caspase 8. (A, B) Cells were transfected with siRNA against caspase 8 and/or the miR-21 inhibitor, and then the apoptotic cell rate was determined by flow cytometry. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the control.

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