

## Original Article

# miR-320 inhibits multidrug resistance of osteosarcoma cells to methotrexate by targeting XIAP

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Received October 12, 2016; Accepted November 30, 2016; Epub January 1, 2017; Published January 15, 2017

**Abstract:** The development of multidrug resistance (MDR) is a hallmark of cancer therapy. Increasing evidence found that many microRNAs (miRNAs), small endogenous noncoding RNAs, are dysregulated in osteosarcoma (OS) and may be a regulator of MDR-related gene expression. To investigate the functional role of miR-320 in the MDR of OS, OS MDR cell lines were first established by pulse exposure SaOS-2 and U2OS to gradually increasing concentrations of MTX. A negative correlation between the expression of miR-320 and X-linked inhibitor of apoptosis protein (XIAP) was observed in established OS MDR cell lines by real-time polymerase chain reaction (RT-PCR). MTT assay and flow cytometry analysis elucidated that overexpression miR-320 or knockdown XIAP sensitized SaOS-2/R and U2OS/R cells to anti-cancer drugs and promoted cell apoptosis. The 3' untranslated region (UTR) of XIAP was predicted to be a direct target of miR-320. Luciferase assay and western blot demonstrated that miR-320 could directly interact with the 3'UTR of XIAP and regulate the expression of apoptosis-related proteins XIAP, caspase-3, bax and bcl-2. Taken together, our data identified miR-320 as an important regulator of OS MDR by directly targeting XIAP and might provide a novel therapeutic strategy for OS treatment.

**Keywords:** miR-320, osteosarcoma, multidrug resistance, XIAP, apoptosis

## Introduction

In osteosarcoma (OS), chemotherapy is the major treatment, with multiple anticancer drugs, including cisplatin, doxorubicin and methotrexate [1, 2]. Since the introduction of combinatorial chemotherapy to OS, the 5-year survival rate has significantly improved over the past few decades to approximately 60-70% [3]. Unfortunately, survival outcomes for OS patients remain unsatisfactory. Due to the development of multidrug resistance (MDR) either intrinsic or acquired, the clinical therapy failure and unfavorable prognosis always occur in recent years [4]. Previous studies have indicated that MDR is a complex process involving drug transport and metabolism, DNA synthesis and repair, cell survival and apoptosis, genetic and epigenetic changes [5-7].

MicroRNAs, a class of 18-24 nucleotides small endogenous noncoding RNAs, play important roles in a wide array of biological processes through inhibiting gene expression by targeting mRNAs for translational repression and/or

cleavage [8, 9]. Recently, increasing studies have suggested that microRNAs are associated with the chemoresistance and act as potential oncogenes or tumor suppressors in a variety of cancer cell types, including OS. For example, high expression of miR-33a promotes OS cell resistance to cisplatin by down-regulating TWIST [10]. Besides, Zhang et al. found that miR-301a promoted HMGCR expression and enhanced resistance of OS cells to doxorubicin by targeting AMP-activated protein kinase alpha 1 [11]. Moreover, overexpression of miR-30a reduced chemoresistance of OS cells through downregulating autophagy by targeting Beclin-1 [12]. Taken together, these studies indicate that miRNA might be a promising candidate target to develop novel therapeutic strategy to overcome drug resistance.

In the present study, we reported that miR-320 was downregulated in both SaOS-2 and U2OS MTX resistant cells compared with normal OS cells. Overexpression of miR-320 could suppress chemoresistance of the OS cells and promote cell apoptosis by targeting XIAP.

## Materials and methods

### Cell lines and cell culture

OS cell lines SaOS-2 and U2OS were purchased from the American Type Culture Collection (ATCC; Manassas, Virginia, USA). Cells were cultured in the Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 U penicillin/ml and 100 µg streptomycin/ml at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Establishment of OS drug resistant cell lines

OS cell lines SaOS-2 and U2OS were pulse exposed to gradually increasing concentrations of MTX until the cells were accommodated in the continuous presence of 2 µg/ml MTX. To maintain the MDR phenotype, 1 µg/ml MTX was added to the culture media. The new OS drug-resistant cell lines generated in this way were named SaOS-2/R and U2OS/R, respectively.

### Real-time polymerase chain reaction (RT-PCR)

The total RNA was isolated using TRIzol reagent (Invitrogen, USA). First-strand cDNA was generated using the reverse-transcriptional kit (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. The qRT-PCR assays were performed in the StepOne™ Plus Real Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR PrimeScript RT-PCR Kit (Takara Bio, Japan). The miR-320 relative expression level of each group was calculated using the 2-ΔΔCt method and normalized using RNU6B as endogenous reference genes. PCR was performed in triplicate.

### Transfection with miR-320 mimics or siRNA specific to XIAP

Cells were plated in six-well plates at a density of 2 × 10<sup>5</sup> cells/well and transfected with Lipofectamine™ 2000 Reagent (Invitrogen, CA, USA) following the manufacturer's protocol. MiR-320 mimics, miRNA negative control, XIAP siRNA as well as siRNA negative control, were designed and synthesized by Shanghai GenePharma Company (Shanghai, China).

### In vitro drug sensitivity assay

After 24 h transfection, 1 × 10<sup>4</sup> SaOS-2/R and U2OS/R cells were seeded into 96-well plates.

According to the peak plasma concentration (PPC) (MTX 1.5, ADM 0.5, DDP 2.5, IFO 5.0, and EPI 0.8 µg/ml), 7 concentration gradient experimental wells and a control well were established of 1000, 100, 10, 1, 0.1, 0.01, 0.001, and 0 PPC. After 24 h incubation, cell viability was assessed by MTT assay. Absorbance value at 490 nm was then measured. The concentration at which each drug produced 50% inhibition of growth (IC<sub>50</sub>) was estimated by the relative survival curve.

### Cell apoptosis analysis

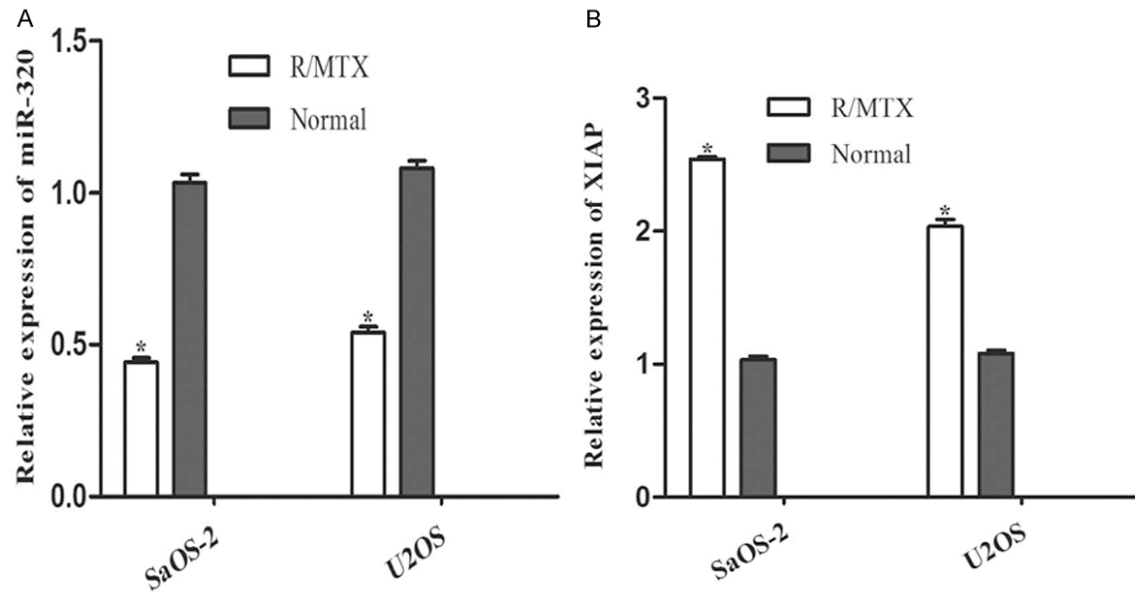
SaOS-2/R and U2OS/R were plated in 6-well plates (6 × 10<sup>5</sup> cells/well). After 24 h transfection, SaOS-2/R and U2OS/R cells were treated with MTX, with final concentration of 320 µg/ml and 100 µg/ml, respectively. Cell apoptosis was analyzed 48 h after the treatment of MTX using staining with Annexin V and PI (BD Bioscience, San Jose, CA, USA) according to the manufacturer's specifications.

### Luciferase reporter assay

A fragment of the 3'-UTR of XIAP containing the putative target sites for the miR-320 was designed and synthesized and then cloned into the pMIR-REPORT luciferase reporter vector (Ambion, San Diego, CA) at the HindIII and SpeI sites. All PCR products were verified by DNA sequencing. For luciferase reporter assays, the constructed luciferase reporter plasmid, β-galactosidase (β-gal) and miR-320 mimics or the negative control were co-transfected into SaOS-2/R and U2OS/R cells using Lipofectamine 2000. After 48 h transfection, luciferase activity was measured according to the manufacturer's protocols. The luciferase activity was normalized to β-gal.

### Western blot analysis

After 72 h transfection, cells were harvested and homogenized with RIPA lysis buffer. Total protein was separated by denaturing 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline Tween (TBST) buffer for 2 h at room temperature and incubated with primary antibodies



**Figure 1.** Expression of miR-320 and XIAP in SaOS-2/R and U2OS/R cells. A: Relative expression of miR-320 in SaOS-2/R and U2OS/R cells, compared with SaOS-2 and U2OS. B: Relative expression of XIAP in SaOS-2/R and U2OS/R cells, compared with SaOS-2 and U2OS. Data are expressed as means  $\pm$  SD of three independent experiments. \* $P < 0.05$  compared with SaOS-2 and U2OS.

against XIAP (Abcam, Cambridge, MA, 1:1000), caspase-3 (Abcam, Cambridge, MA, 1:1000), bax (Abcam, Cambridge, MA, 1:1000), bcl-2 (Abcam, Cambridge, MA, 1:1000) or  $\beta$ -actin (Abcam, Cambridge, MA, 1:5000) overnight at 4°C followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The protein expression levels were normalized using  $\beta$ -actin and then determined by densitometry scans and calculated using Quantity One software (Bio-Rad Co., USA).

#### Statistical analysis

All experimental values were performed independently at least three times. Data are expressed as means  $\pm$  SD. Statistical analysis was performed by using the Graph-Pad Prism Software. The difference between means was analyzed with Student's *t* test. *P* value  $< 0.05$  was considered significant.

#### Results

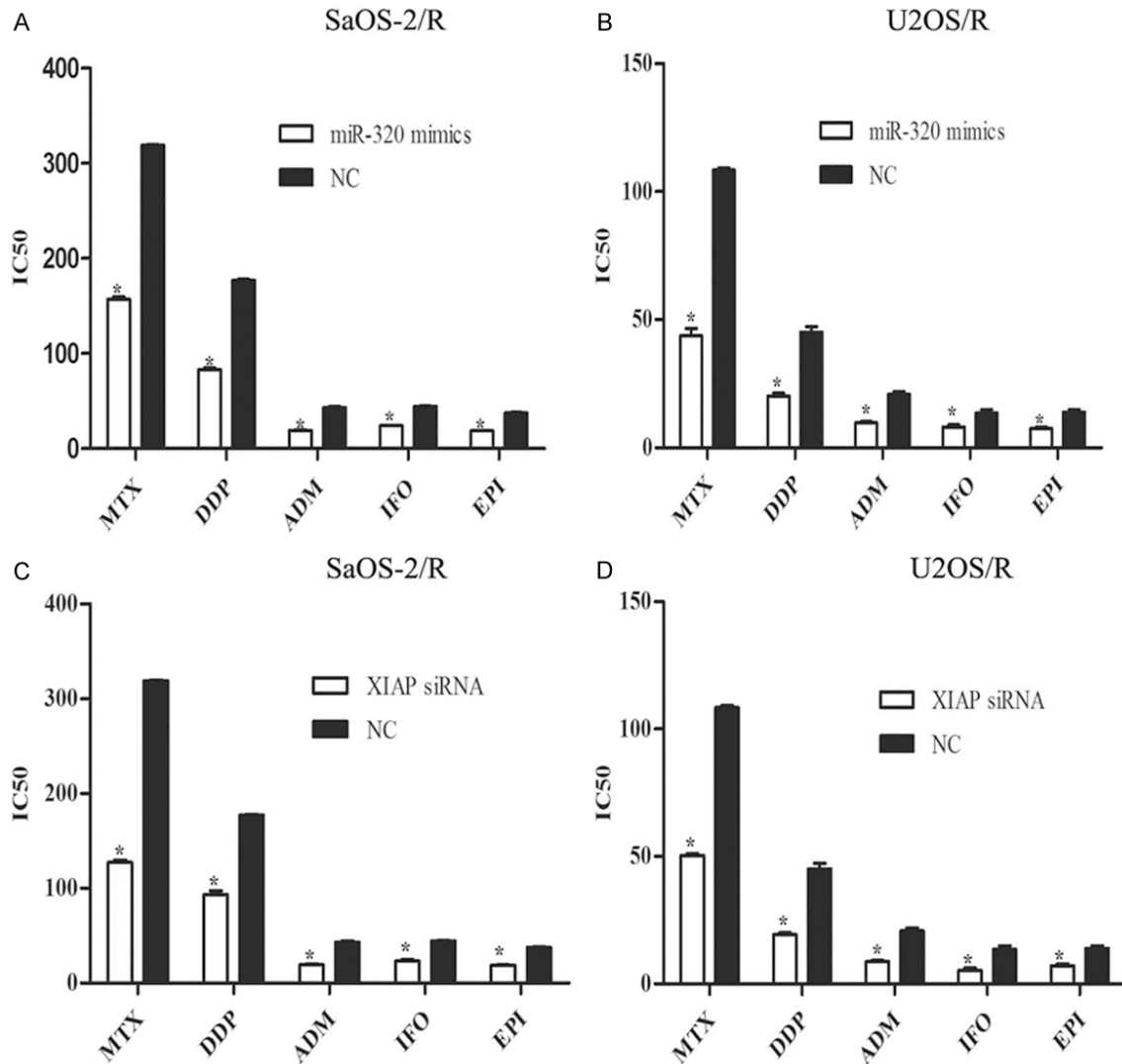
##### *The expression of miR-320 and XIAP in SaOS-2/R and U2OS/R cells*

To examine the possible role of miR-320 in the development of MDR in human OS cell lines, we

first established OS MDR cell lines SaOS-2/R and U2OS/R by pulse exposure of gradually increasing concentrations of MTX. Next we performed qRT-PCR analysis in OS MDR cell lines and parental normal OS cell lines. As shown in **Figure 1A**, the expression levels of miR-320 was significantly downregulated in SaOS-2/R and U2OS/R cells compared with those in SaOS-2 and U2OS, respectively. Contrary to miR-320, XIAP expression level was significantly upregulated in OS MDR cells compared with those of controls (**Figure 1B**).

##### *Both overexpression miR-320 and knockdown XIAP sensitize SaOS-2/R and U2OS/R cells to anti-cancer drugs, respectively*

To assess the effect of miR-320 and XIAP on the drug resistance, MTT assay was performed in SaOS-2/R and U2OS/R cells. As shown in **Figure 2A** and **2B**, miR-320 mimics could greatly enhance drug susceptibility to MTX, ADM, DDP, IFO and EPI in both SaOS-2/R and U2OS/R cells compared with the negative control, respectively. Meanwhile, SaOS-2/R and U2OS/R cells transfected with XIAP siRNA were more sensitive to those anti-cancer drugs, compared with the negative control, respectively (**Figure 2C** and **2D**).



**Figure 2.** Both overexpression miR-320 and knockdown XIAP sensitize SaOS-2/R and U2OS/R cells to anti-cancer drugs, respectively. A: In SaOS-2/R cells those transfected with miR-320 mimics exhibited greatly enhanced sensitivity to MTX, DDP, ADM, IFO and EPI, compared with the miRNA mimic control transfected cells. B: In U2OS/R cells, those transfected with miR-320 mimics exhibited greatly enhanced sensitivity to MTX, DDP, ADM, IFO and EPI, compared with the miRNA mimics control transfected cells. C: Knockdown XIAP sensitized SaOS-2/R cells to MTX, DDP, ADM, IFO and EPI, compared with the negative control. D: Knockdown XIAP sensitized U2OS/R cells to MTX, DDP, ADM, IFO and EPI, compared with the negative control. Data are expressed as means  $\pm$  SD of three independent experiments. \*P < 0.05 compared with the negative control group.

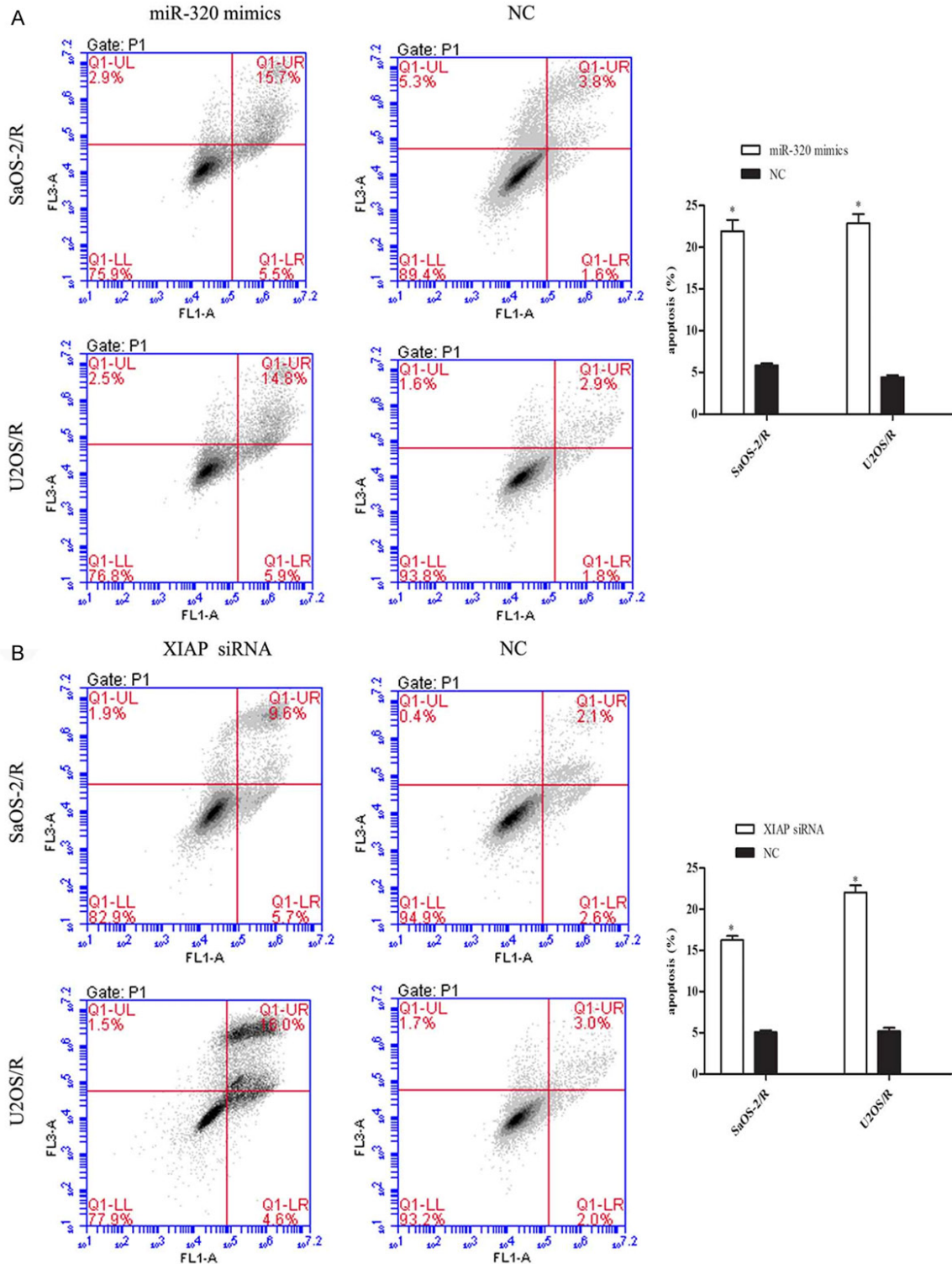
#### Overexpression miR-320 and knockdown XIAP sensitize SaOS-2/R and U2OS/R cells to MTX-induced apoptosis, respectively

To further determine the role of miR-320 and XIAP in cell apoptosis, we analyzed MTX-induced apoptosis after transfection with the miR-320 mimics or XIAP siRNA in SaOS-2/R and U2OS/R cells by flow cytometry. As shown in **Figure 3A**, in both SaOS-2/R and U2OS/R cells, the proportion of apoptotic cells transfected with miR-320 mimics was significantly

increased in comparison with the negative control. Moreover, knockdown of XIAP could promote SaOS-2/R and U2OS/R cell apoptosis compared with the negative control (**Figure 3B**).

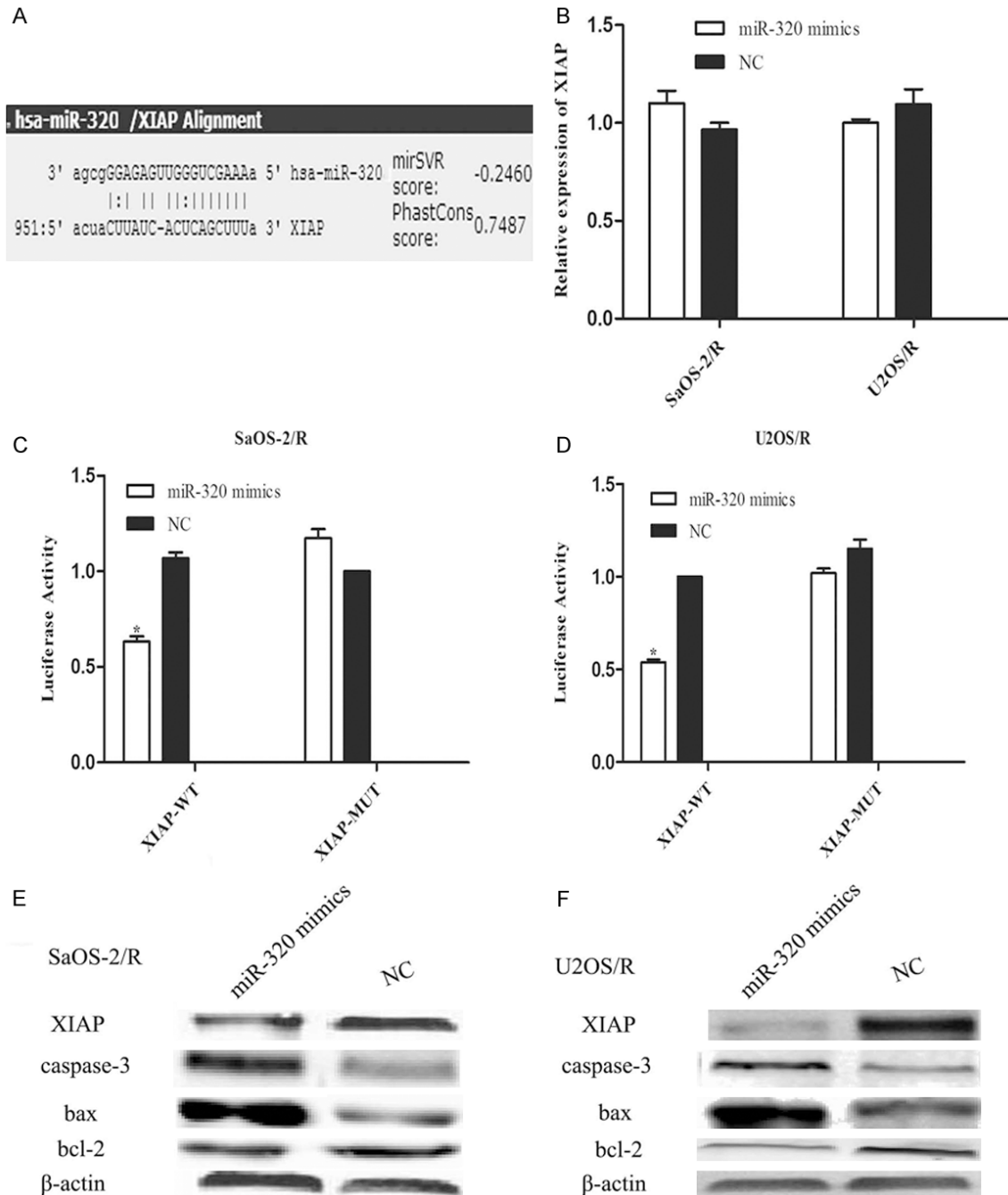
#### miR-320 directly targets the 3'UTR of XIAP and regulates cell apoptosis-related proteins expression in OS MDR cells

To investigate whether XIAP is a direct target of miR-320 in OS MDR cells, the 3'UTR of XIAP was predicted to harbor a potential miR-320



**Figure 3.** Overexpression miR-320 and knockdown XIAP sensitize SaOS-2/R and U2OS/R cells to MTX-induced apoptosis, respectively. A. In both SaOS-2/R and U2OS/R cells, flow cytometry assay showed a marked increase of apoptosis in miR-320 mimics-transfected cells after MTX treatment, compared with the miRNA mimic control transfected cells. B. Knockdown XIAP sensitized SaOS-2/R and U2OS/R cells to MTX-induced apoptosis. The results shown represent the mean  $\pm$  SD from 3 independent experiments. \*P < 0.05 compared with the negative control group.





**Figure 4.** miR-320 directly targets the 3'UTR of XIAP and regulates cell apoptosis-related proteins expression in OS MDR cells. A. The miR-320 targets on XIAP were predicted using microRNA.org. B. The gene expression of XIAP was detected by qRT-PCR in SaOS-2/R and U2OS/R cells. C. Luciferase reporter assays in SaOS-2/R cells. D. Luciferase reporter assays in U2OS/R cells. E. The protein expression of XIAP was determined by Western blot in SaOS-2/R cells. F. The protein expression of XIAP was determined by Western blot in U2OS/R cells. Data are expressed as means  $\pm$  SD of three independent experiments. \*P < 0.05 compared with the negative control group.

binding site by microRNA.org (Figure 4A). Then we constructed a luciferase reporter of the 3'-UTR of XIAP containing the putative target sites for the miR-320. Luciferase reporter vec-

tors together with the miR-320 mimics or the negative control were transfected into SaOS-2/R and U2OS/R cells, respectively. In both SaOS-2/R and U2OS/R cells, a significant de-

crease in relative luciferase activity was observed when pMIR-REPORT-XIAP-3'-UTR was co-transfected with the miR-320 mimics compared with the negative control, respectively (**Figure 4C** and **4D**). However, there was no correlation between miR-320 and XIAP expression in mRNA level (**Figure 4B**). As shown in **Figure 4E** and **4F**, a reduction of XIAP and bcl-2 protein expression was observed in OS MDR cells transfected with miR-320 mimics, while the expression of caspase-3 and bax was increased.

## Discussion

Several studies have demonstrated that miR-320 was involved in various types of disease. It has been reported that miR-320 inhibits cell growth and proliferation in human leukemia cells by targeting Tfr-1 [13], suppresses tumor angiogenesis in oral squamous cell carcinoma by targeting NRP1 [14], regulates the resistance of pancreatic cancer cells to gemcitabine through SMARCC1 [15], modulates endothelial cell migration and tube formation in recipient endothelial cells by targeting Hsp20 [16]. Besides, miR-320 was also shown to inhibit OS cells proliferation by directly targeting fatty acid synthase [17]. However, the relationship between miR-320 and MDR in OS cells has not been extensively studied.

In the present study, we found that there is an inverse relationship between the expression of miR-320 and XIAP in the established OS MDR cell lines SaOS-2/R and U2OS/R. Besides, either overexpression miR-320 or knockdown XIAP could sensitize SaOS-2/R and U2OS/R cells to anti-cancer drugs, respectively. When transfected with the miR-320 mimics or XIAP siRNA in SaOS-2/R and U2OS/R cells, the proportion of apoptotic cells was significantly increased compared with that in the negative control group. To investigate whether XIAP is a direct target of miR-320, we analysed their association through bioinformatics software (MicroRNA.org). The results of luciferase assay and western blot further confirmed that miR-320 could directly interact with XIAP and affect the expression of cell apoptosis-related proteins. Taken together, we reasonably speculate that miR-320 is an important regulator of MDR and cell apoptosis in OS.

The anti-apoptotic ability partially caused by MDR is a hallmark of cancer therapy, which

enables cells to overcome many of the cytotoxic effects of chemotherapeutic agents [18]. XIAP, the most potent caspase inhibitor, belongs to the cellular member of the inhibitor of apoptosis protein family. Previous reports have demonstrated that XIAP was significantly upregulated in several types of human cancers, including breast cancer [19], cervical cancer [20], ovarian cancer [21], and rectal cancer [22]. In addition, McManus et al. found that loss of XIAP protein expression by either RNAi or antisense approaches increases cancer cell susceptibility to functionally diverse chemotherapeutic agents [23]. Moreover, XIAP and its negative regulatory factors such as XIAP-associated factor 1 (XAF1), Smac/DIABLO and HtrA2/Omi, may be a promising therapeutic target for the treatment of malignant tumor [24-27].

In conclusion, for the first time we have shown a close link between miR-320 and XIAP in OS MDR cells, in which overexpression of miR-320 suppressed the MDR and promoted cell apoptosis of OS MDR cell lines by targeting XIAP. Targeting the MDR-related miRNAs, such as miR-320, may be another promising method to enhance the effect of clinical treatment for OS patients.

## Acknowledgements

We thank all the staff in the department of orthopedics of Tianjin Third Central Hospital for their help on the manuscript.

## Disclosure of conflict of interest

None.

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