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Down-regulation of miR-377 contributes to cisplatin resistance by targeting XIAP in osteosarcoma

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Abstract. – OBJECTIVE: Chemoresistance is one of the obstacles for effective treatment of cancers, and recent evidence has shown that microRNAs play critical roles in drug resistance. In this study, we investigated the effect of miR-377 on cisplatin resistance in osteosarcoma (OS).

PATIENTS AND METHODS: Tumor tissues from chemoresistant and control OS patients were subjected to Real-time polymerase chain reaction (PCR) to assess miR-377 expression. The effect and mechanism of miR-377 on cisplatin resistance were assessed using Cell Counting Kit (CCK) 8, flow cytometry, Western blot, and luciferase assays in cisplatin-resistant OS cells lines.

RESULTS: Down-regulation of miR-377 was found in chemoresistant OS tissues and cisplatin resistant OS cell lines. Overexpression of miR-377 re-sensitizes cisplatin-resistant OS cells to cisplatin-induced caspase-3 dependent apoptosis. MiR-377 directly represses X-linked inhibitor of apoptosis protein (XIAP) expression through binding to its 3' untranslated region (UTR) of mRNA. Overexpression of XIAP partially cancelled the cisplatin-sensitizing effect of miR-377.

CONCLUSIONS: These data uncovered an essential function of miR-377/XIAP signaling axis in regulating cisplatin resistance of OS. MiR-377 may have potential therapeutic values in tackling OS chemoresistance.

Key Words:
Osteosarcoma, miR-377, Chemoresistance, Cisplatin, Apoptosis, XIAP.

Introduction

Osteosarcoma (OS) is an aggressive and highly lethal malignancy that commonly affects child and adolescents[1]. Chemotherapy is an effective and conventional adjuvant therapy after surgical treatment; cisplatin is one of the standard drugs that has been widely applied clinically[2]. However, a substantial number of patients respond poorly to cisplatin. This often leads to failures in treatment or recurrence. There is no single molecular mechanism that can fully explain the development of cisplatin resistance. Many factors have been proposed to play pivotal roles, and these factors compose a complex regulatory network[2-4]. There is accumulating evidence that the small non-coding RNAs, namely microRNAs, have indispensable functions in the development of drug resistance[5-9]. MicroRNAs are single-stranded and approximately 18-25 nt in length. They are profoundly involved in the essential cellular processes such as proliferation, differentiation, metabolism, and cell death[10]. Dysregulation of microRNAs can lead to multiple disorders. The context-dependent roles of miR-377 have been studied in various cancers, in most of the studies, miR-377 functions as a tumor suppressor. By contrast, miR-377 promoted tumorigenic activity in colorectal cancer[11]. The exact functions of miR-377 in OS have not been fully explored. A recent study by Wang et al[12] showed that overexpression of miR-377 in OS cells inhibited proliferation and cell invasion by targeting CDK6. It is still elusive whether it regulates another cellular process by various other mechanisms.

In the current study, we focus our attention on the potential role of miR-377 in cisplatin resistance of OS cells. We provide evidence that miR-377 is down-regulated in OS tissues of chemoresistant patients and cisplatin resistant OS cells. Overexpression of miR-377 restored the cisplatin responsiveness in cisplatin resistant OS cells. Moreover, we demonstrate that these effects by miR-377 is due to its direct inhibition of the

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apoptosis inhibitor XIAP. Our study provides a novel insight into the mechanisms of cisplatin resistance of OS cells, which is of vital importance for developing novel strategies to tackle the problem of chemoresistance.

Patients and Methods

Patient Samples

The OS tissue samples from patients were collected between November 2013 and September 2016, 42 patients were enrolled in our study. 21 patients were poor responders and the other 21 patients were good responders. Poor responders were defined by a tumor necrosis rate < 90% after neo-chemotherapy. The good responders took the same methotrexate/doxorubicin/cisplatin-based chemotherapy regimen with that of poor responders. The age, sex, and tumor stages were matched between two groups. Poor responders were taken as chemoresistant, and good responders were taken as control. This study was approved by the Institutional Ethics Committee, and informed consents were obtained from patients.

Cell Culture

The cisplatin resistant OS cell lines MG-63/CDDP, Saos-2/CDDP and their parental cell lines MG-63 and Saos-2 were purchased from Shanghai Cell Bank of Chinese Academy of Sciences. All cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Beyotime, Shanghai, China). Cells were placed in a humidified incubator at 37°C with 5% CO_2.

Transfection

Cells grown in 70% confluence were subjected to transfection. We transfected miR-377 at the final concentration of 150 nmol/L, and the overexpression plasmid pcDNA3.1-XIAP (Addgene plasmid 11833) and luciferase plasmid pmiRGLO-XIAP were transfected at the final concentration of 1 μg/ml. Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used to deliver microRNAs or plasmids into cells. The procedure followed the protocol provided by the manufacturer. Briefly, cells grown in 6-well plates at the confluence of 70% were changed to medium with no serum. For each well, microRNAs (100 pmol) or plasmids (2 μg) were incubated with 10 μl Lipofectamine 2000 reagent for 30 min at room temperature. The transfection mix was then added to the medium. The fresh complete medium was replaced to the well 24 h after transfection.

Real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and RNA was precipitated by ethanol. 1 μg RNA was used for reverse transcription to synthesize the cDNA first strands for each reaction, which was done by the PrimeScript™ RT reagent kit (TaKaRa, Tokyo, Japan). For the final detection of gene expression, the SYBR Premix Ex Taq™ II (TaKaRa, Otsu, Shiga, Japan) kit was used according to the instructions provided by the manufacturer. For miR-377 reverse transcription, a commercially available Bulge-loop primer was obtained from RiboBio (Guangzhou, China). Primers for the amplification step were also purchased from RiboBio (Shanghai, China). U6 small nuclear RNA was used as internal control.

Cell Viability Assay

Cell viability was determined by CCK-8 kit (Beyotime, Shanghai, China) according to the instructions. Cells were treated with geometrically increased doses of cisplatin for 72 h. The viabilities at each dose were plotted. Cells were grown in 96-well plates at 1×10^5/ml. 10 μl CCK-8 reagent was added to each well 2 h before the assay. Absorbance at 450 nm was detected using a spectrophotometer.

Flow Cytometry

The apoptosis was determined by an AnnexinV-FITC/PI apoptosis detection kit (BD Pharmingen, Franklin Lakes, NJ, USA). Briefly, 72 h after transfection and cisplatin treatment, cells were washed with cold phosphate-buffered saline (PBS) and re-suspended in the binding buffer provided in the kit. FITC Annexin V and PI were diluted as demand and added into cells followed by a 15 min incubation at room temperature in the dark. Then, appropriate (400 μl) of binding buffer was added to each sample. The samples were analyzed by a FACS Calibur flow cytometry system (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot

Protein expression was analyzed by Western blot analysis, and the experimental procedures

followed a previously published paper with little modifications[13]. Briefly, protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride (PVDF) membrane followed by blocking with 5% non-fat milk. Membranes were incubated with primary antibodies overnight, and re-probed with HRP secondary antibodies (CWbio, Beijing, China). The bands were visualized by a chemiluminescence detection (Amersham Biosciences, Little Chalfont, UK). Primary antibodies for PARP, Cleaved-caspase-3, and XIAP were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). Actin antibody was purchased from CWbio (Beijing, China).

Luciferase Assay

Luciferase plasmids were constructed, the 3'UTR of XIAP were cloned downstream of the firefly luciferase gene of pmiRGLO plasmid (Promega, Madison, WI, USA). For mutant 3'UTR, a mutagenesis was introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Luciferase assay was performed as follows: Saos-2/CDDP cells were grown in 24-well plate and transfected with miR-377 or miR-NC together with pmiRGLO-XIAP-WT (wild-type) or pmiRGLO-XIAP-Mut (mutant) for 48h; cells were lysed with passive lysis buffer and luciferase activity was measured using the Dual-luciferase detection system (Promega, Madison, WI, USA).

Statistical Analysis

Data were shown as means ± standard deviation (SD), and Student's *t*-test was used to evaluate the difference between groups. $p < 0.05$ was used as the criteria for statistical significance.

Results

MiR-377 Expression in Chemoresistant OS Tissues and Cisplatin Resistant OS Cells

To investigate the role of miR-377 in cisplatin resistance, we first analyzed the expression of miR-377 in chemoresistant OS tissues and OS cell lines. As shown in Figure 1A, miR-377 expression was significantly down-regulated in chemoresistant OS tissues compared with that in control OS tissues. We also assessed cellular expression of miR-377 in cisplatin resistant cell

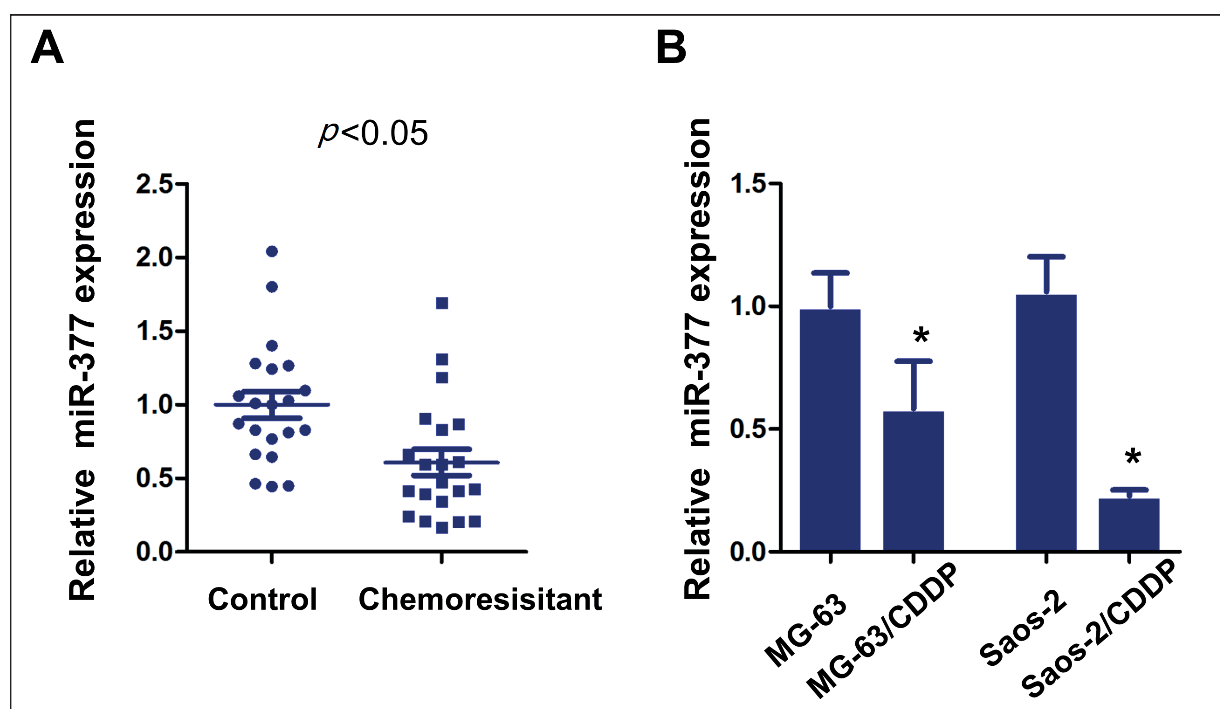

Figure 1. MiR-377 expression in chemoresistant OS tissues and cisplatin resistant OS cells. ***A,*** The expression of miR-377 was downregulated in chemoresistant OS tissues compared with control OS tissue. ***B,*** The expression of miR-377 was downregulated in cisplatin resistant OS cell lines MG-63/CDDP and Saos-2/CDDP compared with their parental cells. *$p < 0.05$. n = 3.

lines, MG-63/CDDP and Saos-2/CDDP, and compared it with that in parental cell lines. MiR-377 expression was also proved to be down-regulated in these cells (Figure 1B).

MiR-377 Alleviates Cisplatin Resistance

The above data suggest that miR-377 may have an important function in cisplatin resistance of OS. To test this idea, we overexpressed miR-377 in cisplatin resistant cells and analyzed its effect on cell viability when treated with various doses of cisplatin. As shown in Figure 2A, miR-377 significantly increased the responsiveness to cisplatin in MG-63/CDDP and Saos-2/CDDP cells. We further treated miR-NC and miR-377 transfected Saos-2/CDDP cells with cisplatin (8 μg/ml) and analyzed apoptosis by flow cytometry assay; as shown in Figure 2B, miR-377 significantly sensitized Saos-2/CDDP cells to apoptotic cell death. Western blot analysis of PARP and cleaved caspase-3 indeed confirmed the occurrence of caspase-3 dependent apoptosis in miR-377 transfected cells (Figure 2C). These data strongly suggest that miR-377 plays a cisplatin-sensitizing role in OS cells.

XIAP is a Target of miR-377 in OS

To understand the mechanism underlying the cisplatin-sensitizing effect of miR-377, we explored potential targets of miR-377 using the online software (www.microrna.org). We found that XIAP was predicted to be targeted by miR-377 with a relatively high probability (Figure 3A). Therefore, we constructed a wild-type or the seed sequence binding site mutated luciferase reporter to test whether miR-377 can bind to the mRNA 3'UTR of XIAP and inhibits its expression. As shown in Figure 3B, miR-377 could reduce the luciferase activity in Saos-2/CDDP cells co-transfected with wild-type XIAP 3'UTR luciferase reporter, but it did not change the luciferase activity in cells co-transfected with mutant XIAP 3'UTR luciferase reporter. Western blot analysis confirmed the repression of XIAP by miR-377 in Saos-2/CDDP cells (Figure 3C). These findings indicate that XIAP is a direct target of miR-377 in OS.

Restoration of XIAP Expression Attenuates the Effect of miR-377 on Cisplatin Resistance

Finally, we tested whether the miR-377/XIAP interaction has a functional significance in regulating cisplatin resistance in OS cells. We co-transfected pcDNA3.1-XIAP or the empty plasmid with miR-377 to restore XIAP levels in these cisplatin resistant cells. We could clearly observe a partial inhibition of cisplatin responsiveness when XIAP was re-expressed (Figure 4A). Of note, XIAP overexpression greatly cancelled the apoptosis promoting the effect of miR-377 in Saos-2/CDDP cells treated with cisplatin (8 μg/ml) (Figure 4B). Consistent with the above data, XIAP overexpression decreased the level of PARP and caspase-3 cleavage, demonstrating a retard of apoptosis (Figure 4C). These data strongly suggest that XIAP is a functional target of miR-377, and the miR-377/XIAP signaling axis is essential in regulating cisplatin-induced apoptosis in OS cells.

Discussion

Chemoresistance is a major reason for the meta stasis and recurrence of OS, which often leads to treatment failure. The unmet demands in clinical practice beg for a deep understanding of the molecular basis underlying chemoresistance in OS. Accumulating evidence has proved that microRNAs are essential regulators in cancer development and progression; some of them also participate in the regulation of chemoresistance. In the current study, we provide the first compelling evidence to show that miR-377 is critically required for cisplatin sensitivity in OS. Down-regulation of miR-377 probably causes the decreased responsiveness of OS cells to apoptotic signals. In contrast, exogenous expression of miR-377 re-sensitizes cisplatin resistant OS cells to undergo caspase dependent apoptotic cell death. Notably, we firstly identified the apoptosis inhibitor, XIAP, as a direct target for miR-377. Reactivation of XIAP reversed the pro-apoptotic effect of miR-377, suggesting that this target is functional in the development of cisplatin resistance in OS cells. Our data uncovered a novel function of miR-377 in regulating drug resistance of OS. Regulation of chemosensitivity is one of the most important aspects of the functions of microRNAs implicated in cancer progression. With the advances of *in vivo* delivery of microRNAs into cancer cells, utilizing microRNA-based therapy would be feasible[14-16]. Uncovering the potential microRNAs that has certain functions in various given context allows a precise management of cancer treatment in different clinical senarios. Recent investigations[17-21] have discovered

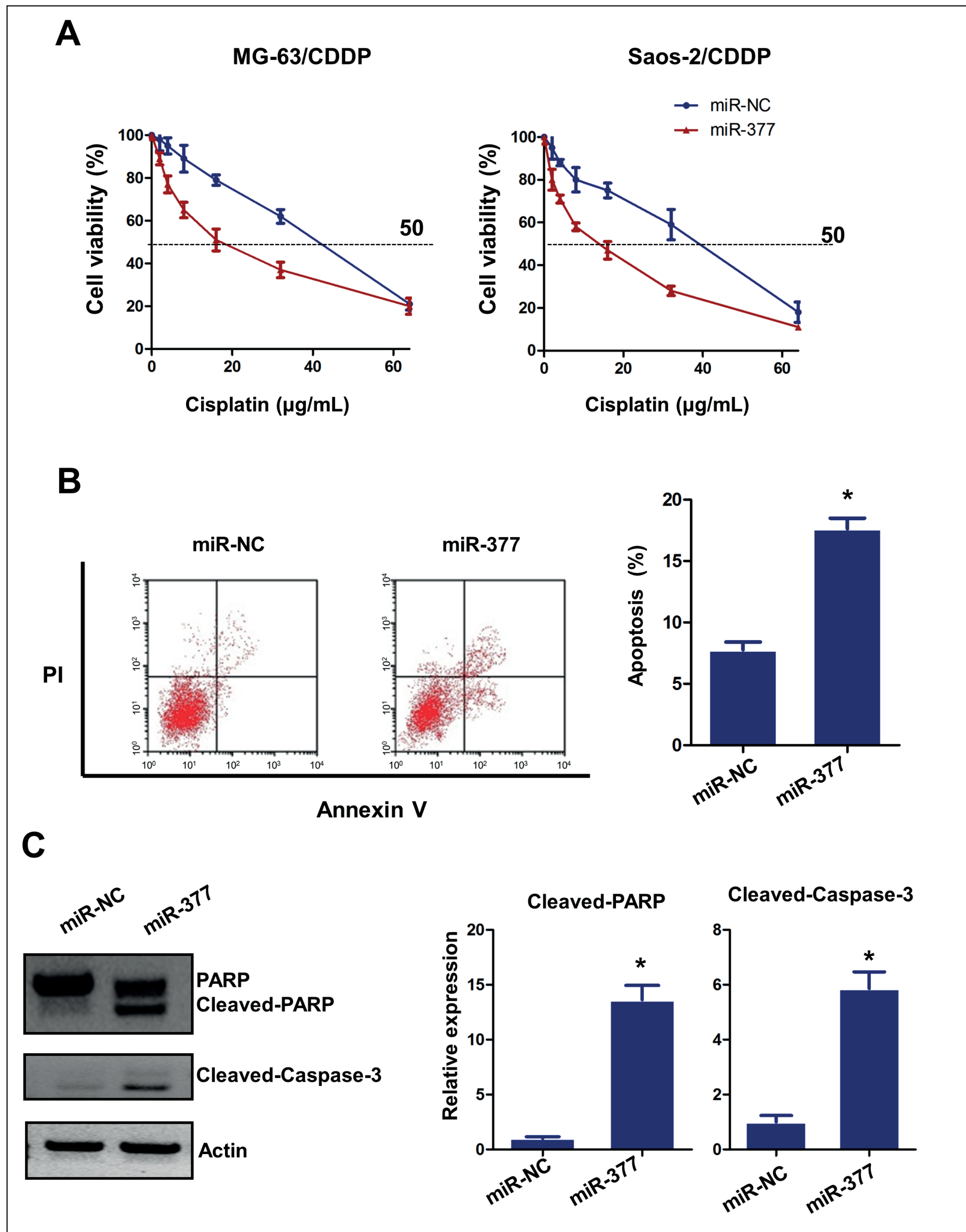

Figure 2. The effect of miR-377 on cisplatin resistance in OS cells. ***A,*** MG-63/CDDP and Saos-2/CDDP cells were transfected with the negative control (miR-NC) and miR-377 and treated with different doses of cisplatin for 72 h, cell viability was measured by CCK8 assay. ***B,*** Saos-2/CDDP cells were transfected with the negative control (miR-NC) and miR-377 and treated with 8 μg/ml cisplatin for 72 h, apoptosis was measured by flow cytometry. ***C,*** Following the same treatment in ***(B)***, PARP and cleaved-caspase-3 were analyzed by Western blot. *$p < 0.05$, n = 3.

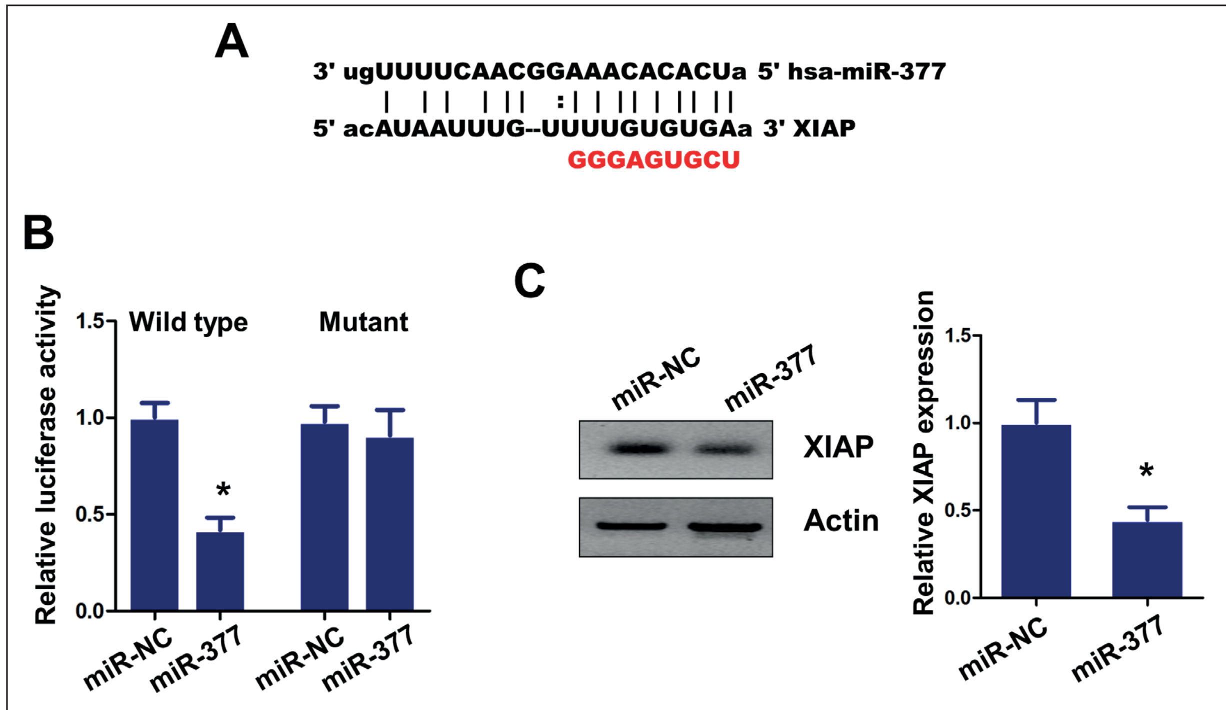

Figure 3. XIAP is targeted by miR-377. ***A,*** The schematic diagram showing the binding between miR-377 and XIAP 3'UTR. The base pairs in red font denote mutagenesis. ***B,*** Saos-2/CDDP cells were transfected with miR-NC or miR-377with either wild type XIAP 3'UTR luciferase construct or mutant XIAP 3'UTR luciferase construct, miR-377 significantly reduced luciferase activity in wild type group, whereas no effect was observed in mutant group. ***C,*** Saos-2/CDDP cells were transfected with miR-NC or miR-377, the expression of XIAP was analyzed by Western blot, the protein level of XIAP was reduced in miR-377 transfected cells. *$p < 0.05$, n = 3.

several microRNAs implicated in chemosensitivity or radiotherapy of OS. For example, miR-21, miR-184, miR-224, and miR-138 are implicated in the sensitivity of several chemotherapeutic drugs in OS by targeting various pro-survival factors. Although we currently do not know whether these microRNAs respond specifically to a certain spectrum of drugs or have general functions in OS, these studies may provide clues for further investigation of clinically applicable molecules to reverse chemoresistance. Wang et al[12] previously showed that miR-377 functions as a tumor suppressor in OS, intriguingly, they observed that miR-377 affected cell proliferation and invasion but had no effect on apoptosis. Adding to the complexity is that miR-377 promotes tumor progression in malignancies of another system such as colorectal cancer and gastric cancer[10,21]. These findings supported the notion that the functions of miR-377 in tumors are site or stage specific. Therefore, the role of miR-377, especially in the chemosensitivity of OS, still warrants further investigation. By comparing the endogenous expression of miR-377 in tissues from poor responders with that from normal patients, we show that miR-377 was significantly down-regulated, our conclusion is conceptually consistent with the study by Wang et al[12], which claims that miR-377 has a favorable role in OS development. Different from this previous study, we show that miR-377 was involved in the regulation of apoptosis in cisplatin resistant OS cells exposed to low dose cisplatin. Several possibilities could explain the discrepancy, first, the cell line used by their study is different from the ones used in our study, which may have different genetic response to miR-377; second, different from their experimental protocol, we treated cells with cisplatin; thus, miR-377 may mediate or amplify the apoptosis triggered by cisplatin, but have very limited capacity to induce apoptosis alone. Nonetheless, we identify a new molecule that exerts apoptosis related function in cisplatin resistant OS cells.

Our study identified the key regulator of apoptosis, XIAP, as a target of miR-377. The RING domain of XIAP has E3 ligase activity, ant it was utilized by cells to stop apoptosis by degrading caspase 3 and 9 via the proteasome

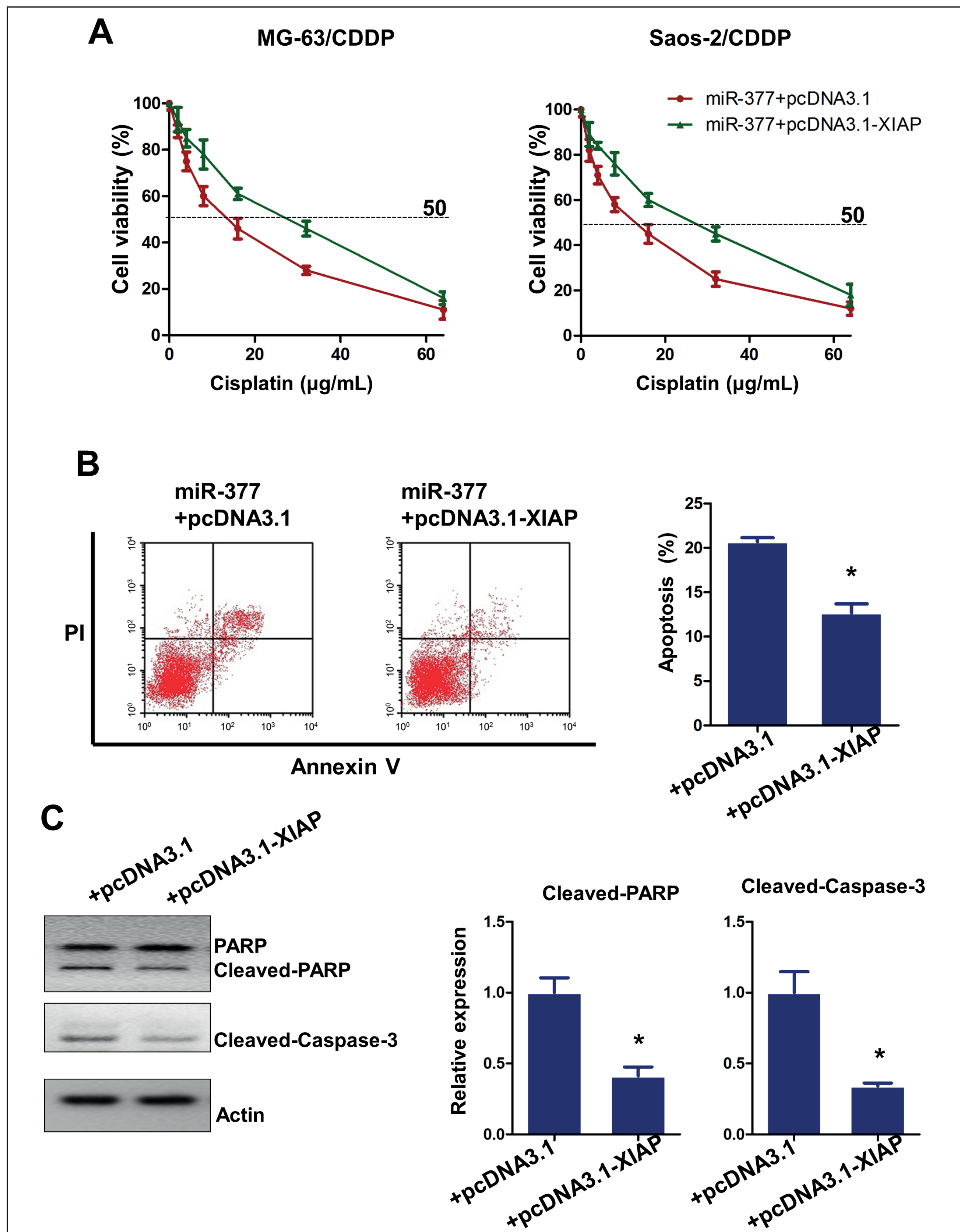

Figure 4. XIAP overexpression attenuates the cisplatin-sensitizing effect of miR-377. ***A,*** MG-63/CDDP and Saos-2/CDDP cells were transfected with miR-377 + pcDNA3.1 or miR-377 + pcDNA3.1-XIAP, and treated with different doses cisplatin for 72 h, cell viability was measured by CCK8 assay. ***B,*** Saos-2/CDDP cells were transfected with miR-377 + pcDNA3.1 or miR-377 + pcDNA3.1-XIAP, and treated with 8 μg/ml cisplatin for 72 h, apoptosis was measured by flow cytometry. ***C,*** Following the same treatment in ***(B)***, PARP and cleaved-caspase-3 were analyzed by Western blot. *$p < 0.05$, n = 3.

pathway[23]. The interactions between microRNAs and XIAP have been closely related to cancers but are seldom reported in chemoresistance. To the best of our knowledge, only two such findings showed that miR-874-XIAP and miR-130a-XIAP axis respectively regulate colorectal cancer and ovarian cancer chemoresistance[24,25]. In cardiomyocytes, XIAP was identified as a target of miR-377[26]. Consistent with this finding, we show that targeting XIAP by miR-377 is conserved in the human OS. Of note, re-expression of XIAP partially cancelled the apoptosis promoting effect of miR-377, suggesting that miR-377-XIAP axis is functional in the regulation of cisplatin sensitivity of OS cells. On the other hand, since the partial loss of chemo-sensitizing function of miR-377 under the XIAP overexpression condition, other factors that may contribute to aberrant XIAP expression should be taken into account.

Conclusions

We demonstrated for the first time that down-regulation of miR-377 contributes to cisplatin resistance in the human OS. Overexpression of miR-377 re-sensitizes OS cells to undergo cisplatin-induced apoptosis, which is achieved by direct inhibition of apoptosis inhibitor XIAP. Therefore. MiR-377 may have potential therapeutic value in tackling OS chemoresistance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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