

# IMPDH2 mediate radioresistance and chemoresistance in osteosarcoma cells

H.-X. LI, Q.-P. MENG<sup>1</sup>, W. LIU, Y.-G. LI, H.-M. ZHANG,  
F.-C. BAO, L.-L. SONG, H.-J. LI

Department of Hematology and Oncology, Children's Hospital of Zhengzhou, Zhengzhou, China

<sup>1</sup>Department of Science Textbooks, Children's Hospital of Zhengzhou, Zhengzhou, China

**Abstract. – OBJECTIVE:** Osteosarcoma (OS) is the most common primary malignant bone tumour in children and adolescents. Despite aggressive therapy, survival outcomes remain unsatisfactory, especially for patients with metastatic disease or patients with a poor chemotherapy response. Previous study founds inosine 5'-monophosphate dehydrogenase type II (IMPDH2) was an independent prognostic factor and observed frequent IMPDH2 overexpression in osteosarcoma patients with poor response to chemotherapy. In the present work, we provide evidence for direct involvement of IMPDH2 in the development of radioresistance and chemoresistance.

**MATERIALS AND METHODS:** The expression of IMPDH2 was examined in OS cells. Stable cell lines overexpressing IMPDH2 and IMPDH2 knock-down cells were generated using the osteosarcoma cell line. The stable transfected cells, alone or in combination with cisplatin or  $\gamma$ -irradiation, was used to treat OS cells. The growth inhibitory and apoptotic effects of IMPDH2 *in vitro* and *in vivo* were examined.

**RESULTS:** Overexpression of IMPDH2 in IMPDH2 poor-expressed U2OS cells induced strong cisplatin chemoresistance and  $\gamma$ -irradiation radioresistance through inhibition of apoptosis *in vitro* and *in vivo*. Knockdown of IMPDH2 in IMPDH2 rich-expressed Saos-2 cells resulted in significant chemosensitivity and  $\gamma$ -irradiation radiosensitivity through inducing of apoptosis *in vitro* and *in vivo*.

**CONCLUSIONS:** IMPDH2 is directly involved in the development of chemoresistance and radioresistance in osteosarcoma cells, suggesting that targeting of IMPDH2 by shRNA in combination with chemotherapy and  $\gamma$ -irradiation might be a promising means of overcoming chemoresistance and radioresistance in osteosarcomas with high IMPDH2 expression.

*Key Words:*

Osteosarcoma, Chemoresistance, Radioresistance, IMPDH2.

## Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumour in children and adolescents. The gold standard for therapy consists of a combination of multi-agent neoadjuvant chemotherapy, followed by radical surgery and adjuvant chemotherapy. With this aggressive regimen, 5 year survival rates of approximately 65% are obtained in patients with localized disease. However, in the case of metastatic or recurrent disease, 5 year survival rates are reduced to only 20%<sup>1-4</sup>. The remainder of patients respond poorly to chemotherapy with an increased risk of relapse and the development of metastasis. Further efforts to improve patient outcome, for example by means of novel treatment protocols, have not significantly affected overall and disease-free survival of osteosarcoma patients over the past 20 years<sup>5,6</sup>. The lack of responsiveness to chemotherapy due to intrinsic or acquired chemoresistance is the major reason for poor survival and disease relapse of osteosarcoma patients. However, the mechanisms underlying osteosarcoma chemoresistance remain largely unknown. Therefore, the identification of prognostic factors that allow risk stratification at the time of diagnosis and elucidation of the mechanisms underlying chemoresistance will be pivotal in the development of new therapeutic strategies.

Radiotherapy as a treatment modality for cancer has evolved over the past decades, but its use in OS treatment is controversial because OS is considered to be a relatively radioresistant tumour<sup>7-10</sup>. At present, radiotherapy is applied only in a select group of patients with OS, namely those who suffer from inoperable (advanced extremity, axial or head-and-neck) OS, patients with painful bone metastases and patients who refuse surgery. Therefore, the development of new therapeutic strategies to sensitize OS cells to irradiation treatment is essential.

Inosine 5'-monophosphate dehydrogenase type II (IMPDH2) encodes the rate-limiting enzyme in the de novo guanine nucleotide biosynthesis and has been linked to cell growth, differentiation, and malignant transformation<sup>11,12</sup>. Zhou et al<sup>13</sup> has found enhanced expression of IMPDH2 may promote the tumor metastasis and the advanced tumor progression in patients with PCa. He et al<sup>14</sup> has found IMPDH2 may be a protein biomarker and novel therapeutic target in colorectal cancer. IMPDH2 has also been shown to be overexpressed in methotrexate (MTX)-resistant erythroleukemia K562 and human colon cancer cells. Pharmacological inhibition of IMPDH2 sensitized these cells to MTX treatment, suggesting that IMPDH2 might be a target for the modulation of chemosensitivity<sup>15,16</sup>.

Previous study has identified IMPDH2 as independent prognostic factor for the response to chemotherapy in osteosarcoma patients. IMPDH2 gene expression was significantly elevated in patients with poor response and significantly associated with poor event-free survival<sup>17</sup>. In the present report, we investigated whether IMPDH2 is directly involved in the development of chemoresistance and radioresistance in osteosarcomas and whether inhibition of IMPDH2 expression might usefully enhance the therapeutic responses of OS cells to chemotherapeutic agents and irradiation.

## Materials and Methods

### Cell Lines and Reagents

U2OS, MG-63, Saos-2 and MNNG were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cell lines, were cultured in Dulbecco's modified Eagle's medium (4.5 g/l glucose)/Ham F12 (1:1) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS). All cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. cisplatin was purchased from Sigma (Buchs, Switzerland).

### Transfection and Generation of Stable Cell Lines

For overexpression of IMPDH2, the full coding sequence of IMPDH2 was cloned into the mammalian expression vector pcDNA3.1-EGFP (Shanghai, China) and verified by sequencing. For transfection studies, IMPDH2 poor-expressed U2OS cells were plated at a density of

1×10<sup>6</sup> cells per well in six-well plates and incubated for 24 h in complete medium. The cells were then transfected with 2-4 ug of the IMPDH2 construct (pcDNA3.1-IMPDH2) by using Lipofectamine method (Invitrogen). For controls, the same amount of empty vector, pcDNA3.1, and GFP vector (as positive control for transfection) was also transfected). Gentamicin (G418; Invitrogen) was added to 500 microg/mL for the selection of stable transfectants.

For gene knockdown, a commercially available shRNA expression vector expressing the following IMPDH2-specific 29-mer shRNA was used: 5'-ATAGCCTCCATTCGTATGAGAAGCGGCTT-3' (Origene, Shanghai, China). For transfection studies, IMPDH2 rich-expressed Saos-2 cells (10<sup>5</sup>/cells) were transfected with 0.5 mg of the IMPDH2 plasmid (shRNA-IMPDH2) by using Lipofectamine method (Invitrogen). Forty-eight hours after transfection, medium was replaced by fresh medium containing 600 mg/ml gentamicin for cells transfected with the shRNA-IMPDH2 vector.

Stable clones were selected for at least 2 weeks before single colonies were picked and analyzed for IMPDH2 expression by western blot.

### Western Blotting

Total cellular protein was extracted in RIPA lysis buffer and estimated using ABC protein estimation kit (Bio-Rad, Hercules, CA, USA). Protein was denatured by heating at 95°C for 2 minutes, resolved on 10%-15% denaturing SDS-polyacrylamide gel electrophoresis, transferred onto a Hybond nitrocellulose membrane (semi-dry transfer; 20 V for 45 minutes), blocked, and incubated overnight with primary IMPDH2 antibody. After three buffer washes, the blot was exposed to horseradish peroxidase-conjugated secondary antibody for 3 hours, washed, and developed using Pierce SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL, USA).

### Drug and $\gamma$ -Irradiation Exposure *in vitro*

Stably transfected cells and its controls were exposed to cisplatin for 6 h to reproduce the clinical conditions of OS treatment. Taking into account that the peak plasma level is 3  $\mu$ g/ml, we tested 3  $\mu$ g/ml concentrations for cisplatin. Evaluation of the cytotoxic effect was performed 48 h after the end of drug exposure. For chemosensitivity assay, stably transfected cells and its controls were exposed to  $\gamma$ -irradiation at 6 Gy.

### **Apoptosis and Growth Assays *in vitro***

After exposure to cisplatin or  $\gamma$ -irradiation, cells were trypsinized, fixed in 1% paraformaldehyde in phosphate buffered saline (PBS) on ice for 15 min, suspended in ice cold ethanol (70%) and stored overnight at  $-20^{\circ}\text{C}$ . Cells were then washed twice in PBS and incubated with 50  $\mu\text{l}$  of solution containing Terminal Deoxynucleotidyl Transferase (TdT) and FITC-conjugated dUTP deoxynucleotides 1:1 (Roche Diagnostic GmbH, Mannheim, Germany) in a humidified atmosphere for 60 min at  $37^{\circ}\text{C}$  in the dark. Samples were then washed in PBS containing 0.1% Triton X-100, counterstained with 3  $\mu\text{g/ml}$  of propidium iodide (Sigma Aldrich, St. Louis, MO, USA) and 10 Kunits/ml of RNase (Sigma-Aldrich) for 30 min at  $4^{\circ}\text{C}$  in the dark, and finally analyzed by flow cytometry using a FACS Vantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data acquisition and analysis were performed using CELLQuest software (Becton Dickinson). For each sample, 10,000 events were recorded.

Cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay in 96-well plates (2,500 cells per well) using the CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA) following the instructions of the manufacturer. A490 nm was measured using a Victor III (Perkin-Elmer/Wallace, Akron, OH, USA) plate reader. Each experiment was done in triplicate and repeated at least twice.

### ***In vivo* Tumor Study**

For chemosensitivity assay, stably transfected cells and its controls were injected into the flanks of 4- to 6-week-old female nude mice ( $1 \times 10^8/100$   $\mu\text{l}$  serum-free culture medium). Once the tumor reached 5 mm in size, cisplatin (3 mg/kg/d) was injected intraperitoneally into the mice on days 14, 21, 28. Mock transfected cells were injected into separate tumors in the same animals.

For radiosensitivity assay, once the tumor reached 5 mm in size, the animals were irradiated with two doses of 6 Gy on alternate days. The regression in the tumor growth was followed for up to 42 days.

Tumor growth was monitored every other day with calipers for 42 days to calculate tumor volume according to the formula  $[\text{length} \times \text{width}^2]/2$ .

All mice were sacrificed at 42 days. Sections (5 mm) from paraffin-embedded tumor were subjected to hematoxylin and eosin (H&E) staining

for histological analysis. TUNEL staining was performed using an ApopTag Kit (Chemicon international, Temecula, CA, USA) according to the manufacturer's instructions. The data was reported as mean  $\pm$  SEM. Six mice were used in each group.

### **Statistical Analysis**

Data were analyzed by unpaired t test or ANOVA in which multiple comparisons were performed using the method of least significant difference. The survival data was analyzed by log-rank test using GraphPad Prism 4 software. Differences were considered significant if the probability of the difference occurring by chance was  $p < 0.05$ .

## **Results**

### **IMPDH2 Expression**

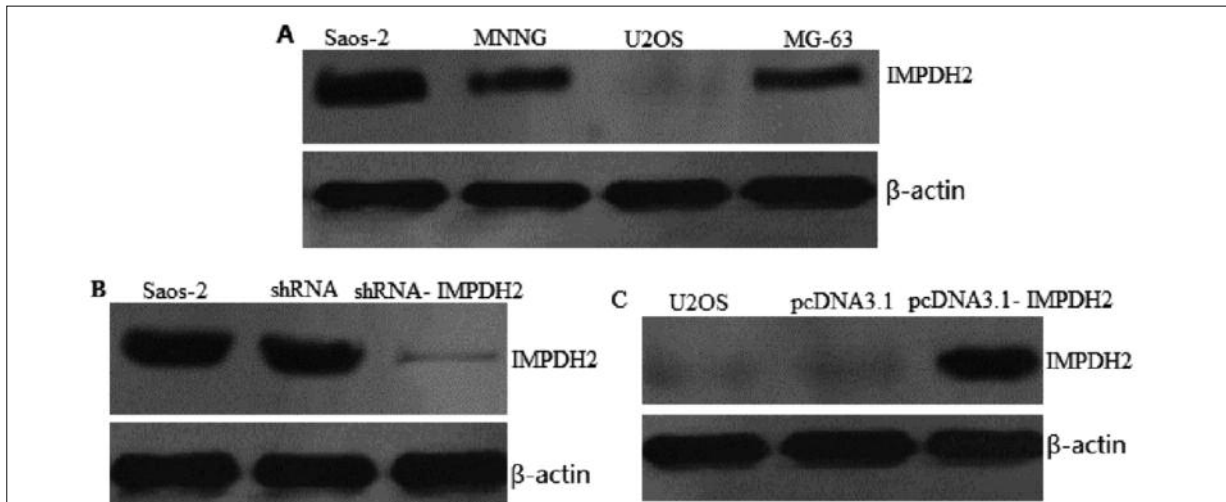
IMPDH2 protein levels were evaluated in U2OS, MG-63, Saos-2 and MNNG OS cell lines. MG-63, Saos-2 and HOS cell lines expressed detectable levels of IMPDH2 protein, and Saos-2 expressed the highest IMPDH2 protein (Figure 1A). U2OS cells expressed the undetectable levels of IMPDH2 protein (Figure 1A). In the present study, U2OS and Saos-2 cells were used for further study.

When the Saos-2 cell lines were stably transfected into the IMPDH2-specific 29-mer shRNA (shRNA- IMPDH2), a downregulation of the IMPDH2 protein was observed with respect to exposure to an appropriate control shRNA (Figure 1B). When the U2OS cell lines were stably transfected into the pcDNA3.1- IMPDH2, a upregulation of the IMPDH2 protein was observed with respect to transfection of an appropriate control pcDNA3.1-EGFP (Figure 1C).

### **Overexpression of IMPDH2 Increases U2OS Cell Chemoresistance and Radioresistance**

U2OS, pcDNA3.1-EGFP and pcDNA3.1-IMPDH2 transfected U2OS cells were then exposed to cisplatin,

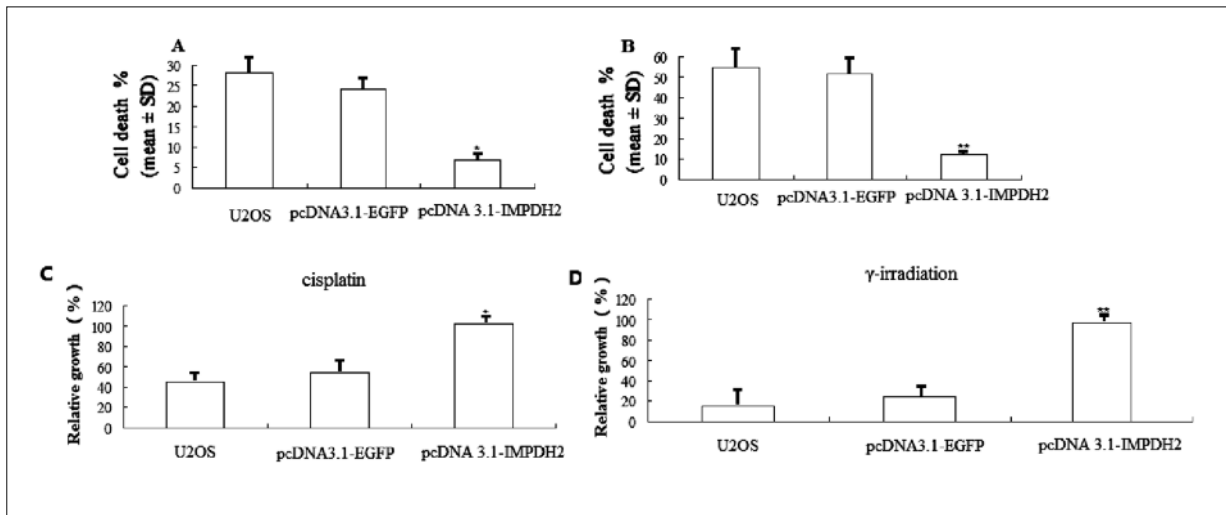
a drug currently used in OS therapy. Drug concentrations were based around *in vivo* peak plasma levels, and the 6-h exposure time was chosen on the basis of the drug's plasmatic half-life. Cell death analysis performed by FCM assay showed,



**Figure 1.** IMPDH2 expressed in OS cell lines. **A**, U2OS, MG-63, Saos-2, HOS and MNNG OS cells. Western blot (WB) analysis of IMPDH2 and  $\beta$ -actin. **B**, Saos-2 cells stably transfected with either IMPDH2-specific 29-mer shRNA (shRNA-IMPDPH2) or control shRNA. WB analysis of IMPDH2 and  $\beta$ -actin. **C**, U2OS cell lines were stably transfected into the pcDNA3.1-IMPDPH2 and control pcDNA3.1-EGFP. WB analysis of IMPDH2 and  $\beta$ -actin.

at both concentrations (3  $\mu$ g/ml), a higher cell death in U2OS and control pcDNA3.1-EGFP transfected U2OS cells compared to pcDNA3.1-IMPDPH2 transfected U2OS cells (Figure 2A). MTT assay has the same results as the above (Figure 2B).

The TUNEL assay showed reduced apoptosis rate of pcDNA3.1-IMPDPH2/U2OS cells with 6 Gy  $\gamma$ -irradiation compared to U2OS and pcDNA3.1-EGFP/U2OS cells with 6 Gy (Figure 2C). MTT assay has the same results as the above (Figure 2D).



**Figure 2.** Overexpression of IMPDH2 decreased cisplatin and  $\gamma$ -irradiation-induced cell death in U2OS cells. **A**, U2OS, pcDNA3.1-EGFP and pcDNA3.1-IMPDPH2 transfected U2OS cells were exposed to cisplatin (1.5 and 3  $\mu$ g/ml). **B**, U2OS, pcDNA3.1-EGFP and pcDNA3.1-IMPDPH2 transfected U2OS cells were exposed to 6 Gy  $\gamma$ -irradiation. TUNEL assay (representative experiments), with percentage of dead cells ("D" refers to debris, which never exceeded 5%) and median values of each experiment, which was repeated 3 times (\* $p$  < 0.01, \*\* $p$  < 0.001). **C**, Cell growth was measured by MTT assay following exposure to cisplatin at 48 hours. **D**, Cell growth was measured by MTT assay following exposure to 6 Gy  $\gamma$ -irradiation. Compared to untreated cells, \* $p$  < 0.01, \*\* $p$  < 0.001.

### Knockdown of IMPDH2 Increases Saos-2 Cell Chemosensitivity and Radiosensitivity

Saos-2, shRNA and shRNA-IMPDH2 transfected Saos-2 cells were then exposed to cisplatin at both concentrations (3  $\mu\text{g}/\text{ml}$ ). A higher cell death in shRNA-IMPDH2 transfected Saos-2 cells was found compared to Saos-2 and shRNA transfected Saos-2 cells (Figure 3A). MTT assay has the same results as the above (Figure 3B)

The TUNEL assay showed increased apoptosis rate of shRNA-IMPDH2 transfected Saos-2 cells with 6 Gy  $\gamma$ -irradiation compared to Saos-2 and shRNA transfected Saos-2 cells with 6 Gy (Figure 3C). MTT assay has the same results as the above (Figure 3D).

### Knockdown of IMPDH2 Augments Therapeutic Effect of Cisplatin and $\gamma$ -irradiation on Implanted Tumor

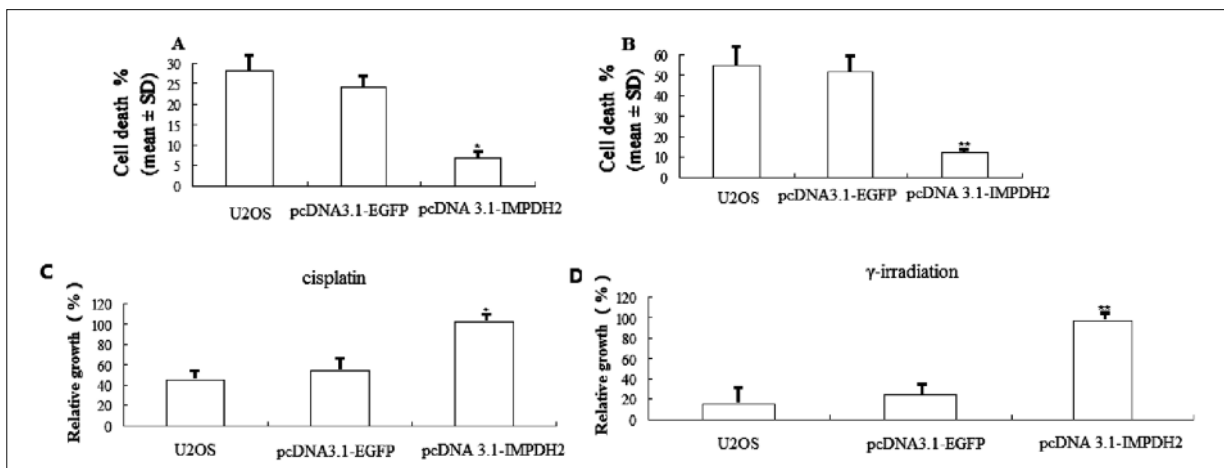
Experiments were done to investigate the therapeutic utility of shRNA-IMPDH2 and cisplatin or  $\gamma$ -irradiation combination in SCID mice bearing implanted OS cells, Saos-2. In shRNA-IMPDH2 transfected tumor, treatment with cisplatin or  $\gamma$ -irradiation resulted in significant reduction in the mean tumor volume compared with the Saos-2 and control shRNA-transfected tumor (Figure 4A and B). shRNA-IMPDH2 treatment alone did not cause any reduced volume compared to the Saos-2 tumor, suggesting that IMPDH2 silencing does not induce any deleterious effect under the present experimental conditions.

Analysis of the apoptotic index in the different treatment groups showed that combination with shRNA-IMPDH2 and cisplatin or  $\gamma$ -irradiation caused significant cell apoptosis compared with control tumors (data not shown).

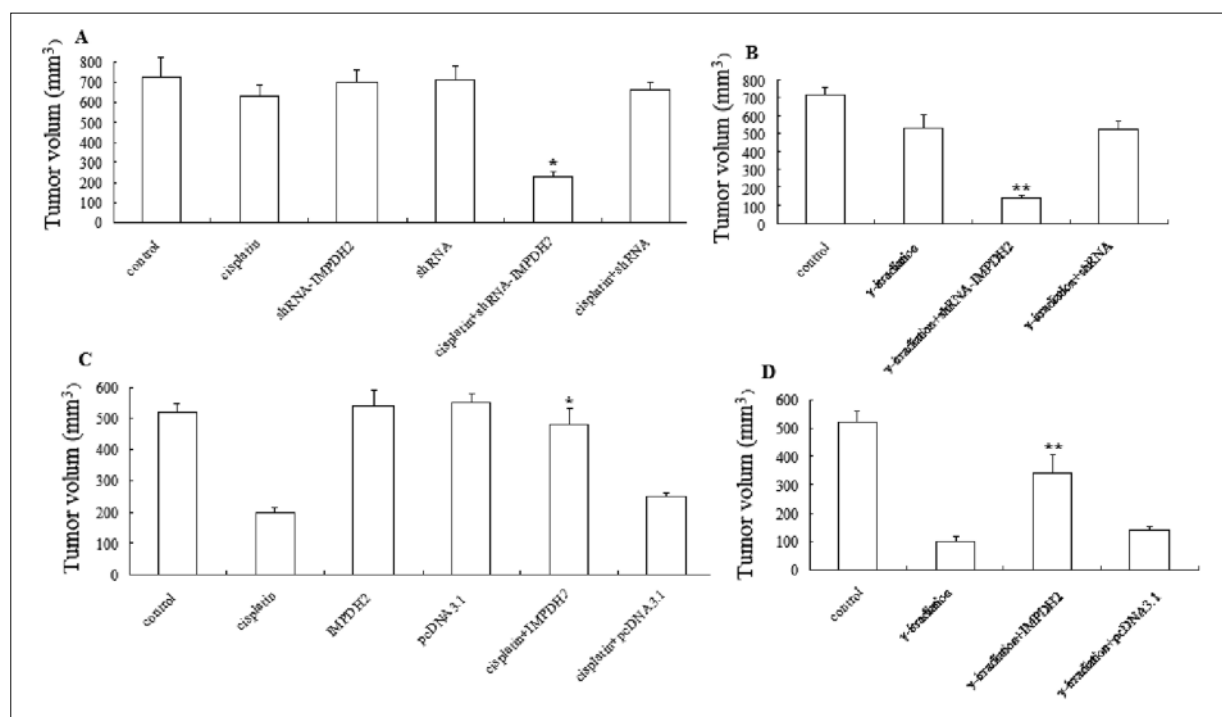
To further investigate the therapeutic potential of IMPDH2 overexpression combined with cisplatin or  $\gamma$ -irradiation, pcDNA3.1-IMPDH2 transfected tumor were treated with cisplatin or  $\gamma$ -irradiation. In IMPDH2 transfected tumor, treatment with cisplatin or  $\gamma$ -irradiation resulted in significant increase in the mean tumor volume compared with the U2OS and control-transfected tumor (Figure 4C and D). IMPDH2 treatment alone did not cause any increased volume compared to the U2OS tumor, suggesting that IMPDH2 overexpression does not have any effect under the present experimental conditions. Analysis of the apoptotic index in the different treatment groups showed that combination with IMPDH2 and cisplatin or  $\gamma$ -irradiation caused fewer cell apoptosis compared with control tumors (data not shown).

## Discussion

Due to the emergence of adjuvant and neoadjuvant chemotherapy, the survival rate has been greatly improved in osteosarcoma (OS) patients with localized disease. However, this survival rate has remained unchanged over the past 30



**Figure 3.** Knockdown of IMPDH2 increases Saos-2 cell chemosensitivity and radiosensitivity. **A**, Saos-2, shRNA and shRNA-IMPDH2 transfected Saos-2 cells were exposed to cisplatin (3  $\mu\text{g}/\text{ml}$ ). **B**, Saos-2, shRNA and shRNA-IMPDH2 transfected Saos-2 cells were exposed to 6 Gy  $\gamma$ -irradiation. TUNEL assay was used to detect percentage of dead cells which was repeated 3 times (\* $p < 0.01$ , \*\* $p < 0.001$ ). **C**, Cell growth was measured by MTT assay following exposure to cisplatin at 48 hours. **D**, Cell growth was measured by MTT assay following exposure to 6 Gy  $\gamma$ -irradiation. Compared to untreated cells, \* $p < 0.01$ , \*\* $p < 0.001$ .



**Figure 4.** IMPDH2 regulates therapeutic effect of cisplatin and  $\gamma$ -irradiation on implanted tumor. **A**, Effect of IMPDH2 knockdown and cisplatin combination on SCID mice bearing implanted Saos-2 cells. **B**, Effect of IMPDH2 knockdown and  $\gamma$ -irradiation combination on SCID mice bearing implanted Saos-2 cells. **C**, Effect of IMPDH2 overexpression and cisplatin combination on SCID mice bearing implanted U2OS cells. **D**, Effect of IMPDH2 overexpression and  $\gamma$ -irradiation combination on SCID mice bearing implanted U2OS cells. vs cisplatin, \* $p < 0.05$ ; vs  $\gamma$ -irradiation, \*\* $p < 0.01$ .

years, and the long-term survival rate for OS patients with metastatic or recurrent disease remains poor. To a certain extent, the reason behind this may be ascribed to the chemoresistance to anti-OS therapy<sup>18</sup>. Knowledge of the mediators that contribute to chemoresistance is pivotal to the identification of high-risk patients and the development of new therapeutic strategies.

Radiation has been a cancer therapy for more than a hundred years. Nowadays, more than 50-60% of cancer patients need radiotherapy as a part of their cancer treatment. The rationale of radiotherapy in cancer treatment is based on the observation that radiation can inhibit cell proliferation or induce apoptotic cell death *in vitro*, and suppress tumor growth *in vivo*<sup>19</sup>. However, OS is considered to be a relatively radioresistant tumor<sup>7-10</sup>, however to develop the new therapeutic strategies to sensitize OS cells to irradiation treatment is essential.

Yue et al<sup>20</sup> has recently reported NRP-1 overexpression in MG-63 cells increased survival of cells after exposure to doxorubicin. In contrast, downregulation of NRP-1 expression in SaOS-2 cells markedly increased chemosensitivity after exposure to doxorubicin, which suggest that

NRP-1 could be used as a biomarker for OS progression and a novel therapeutic or chemopreventive target for human OS treatment.

Previous study of frequent IMPDH2 overexpression in osteosarcoma patients with poor response to chemotherapy and the identification of IMPDH2 as an independent prognostic marker for chemotherapy response suggest that IMPDH2 might be directly involved in the development of chemoresistance<sup>17</sup>. To verify this hypothesis we established osteosarcoma cell lines with modulated IMPDH2 expression either by overexpression of the IMPDH2 coding sequence in U2OS cells or by IMPDH2 knock-down using an shRNA construct specific for IMPDH2 in Saos-2 cells. The analysis of chemosensitivity and radioresistance revealed a strong resistance of IMPDH2-overexpressing U2OS /MPDH2 cells against cisplatin and  $\gamma$ -irradiation. Contrary to our expectations, IMPDH2 knock-down significantly enhance the chemosensitivity and radiosensitivity of Saos-2 cells.

The identification of genes contributing to chemoresistance and radioresistance is a crucial first step in overcoming the hurdle of OS patient

chemoresistance, radioresistance and improving overall OS patient survival. As described, we have identified IMPDH2 genes that may be involved in OS chemoresistance and radioresistance. Although a direct correlation between IMPDH2 and chemoresistance and radioresistance has yet to be established, further investigation is warranted in order to determine its role.

## Conclusions

Based on our results, we propose MPDH2 as a potential new drug target for OS to enhance the efficacy of chemotherapies and radiotherapies. Prior to its use in clinical trials, further clinical studies are required to assess the effect of MPDH2 in OS patients either used alone or in combination with other anti-OS drugs. Further studies should be focused on the precise mechanism of the interaction between conventional chemotherapy and MPDH2 inhibition.

## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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