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MicroRNA-29b sensitizes osteosarcoma cells to doxorubicin by targeting matrix metalloproteinase 9 (MMP-9) in osteosarcoma

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Abstract. – OBJECTIVE: To discover the effect and mechanism of exogenous microRNA-29b (miR-29b) on proliferation, apoptosis and the sensitivity to chemotherapy of osteosarcoma (OS) cells.

PATIENTS AND METHODS: We assessed the expression of microRNA-29b in osteosarcoma tissues evaluating the regulation of in on the OS cell growth and drug sensitivity in human osteosarcoma MG-63 cell model. Firstly, quantitative RT-PCR (reverse transcription-PCR, RT-PCR) was used to measure the expression of miR-29b and matrix metallopeptidase 9(MMP-9) in primary osteosarcoma samples, and to evaluate the correlation between the two molecules. Secondly, miR-29b mimics or mimics were used to modify its expression in MG-63 cells. Luciferase reporter assay, Western blotting, cell viability, colony forming assay and apoptosis examination were performed to assess the regulation by manipulated miR-29b in the osteosarcoma-derived cells.

RESULTS: We found that miR-29b is down-expressed, whereas the MMP-9 level was markedly higher in primary osteosarcoma tissues and osteosarcoma-derived cells. We also found that exogenous miR-29b reduces the proliferation, promotes the apoptosis and upregulates the sensitivity to chemotherapy (doxorubicin) of osteosarcoma cells via direct targeting of the MMP-9.

CONCLUSIONS: Our data suggest that the reduced miRNA-29b may serve as a predictor of response to chemotherapy and as a therapeutic target in human osteosarcomas.

Key Words:
MicroRNA-29b, Osteosarcoma, Doxorubicin, Matrix metalloproteinase 9 (MMP-9).

Abbreviations

MMP-9: Matrix metalloproteinase 9; OS: Osteosarcoma; MMPs: Matrix metalloproteinases; RT-PCR: Real-time reverse transcriptase PCR; PBS: Phosphate-buffered saline; GLuc: Gaussia Luciferase; SEAP: Secreted Alkaline Phosphatase; CASP 3: cleaved caspase 3; PARP: Poly ADP ribose polymerase.

Introduction

Osteosarcoma is a common primary malignant tumor of bone that occurs most frequently in children and adolescents[1]. Osteosarcoma usually develops in the long bones such as the femur, the tibia or the humerus in 80% of patients[2]. Clinically metastatic evidence presents in 10-20% of diagnosed patients, and over 30% of patients will suffer relapsed osteosarcomas with metastatic tumors in the lung, the kidney and the heart. The 5-year survival rates of osteosarcoma are lower than 60%[3]. Surgical treatment combined with chemotherapy only have a moderate effect on early stage patients, but show limited effects on advanced patients[4]. Moreover, there is no established second-line chemotherapy for relapsed osteosarcomas[5]. Thus, understanding the molecular carcinogenesis of osteosarcoma will be of great help to prevent and cure the disease. miRNAs are small non-coding RNA molecules (containing about 22 nucleotides) that function in gene expression at the translational or post-transcriptional level by binding to the 3' untranslated region (3'-UTR) of the complementary mRNA sequence, suppressing mRNAs translation or degrading target mRNAs[6,7]. Many

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studies have shown that miRNAs are abnormally regulated in human cancers, suggesting a pivotal role in suppressing oncogenes/tumor genes[8]. miRNA expression profiles differ between tumors and normal tissues, and also reflect their differentiation states. MiRNAs deregulation was recently reported in human osteosarcomas. It has been observed that miRNA-29 family members are frequently down-regulated in osteosarcomas[9], and over-expression of miR-29s in osteosarcoma cells promotes cell apoptosis[10]. However, to date little is known about the functions of miR-29s in human osteosarcomas. Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases consisting of at least 20 different members, and they play an essential role in various cellular metabolic processes[11,12]. MMPs are known to be closely related to tumor growth and metastasis[13]. Among the human MMPs, it was reported that gelatinase B (92-kDa type VI collagenase, MMP-9) can degrade type IV collagen and fibronectin, and are closely correlated with the invasion and metastasis of tumor cells[14,15]. Although MMP-9 is associated with invasion and metastasis, the mechanisms of MMP-9 expression in tumor cells and the interaction between miRNA-29b and MMP-9 remain mostly unknown. In our study, we tried to assess the expression of microRNA-29b (miR-29b) in osteosarcoma tissues and evaluate the regulation of it on the cell growth and drug sensitivity in human osteosarcoma MG-63 cell model. We will discover the effect and mechanism of exogenous miR-29b on proliferation, apoptosis and the sensitivity to chemotherapy of osteosarcoma cells.

Materials and Methods

Cell Lines and Culture Conditions

Human osteosarcoma cell line MG-63 was obtained from ATCC (Manassas, VA, USA) in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 IU/mL penicillin and 50 μg/mL streptomycin (Northumbria Biologicals, Cramlington, UK) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell Transfection

MG-63 cells (1.5 x 10^5) were seeded in 12-well plates 16 hours before transfection and were transfected with 30 nM miR-29b mimics or the negative control (Scramble RNA) using the lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions.

RNA Isolation

Total RNA was extracted from tumor tissue or healthy tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, the tissue was frozen in liquid nitrogen and ground into powder. Next, the powder was dissolved in TRIzol reagent and performed consequently according to the protocol. For cancer cell lines, RNA was extracted from 1.5 x 10^5 cultured osteosarcoma cancer cells using TRIzol reagent. RNA integrity was evaluated by denaturing agarose gel electrophoresis.

Quantitative Real-time RT-PCR

The expression level of miR-29b in each sample was quantified by Real-time reverse transcriptase PCR (RT-PCR) using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) and Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression level of miR-29b in each sample was normalized by the expression level of 5S RNA. The fold-change was calculated with the ΔΔCt method. For measuring MMP-9 RNA level, primers were designed as follows: forward primer, ACGCAGACATCGTCATCCAGT, and reverse primer, GGACCACAACTCGTCATCGTC[16]. For GAPDH, primers were designed as previously described: 5′-ATGGGGAAGGTGAAGGTCG-3′ (forward) and 5′-GGGGTCATTGATGGCAACAATA-3′ (reverse)[17]. The expression level of MMP-9 RNA level was measured by SYBR® Premix Ex Taq™ GC (Perfect Real-Time) (TaKaRa, Otsu, Shiga, Japan) in a 7500 Fast PCR instrument (Applied Biosystems, Foster City, CA, USA), and was normalized by the expression level of GAPDH[18]. Cycling conditions were: 10 min of denaturation at 94°C and 40 cycles at 94°C for 15 s and 55°C for 1 min. Quantitative values were expressed as $2^{-\Delta Ct}$, wherein the ΔCt value of each sample was calculated by subtracting the average Ct value of the target gene from the average Ct value of the GAPDH gene.

Western Blotting

The tissues and cell samples were immediately homogenized in a lysis buffer containing complete protease inhibitor cocktail (Kangweishiji, China) and then centrifuged at 12,000 g, 4°C for 15 min to collect the supernatant. Protein concentrations were quantified using a BCA protein

assay kit (Kangweishiji, Beijing, China). Protein samples were resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 4-12% gels (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 10% skim milk/TBST overnight. For detecting GAPDH and MMP-9, the membrane was incubated with either anti-GAPDH mouse polyclonal antibody (1:1,000, Abcam, Cambridge, MA, USA) or anti-MMP-9 mouse monoclonal antibody (1:10,000, Abcam, Cambridge, MA, USA). For β-actin, Caspase 3 and PARP, the membrane was probed with anti-β-actin mouse monoclonal antibody, anti-Caspase 3-mouse monoclonal antibody, and anti-PARP mouse monoclonal antibody (1:1000, Kangweishiji, Beijing, China). Detection was performed with horseradish peroxidase-linked anti-mouse-IgG (1:10,000; Boaolong, Beijing, China) secondary antibodies with Pierce™ ECL Western Blotting Kit (Pierce, Rockford, IL, USA) and the membrane was imaged in a Molecular Imager CheminDocTMXRS+ Systems (Bio-Rad, Hercules, CA, USA).

Cell Apoptosis Assay

Cells apoptotic percentage was performed using the Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, MA, USA) as the tutorial described. Briefly, two days post-treatment (doxorubicin treatment plus miRNA transfection), the MG-63 cells were harvested and washed in phosphate-buffered saline (PBS). Cells were suspended in the 400 μl 1 x Binding Buffer at a concentration of 1 x 10^5 cells/ml, and were added with five μl of Annexin V-FITC and propidium iodide (PI) and were mixed thoroughly. The treated cells were placed in the dark at RT for 5-15 minutes to perform flow cytometry analysis in a FACScalibur (Becton Dickinson Flow Cytometer, Brea, CA, USA). Each experiment was independently performed in triplicate, and data were presented as mean ± SD.

Assay of Cell Proliferation and Colony Formation

Cells were trypsinized and seeded into 96-well plates at a density of 5 x 10^3 cells/well at different time points after miR-29b transfection. Next, cell proliferation was measured using the Cell Counting Kit-8 (Donjindo Molecular Technologies, Inc., Tokyo, Japan). After adding 10 μl of CCK-8 solution into each well, the plate was incubated at 37°C for 1 hour, and OD450 was measured using a microplate reader. For colony formation assay[19], MG-63 cells were plated in triplicate at 1 x 10^3/well in 6-well plates. After two weeks, the colonies were fixed and stained with 1% crystal violet/ethanol (Sigma-Aldrich, St. Louis, MO, USA), documented with an Olympus Stylus SH-50 camera (Olympus, Tokyo, Japan), and counted manually using ImageJ 1.47 software.

Dual Luciferase Reporter Assay

Cells were plated in 24-well plate at 70-80% confluency in triplicate and were co-transfected with GLUC luciferase reporter plasmid and SEAP luciferase plasmid by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after transfection, the activities of Gaussia Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP) from cell culture medium were analyzed using a Secrete-Pair™ Dual Luminescence Assay kit according to the manufacturer's instructions. The ratio of luminescence intensities of the GLuc over SEAP was calculated.

Statistical Analysis

Data were expressed as the means ± SD from at least three separate experiments. Statistical analyses were performed using Student's two-tailed *t*-test. Differences with *p*-values of less than 0.05 are considered significant.

Results

A Negative Correlation was Observed Between the miR-29b Level and the MMP-9 Level in Human Osteosarcoma Tissues

At first, we studied the correlation of miRNA-29b level with the clinicopathological characteristics of osteosarcoma patients. It was found that miRNA-29b correlates with clinical stage and distance metastasis (Table I). Notably, patients in clinical stage I and II had a higher miRNA-29b level than those in clinical stage III, and patients with distance metastasis also presented a higher miRNA-29b level in comparison with those without distance metastasis. We have detected the miR-29b and the MMP-9 mRNA level in human osteosarcoma tissues. As shown in Figure 1A, relative miR-29b has a lower level in osteosarcoma than in control. On the contrary, relative MMP-9 RNA level in osteosarcoma is much higher than that in control. Besides, we analyzed the correlation between the relative miRNA-29b and the re-

Table I. Correlation of miRNA-29b level with the clinicopathological characteristics of osteosarcoma patients.

Characteristics	Patients (N)	miRNA-29b	p-value
Gender			0.4723
Male	31	0.6384±0.1805	
Female	26	0.5399±0.1783	
Age (years)			0.295
≤ 20	37	0.6729±0.1866	
> 20	20	0.4818±0.1728	
Size (cm)			0.1728
≤ 5.0	32	0.6578±0.1669	
> 5.0	25	0.5248±0.1689	
Clinical stage			0.0126
I+II	30	0.8425±0.2094	
III	27	0.4447±0.1347	
Distant metastasis			0.0047
Yes	19	0.9297±0.2483	
No	38	0.4991±0.1583	

HWE: Hardy-Weinberg equilibrium.

lative MMP-9 mRNA level and found a negative relationship between two parameters (Figure 1C).

miR-29b Efficiently Downregulates MMP-9 Expression Level in Osteosarcoma MG-63 Cells

MG-63 cells were transfected with 25 nM or 50 nM miR-29b mimics or scramble RNA (as control) for 24 hours, then the relative miR-29b level to 5 s rRNA and the relative mRNA level of MMP-9 to β-actin were examined by Real-time RT. As presented in Figure 2A, the relative miR-29b levels were significantly improved by 100-fold or 150-fold after transfection of 25 or 50 nM miR-29b mimics, respectively. On the other hand, the relative MMP-9 levels were decreased by 40% or 50% by transfection of 25 or 50 nM miR-29b mimics. The expression of MMP-9 was measured by Western Blotting, and it was found that MMP-9 expressions were significant suppressed (Figure 2C). Similar to Figure 2B, the relative levels of MMP-9/GAPDH were down-regulated by 57% and 65%, when transfected with 25 and 50 nM miR-29b mimics, separately (Figure 2D). To investigate the regulatory role of manipulated miR-29b in the osteosarcoma-derived cells, we constructed a luciferase reporter with the wild or mutant 5' UTR of MMP-9, wild or mutant 5' UTR of homo sapiens MMP-9 were inserted in the reporter plasmid just behind a luciferase gene (Figure 3B). MiR-29b was designed to target a site in the 5' UTR of homo-sapiens MMP-9 (Figure

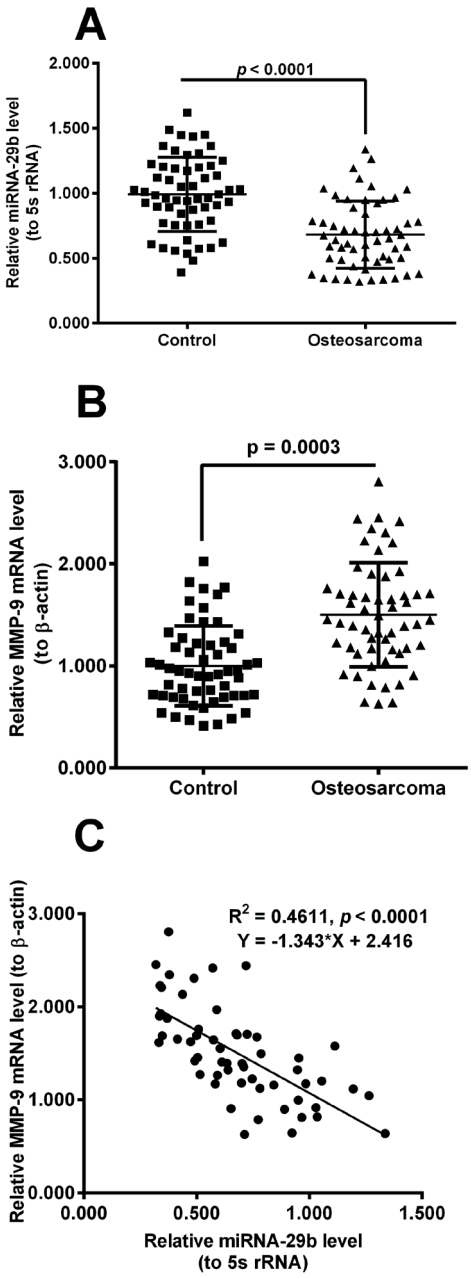

Figure 1. Reduced miR-29b and increased MMP-9 level in human osteosarcoma tissues. ***A,*** Relative miR-29b level to 5s rRNA in human osteosarcoma tissues (N = 57), with peri-tumor tissues (N = 57) as control; ***B,*** Relative mRNA level of MMP-9 to β-actin in osteosarcoma (N = 57) or in peri-tumor tissues (N = 57); ***C,*** Correlation of the increased miR-29b level with the reduced MMP-9 level in osteosarcoma tissues. Statistical significance was considered when *p*-value < 0.05 or less.

3A). After transfection, we found that miR-29b transfection greatly suppressed the luciferase activity by 40% and 60%, respectively, in the

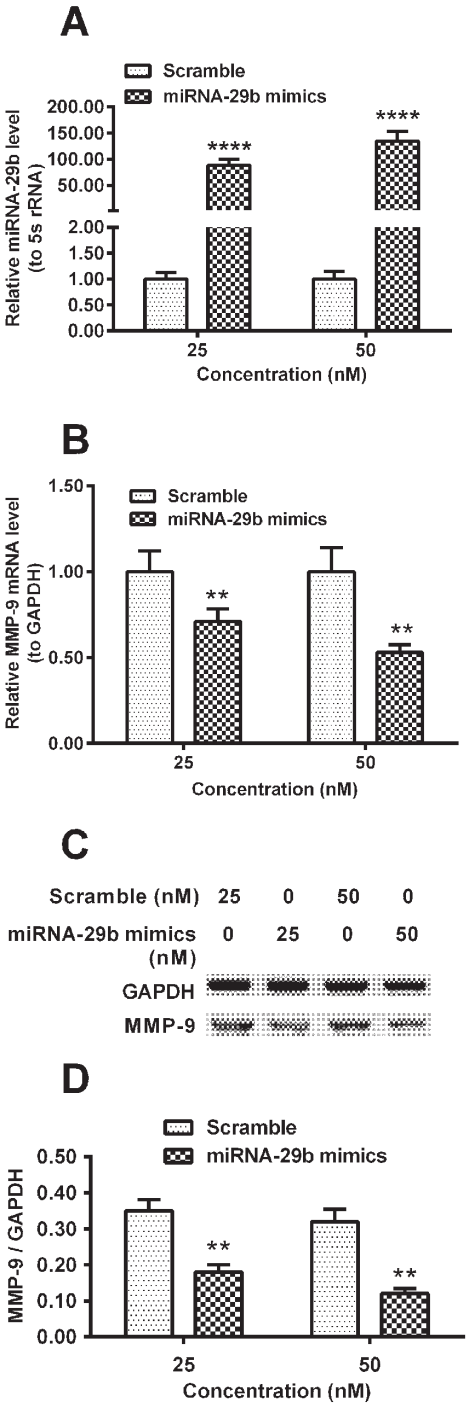

Figure 2. miR-29b downregulates MMP-9 expression in osteosarcoma MG-63 cells. MG-63 cells were transfected with 25 or 50 nM miR-29b mimics or scramble RNA (as control) for 24 hours. Then the relative miR-29b level to 5s rRNA ***(A)*** and the relative mRNA level of MMP-9 to β-actin ***(B)*** were examined; ***C*** and ***D*** Western blot analysis of ***(C)*** and the MMP-9 protein level ***(D)*** in the miR-29b mimics- or the Scramble RNA-transfected (for 24 hours) MG-63 cells. Results were averaged for triple independent experiments; Statistical significance was considered when $p<0.05$ or less, *$p<0.05$, **$p<0.01$ or ****$p<0.0001$.

presence of 25 and 50 nM miR-29b mimics (Figure 3C). However, miR-29b did not affect the expression of luciferase of mutant 5' UTR of MMP-9 (Figure 3D).

MiR-29b Inhibits the Growth of MG-63 cells in vitro

To investigate the inhibition of miR-29b on the growth of MG-63 cells *in vitro*, we transfected 50 nM scramble or miR-29b mimics into MG-63 cells and examined the cell growth by CCK-8 assay at different time points. As indicated in Figure 4A, cells transfected by miRNA-29b mimics presented the lowest cellular viability at all the time points. In the following step, MG-63 cells were seeded in 12-well plate (50 cells per well) for 12 hours, then were transfected with 50 nM scramble or miR-29b mimics. Next, the colony forming of each group of MG-63 cells was observed. We found that miRNA29b mimics efficiently reduced the colony size by nearly 50% when compared with control (Figure 4B and 4C).

MiR-29b Inhibitor Downregulates the Doxorubicin-Induced Apoptosis of MG-63 Cells

At last, we discovered the effect of miR-29b on the doxorubicin-induced apoptosis in MG-63 cells. Using flow cytometry detection (Figure 5 A, B, C, and D), we found that the doxorubicin-induced apoptotic rate was improved by 37.5% by miR-29b transfection (Figure 5 E). Sequentially, cleaved caspase 3 (CASP 3) or lyzed Poly ADP ribose polymerase (PARP) of different groups were quantified using Western blotting. Intuitively, cells treated by doxorubicin plus 50 nM miR-29b presented the highest expression level of cleaved caspase 3 (Figure 5 F). In detail, miR-29b transfection improved the ratio of cleaved caspase 3 and lyzed PARP by 40% and 27%, respectively (Figure 5 G).

Discussion

During this research, we found that miR-29b was down-expressed, whereas the MMP-9 level was markedly higher in primary osteosarcoma tissues and osteosarcoma-derived cells. We also found that exogenous miR-29b reduced the proliferation, and promoted the apoptosis and up-regulated the sensitivity to doxorubicin of osteosarcoma cells via directly targeting MMP-9. These data indicate that miRNA-29b may ser-

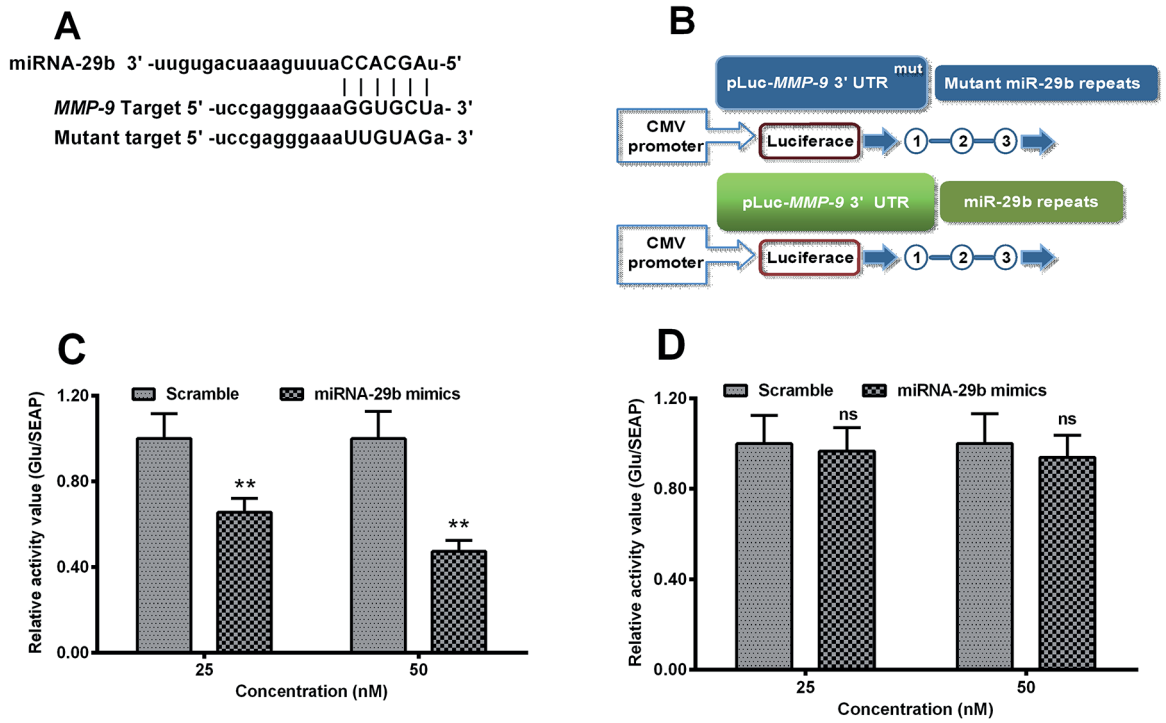

Figure 3. miR-29b regulates the luciferase reporter with the 5' UTR of *MMP-9* in MG-63 cells. ***A*** and ***B:*** Target site in the 5' UTR of homo sapiens *MMP-9* by miR-29b ***(A)*** and the sketch of the luciferase reporter with the wild or mutant 5' UTR of *MMP-9*. Wild or mutant 5' UTR of homo sapiens *MMP-9* with triple replicates were inserted in the reporter plasmid just behind the human cytomegalovirus (CMV) promoter. ***C*** and ***D:*** Relative luciferase activity in MG-63 cells of the reporter with the 5' UTR of *MMP-9* ***(C)*** or the reporter with mutant 5' UTR of *MMP-9* ***(D)***, in the presence of 25 or 50 nM miR-29b mimics or scramble RNA. Triple experiments were independently performed. ns: no significance, $^{**}p<0.01$.

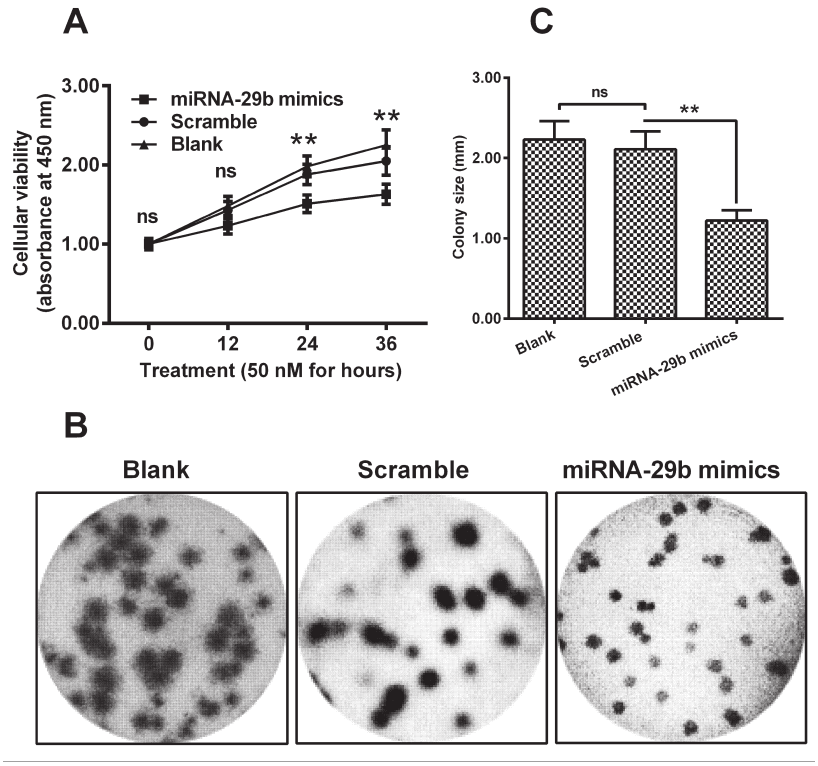

Figure 4. miR-29b inhibits the growth of MG-63 cells *in vitro*. *A*, Growth curve of blank MG-63 cells, or MG-63 cells, which after transfected with 50 nM scramble or miR-29b mimics. Cell growth was examined by CCK-8 assay. ***B*** and ***C*,** MG-63 cells were seeded in 12-well plate (50 cells per well) for 12 hours, then were transfected with 50 nM scramble or miR-29b mimics for 6 hours. Then cells were updated with growth medium (10% FBS) for another four days, and then the colony forming of each group of MG-63 cells was observed ***(B)***, and the colony size was measured ***(C)***. Experiments were performed independently in triplicate. Statistical significance was shown as * $p<0.05$, ** $p<0.01$ ns: no significance.

ve as a predictor of response to chemotherapy and as a therapeutic target in human osteosarcomas. Previous studies[20,21] have demonstrated that MMPs play critical roles in the tumor's growth, metastasis, and invasion. It was reported that elevated MMP levels are associated with poor prognosis in several types of tumors, such as breast cancer, gastric cancer and osteosarco-

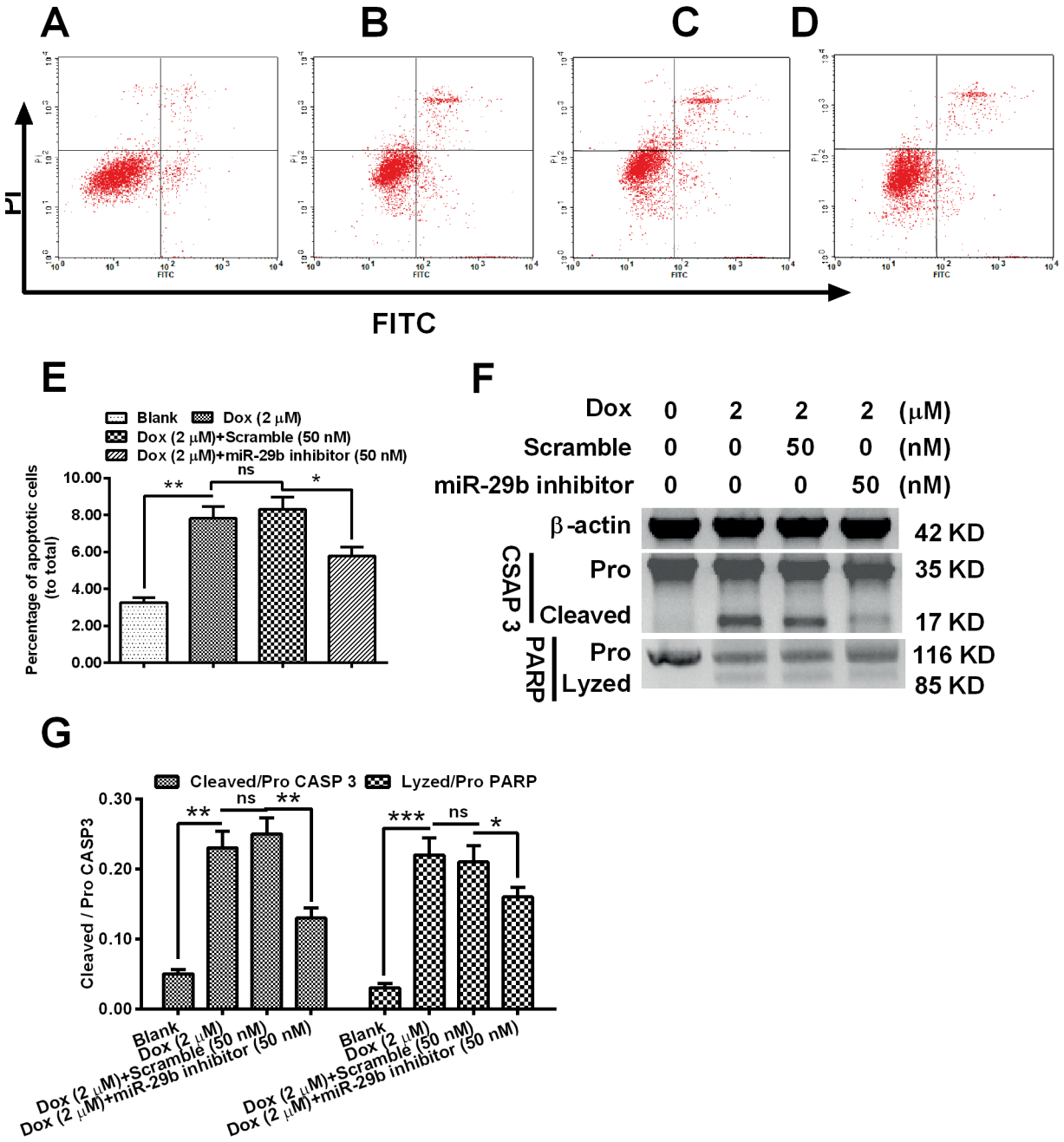

Figure 5. Influence by the miR-29b inhibitor on the doxorubicin-induced apoptosis in MG-63 cells. ***A-D***, Flow cytometry analysis for apoptosis in blank MG-63 cells (A), or in the MG-63 cells, post the treatment with 2 μM doxorubicin ***(B)***, or in the MG-63 cells, post the transfection with 50 nM scramble ***(C)*** or miR-29b inhibitor ***(D)***, in the presence of 2 μM doxorubicin ***(E)***. ***F:*** Percentage of apoptotic cells in the groups mentioned above. ***G*** and ***H:*** Western blotting ***(G)*** and the relative levels (H) of cleaved caspase 3 (CASP 3) or lyzed Poly (ADP ribose) polymerase (PARP) in each group. Data were averaged for triple independent experiments. *p<0.05, **p<0.01, ***p<0.001 or ****p<0.0001.

ma[22-24]. Up-regulation of MMP9 has been shown to improve the metastatic ability of osteosarcoma cells[25]. In our research, we found that miR-29b transfection efficiently decreased the MMP expression level, suppressed the cellular viability and reduced the colony size sequentially. Our results found that miRNA-29b could be an ideal biomarker to predict the progress of osteosarcoma. It has been reported that miR29s is frequently down-regulated in osteosarcoma tissues and that its over-expression in osteosarcoma cells can inhibit tumor cell's proliferation and promotes tumor cell's apoptosis[26]. In our research, we also found that miRNA-29b levels varied in groups of different clinical stages or metastasis status. The exciting thing is that miRNA-29b level was higher in clinical stage I or II than in clinical stage III and that its level in patients with distant metastasis presented a higher level than those without distant metastasis. Here we also show that miR-29b over-expression sensitized MG-63 cells to Dox-induced apoptosis. These results indicated that miRNA-29b might play an essential role in carcinogenesis; however, its biological functions and possible mechanisms of action in osteosarcoma should be elucidated in the next research.

Conclusions

Down-regulation of the miR-29b level and elevated MMP level are observed in osteosarcoma. Furthermore, miR-29b directly regulates MMP that implicated in the tumorigenesis of osteosarcoma. Restoration of miR-29b in MG-63 cells suppresses cells' proliferation and induces apoptosis. miR-29b-MMP pathway contributes to the pathogenesis of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LJL and HFH wrote the manuscript, DMJ and HWC revised it critically for important intellectual content. DJL, HWC, and DMJ conceived and preformed the experiments. DMJ and XQL contributed to the analysis the data. DMJ and XQL contributed to the acquisition of funding. All authors have read and approved the final version of this manuscript.

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