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Knockdown of USP9X reverses cisplatin resistance by decreasing β-catenin expression in nasopharyngeal carcinoma cells

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In various cancers, abnormal USP9X expression is involved in tumorigenesis, progression, apoptosis, and metastasis. However, the relationship between USP9X abnormal expression and cisplatin resistance in nasopharyngeal carcinoma (NPC) cells remains unclear. Using qRT-PCR and western blot, we detected the expressions of USP9X and β-catenin in NPC cells. The effects of USP9X on cisplatin resistance, proliferation, apoptosis, and metastasis were examined by CCK-8 assay, flow cytometry, wound-healing assay, and Transwell chamber assay. Co-IP assay, qRT-PCR, and western blot were performed to explore the detailed molecular mechanism of USP9X-β-catenin and its effect on the protein levels of MDR1, MRP2, Bcl-2, Bax, MMP2, and MMP9. We found that USP9X and β-catenin expressions in cisplatin-resistant cell lines (HNE1/DDP) were much higher than cisplatin-sensitive cell lines (HNE1) at both mRNA and protein levels. Co-IP assay demonstrated that USP9X was immunoprecipitated with β-catenin in NPC cells. Knockdown of USP9X was able to partially reverse cisplatin resistance, increased cisplatin-induced apoptosis, and decreased the capacities of proliferation, migration, and invasion. Overexpression of USP9X can increase cisplatin resistance in NPC cells. Moreover, knockdown of USP9X expression can significantly reduce the expressions of MDR1, MRP2, Bcl-2, MMP2, and MMP9, but significantly increased the expression of Bax. These findings indicate that USP9X high expression plays a significant part in cisplatin resistance of NPC. This study elucidated the possible mechanism of cisplatin resistance in NPC cells and may have implications for the therapeutic reversal of cisplatin resistance.

Key words: nasopharyngeal carcinoma, USP9X, β-catenin, cisplatin resistance, metastasis

Nasopharyngeal carcinoma (NPC) is an epithelial carcinoma occurring from the nasopharyngeal mucosal lining. It has apparent geographical distribution characteristics and is especially prevalent in East and Southeast Asia (particularly southern China) [1]. According to the International Agency for Research on Cancer, in 2018, there were 129,079 new cases of NPC and 72,987 deaths around the world [2]. Besides, approximately 10% of patients have residual or recurrence in the primary site and area [3], whereas distant metastasis occurs in 15–30% of patients with NPC after radical treatment [4]. Chemotherapy combined with radiotherapy is the main curative treatment for treating locoregionally advanced NPC, and cisplatin is generally preferred for chemoradiation (CRT) [5]. Although the addition of CRT significantly improved overall survival, resistance to platinum-based chemotherapy impeded noticeable improvement of NPC prognosis [6–8]. Therefore, the identification of biomarkers that can improve the metastasis of NPC and chemotherapeutic resistance is crucial.

While cisplatin exerts anticancer effects, tumor cells often develop resistance to cisplatin chemotherapy via multiple mechanisms [9, 10]. β-catenin is a crucial regulator in the Wnt signaling pathway, regulating various vital processes for cancer progression [11]. The Wnt/β-catenin signaling pathway is implicated in cisplatin resistance in several human malignancies, including lung adenocarcinoma [12], ovarian cancer [13], oral carcinoma [14], and leukemic stem cells [15]. Recently, the activation of Wnt/β-catenin has been identified to mediates cisplatin chemoresistance in NPC [16]. However, the mechanisms of β-catenin overexpression in NPC cisplatin-resistant cells remain elusive.

USP9X is a crucial member of the ubiquitin-specific proteases (USPs) family of deubiquitinating enzymes (DUBs), which can remove ubiquitin molecules from large

proteins, thus inhibiting the degradation of target proteins and playing an essential role in protein regulation [17–19]. In our previous experiments, we found that USP9X could deubiquitize β-catenin, thereby activating the Wnt/β-catenin signaling pathway to promote the proliferation of glioma cells [20] and mediate TRAIL-resistance in breast cancer cells [21]. Similarly, USP9X promotes neural progenitor and hepatoma cells' proliferation by regulating the expression of β-catenin [22, 23]. More recently, some evidence suggests that DUBs are involved in regulating cell proliferation, apoptosis, and drug resistance [24]. However, the underlying mechanisms of USP9X activity in NPC cells remain delineated, and it is unclear whether knockdown of USP9X could attenuate cisplatin resistance.

In the present study, we investigated whether knockdown of USP9X can reverse cisplatin resistance and explored aberrant signaling pathways mediating cisplatin resistance in NPC cells, thus providing possible targets to overcome chemoresistance in the treatment of NPC. Here, our findings suggest that USP9X which is highly expressed in cisplatin-resistant NPC cells interacts with β-catenin to affect the cells' cisplatin sensitivity, apoptotic, invasion, and migration abilities.

Materials and methods

Cell lines. Human NPC cell lines, HNE1 and HNE1/DDP (a cisplatin-resistant cell), were obtained from Xiangya Hospital of Central South University (Changsha, China) maintained in our laboratory. Cells were cultured in DMEM medium (Hyclone, Logan, Utah, USA) containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml) in 5% CO_2 at 37 °C. The DMEM medium for culturing HNE1/DDP cells was supplemented with cisplatin (2 μmol/l). All experiments were performed with cells grown in the absence of cisplatin for at least 4 d to avoid drug-associated secondary effects.

Cell viability assay. HNE1 and HNE1/DDP cells (3×10^3/well) were seeded in 96-well culture clusters (Costar, Corning, NY, USA). After 24 h starvation in serum-free medium, cells were treated with different concentrations of cisplatin (0, 1, 2, 4, 8, 16, or 32 μmol/l) for subsequent 24 h, 48 h, or 72 h incubation. Then, a fresh complete medium containing Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added to the plates for 1–3 h incubation. The cell viability values were measured by absorbance at 450 nm using a microplate reader (Rayto, RT-6000, USA). After calculating cell viability percentages, IC50 values of cisplatin were determined using the GraphPad Prism 7.0 software. The OD values of differently treated cells after 0, 24, 48, and 72 h were measured to indirectly reflect the effect of cisplatin on cell proliferation.

siRNA and lentivirus transfection. USP9X specific siRNAs (USP9X si-1, USP9X si-2, and USP9X si-3), β-catenin specific siRNAs (β-catenin si-1, β-catenin si-2, and β-catenin si-3), and negative control siRNA (for sequences, see Table 1) were synthesized by GenePharma Company (Shanghai, China). Transfections were performed with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA was prepared at 48 h post-transfection for qRT-PCR analysis and total protein was prepared at 72 h post-transfection for western blot analysis. The full-length USP9X gene was delivered into the pLVX-Puro vector to construct a USP9X overexpression vector. pLVX-USP9X or pLVX-NC vector with lentiviral packaging plasmid was then transfected into 293T cells to construct the target lentivirus. Subsequently, resulting lentiviruses were collected and used to transduce HNE1 and HNE1/DDP cells.

Apoptosis assay. HNE1 and HNE1/DDP cells were seeded in six-well culture clusters (Costar) at a density of 1×10^5/well. After 24 h incubation, cells were treated with 6 μmol/l cisplatin. After 48 h incubation and washing twice with phosphate-buffered saline (PBS), the cells were resuspended in 200 μl binding buffer and incubated with an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN, Suzhou, China) according to the manufacturer's instructions. Flow cytometry samples were measured on FACSCalibur (BD Biosciences, USA), and the data were analyzed using FlowJo software (TreeStar, Ashland, USA).

Cell migration assay. A wound-healing assay was performed to detect changes in cell migration. The HNE1 and HNE1/DDP cells were seeded in 6-well plates, grew to confluent, and then scraped with a 200 μl pipette tip to create a gap of standard width. After rinsing with serum-free medium, cells were incubated in a condition of 37 °C under 5% CO_2 for 24 h. The gap closure was observed under an inverted microscope (magnification, 100×) at 0 h and 24 h post-wounding, and measured to calculate healing percentages by using ImageJ software.

Cell invasion assay. Cell invasion assay was performed using 24-well Transwell plates (Costar, Corning, NY, USA) invasion assays. The membranes undersurface was coated

Table 1. Transfected gene sequences.

Gene	Sequence (5'→3')
Negative control	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
USP9X si-1	CCCGCACUGAAACAAAUUATT UAAUUUGUUUCAGUGCGGGTT
USP9X si-2	GACCUCAUCAGAUUAUUAUTT AUAAUAAUCUGAUGAGGUCTT
USP9X si-3	GGGCAAUGGAGAUCUUAAATT UUUAAGAUCUCCAUUGCCCTT
β-catenin si-1	CCUUCACUCAAGAACGGGUTT ACUUGUUCUUGAGUGAAGGTT
β-catenin si-2	GUCAAGUCUUGUUCAGAATT UUCUGAACAAGACGUUGACTT
β-catenin si-3	GCUCAUCAUACUGGCUAGUTT ACUAGCCAGUAUGAUGAGCTT

with 50 µl of Matrigel (BD Biosciences, USA) mixed with a serum-free medium at a 1:8 dilution and subsequently applied to the top side of the filter. In the lower chamber of Transwell inserts, 700 µl of DMEM medium containing 30% FBS was added. In the upper chamber of Transwell inserts, 1×10^5 cells in 200 µl serum-free medium were loaded. After 24 h incubation, the Transwell inserts were washed twice with PBS. The membranes were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet dye, and observed by using an inverted microscope (magnification, 200×; CKX41, Olympus, Japan). The OD value was measured at 570 nm, which indirectly reflected the cell invasion.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The complementary DNA (cDNA) was synthesized by the 1st strand cDNA Synthesis Kit (Vazyme, Nanjing, China). qRT-PCR was performed using SYBR Green qPCR Super Mix (TransGen, Beijing, China) in a total volume of 20 µl on Roche Light Cycler 96 system (Roche, Basel, Switzerland). The relative mRNA expressions in the various groups were calculated using the ΔΔCt method by normalizing to GAPDH. The sequences of primer pairs can be viewed in Table 2.

Western blot analysis. HNE1 and HNE1/DDP cells were lysed with RIPA Lysis Buffer (Beyotime, Shanghai, China). Then, protein concentrations were quantified with Easy BCA Protein Quantitative Kit (TransGen Biotech, Beijing, China). Equal amounts of proteins were separated by 10% SDS-PAGE (Boster, Wuhan, China) and then transferred onto 0.45 µm PVDF membranes (Millipore, Bedford, USA). After blocking with 5% skim milk, the membranes were incubated with one of the following antibodies: anti-GAPDH antibody (1:1000, 10494-1-AP, Proteintech, Wuhan, China), anti-USP9X antibody (1:500, 55054-1-AP, Proteintech), anti-β-catenin antibody (1:5000, 51067-2-AP, Proteintech), anti-MDR1 antibody (1:1000, 22336-1-AP, Proteintech), anti-MRP2 antibody (1:1000, 24893-1-AP, Proteintech), anti-MMP2 antibody (1:1000, ab86607, Abcam, Cambridge, UK), anti-MMP9 antibody (1:1000, ab76003, Abcam), anti-Bcl-2 antibody (1:1000, #15071, Cell Signaling Technology, USA), or anti-Bax antibody (1:1000, #5023, Cell Signaling Technology) at 4 °C overnight. Then the membranes were incubated in HRP-labeled Goat Anti-Rabbit IgG (1:5000, A0208, Beyotime) or HRP-labeled Goat Anti-Mouse IgG (1:5000, A0216, Beyotime) at 37 °C for 1 h. Finally, the membrane was incubated in ECL solution (Boster) and imaged using a Tanon 5200 digital imaging system (Tanon, Shanghai, China).

Table 2. Primer pairs sequences.

Gene	Sequence (5'→3')
GAPDH	GGAGTCCACTGGCGTCTTCA GTCATGAGTCCTTCCACGATACC
USP9X	AGGCTCCTGATGGACAGTCT AACTGGAACCACCCATCGAG
β-catenin	TTGTCCCGCAAATCATGCAC GCTAGGATGTGAAGGGCTCC
MDR1	CCTCATTATCCCGCTCCGTG AGGTCATCCTGTTTCGGGTG
MRP2	TCACTGCGGCTCTCATTCAG ATGAGCTTCTGGGCATCCAC
Bcl-2	TCCGATCAGGAAGGCTAGAGTT TCGGTCTCCTAAAAGCAGGC
Bax	CCGCCGTGGACACAGAC CAGAAAACATGTCAGCTGCCA
MMP2	AGCGAGTGGATGCCGCCTTTAA CATTCCAGGCATCTGCGATGAG
MMP9	GCCACTACTGTGCCTTTGAGTC CCCTCAGAGAATCGCCAGTACT

Co-immunoprecipitation assay. The Co-IP analysis was used for examination of the change in the association between USP9X and β-catenin in the HNE1 and HNE1/DDP cells. Cell lysates were extracted with RIPA buffer and incubated with antibodies, such as anti-USP9X (55054-1-AP, Proteintech), anti-β-catenin (51067-2-AP, Proteintech), and the negative control rabbit immunoglobulin G (#2729, Cell Signaling Technology, USA), overnight at a constant speed at 4 °C. After that, the complex was mixed with Protein A/G PLUS-Agarose beads (Santa Cruz, CA, USA) and shaken for 2 h at 4 °C to harvest the antigen-antibody complex. The beads were washed three times with RIPA buffer and boiled in SDS-loading buffer (Boster). The eluted proteins were detected by western blot.

Analysis of data. All data in the present study were analyzed with GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA) and expressed as the means ± standard deviation (SD). Statistical analysis was conducted using a two-tailed unpaired Student's t-test. Differences were considered statistically significant at $p<0.05$.

Results

Expression of USP9X and β-catenin in cisplatin-resistant HNE1/DDP cell line. HNE1 and HNE1/DDP cells were treated with different concentrations of cisplatin (0, 1, 2, 4, 6, 8, 16, or 32µmol/) for 24 h, 48 h, or 72 h to determine the IC50 (inhibitory concentration 50%) by the Cell Counting Kit-8 (CCK-8) assay. HNE1/DDP showed cell resistance to cisplatin as opposed to the parental HNE1 cell line. The IC50 of cisplatin were 7.11, 5.17, and 4.28 µmol/l in the cisplatin-sensitive HNE1 cell line (Figure 1A). The IC50 of cisplatin were 33.63, 29.44, and 22.75 µmol/l in the cisplatin-resistant HNE1/DDP cell line (Figure 1B). Compared with the parental cells, HNE1/DDP showed 5.7-fold enhanced resistance to cisplatin. In order to confirm whether USP9X and β-catenin were involved in the potential mechanism of cisplatin resistance, we examined the expression of USP9X and β-catenin in the cisplatin-resistant HNE1/DDP cell

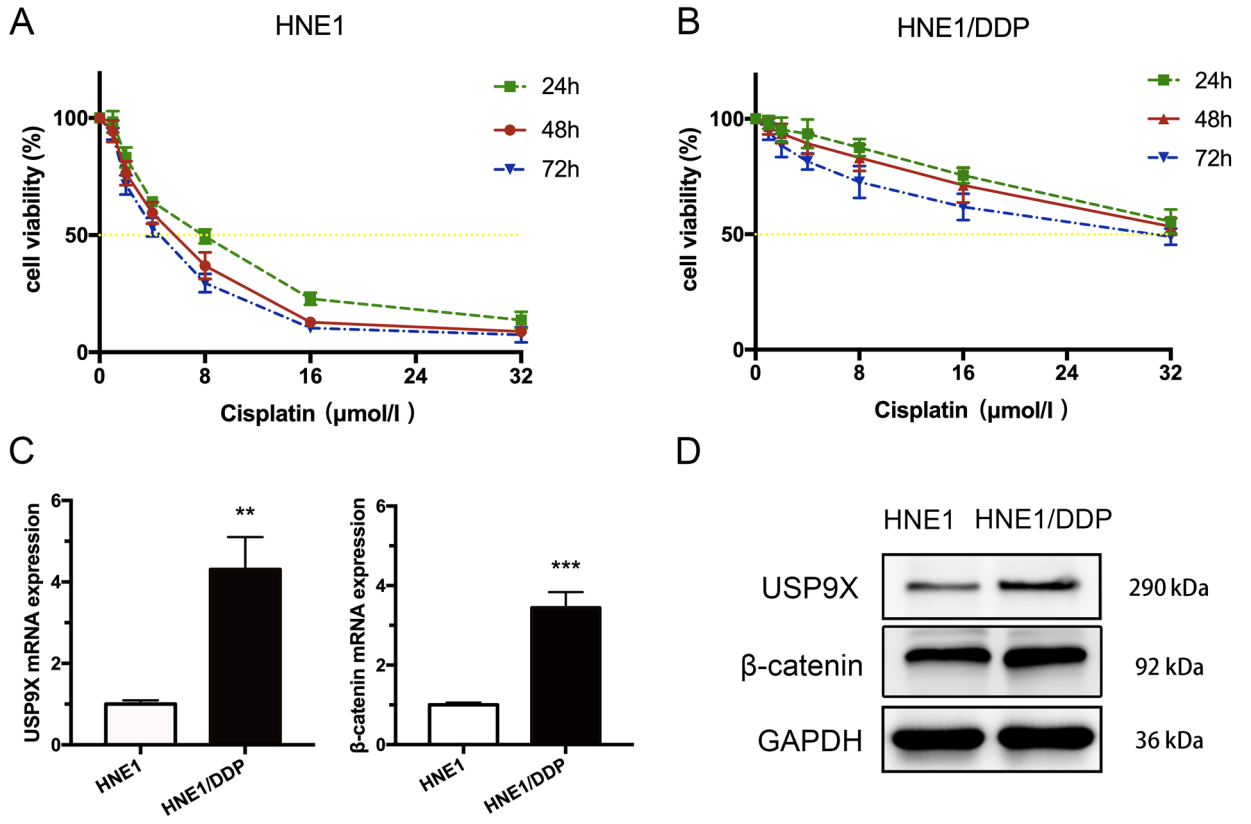

Figure 1. Expression of USP9X and β-catenin in cisplatin-resistant HNE1/DDP cell line. A, B) CCK-8 was used to determine HNE1 and HNE1/DDP cell viability following treatment with 0, 1, 2, 4, 6, 8, 16, or 32 μmol/l cisplatin. HNE1/DDP shows cell resistance to cisplatin as opposed to the parental HNE1 cell line. C) qRT-PCR analysis of USP9X and β-catenin mRNA expressions in HNE1 and HNE1/DDP cells. **p<0.01, ***p<0.001 compared to parental HNE1 cells. D) Western blot analysis of USP9X and β-catenin protein expressions in HNE1 and HNE1/DDP cells. Western blot and qRT-PCR verified that USP9X and β-catenin expressions were higher in HNE1/DDP cells. The mRNA and protein expressions were normalized by GAPDH.

line compared with the parental HNE1 cell line. We found that USP9X was upregulated about 4.3-fold in HNE1/DDP cells compared with HNE1 cells by qRT-PCR (Figure 1C). Moreover, β-catenin was upregulated about 3.4-fold in HNE1/DDP cells compared with HNE1 cells. While at the same time, western blot results showed that the levels of USP9X and β-catenin were significantly higher in HNE1/DDP cells (Figure 1D). These results indicated that USP9X and β-catenin may be related to the cisplatin resistance in HNE1/DDP cells.

Expression of USP9X and β-catenin can regulate cisplatin resistance in NPC cells. To determine whether knockdown of the expression of USP9X or β-catenin could reverse the cisplatin-resistance of HNE1/DDP cells, we used an RNA interference system to explore the role of USP9X and β-catenin in cisplatin resistance. In terms of Figures 2A and 2B, we transfected USP9X siRNAs and β-catenin siRNAs into HNE1 and HNE1/DDP cells respectively, to select the most efficient one (USP9X si-1 and β-catenin si-3) for subsequent experiments. After Negative Control (NC), USP9X, and β-catenin siRNAs were transfected into HNE1 and HNE1/DDP cells separately, IC50 of cisplatin was determined by the same method as in the former experiment. CCK-8 assay data confirmed that siRNA transfection targeting USP9X or β-catenin cut down markedly IC50 of HNE1 cells compared with NC (2.76 or 2.73 vs. 5.72 μmol/l for 24 h, 2.25 or 2.1 vs. 4.58 μmol/l for 48 h, 1.92 or 1.72 vs 3.67 μmol/l for 72 h) (Figure 2C), and IC50 of HNE1/DDP cells compared with NC (10.88 or 10.76 vs. 32.43 μmol/l for 24 h, 9.3 or 8.75 vs. 27.93 μmol/l for 48 h, 8.28 or 7.83 vs. 23.24 μmol/l for 72 h) as well (Figure 2D). Knockdowns of USP9X and β-catenin were able to partially reverse cisplatin resistance in HNE1/DDP cells, and enhance the sensitivity of HNE1 cells to cisplatin. USP9X-overexpressing HNE1 and HNE1/DDP cell lines were constructed by lentiviral transfection. Compared with the pLVX-NC group, USP9X and β-catenin expressions on mRNA and protein levels were significantly increased in the pLVX-USP9X group (Figures 3A, 3B). The overexpression of USP9X mainly upregulated the expression of β-catenin through the protein level but also had a partial effect on the mRNA level. Overexpression of USP9X can effectively enhance IC50 of HNE1 cells compared with

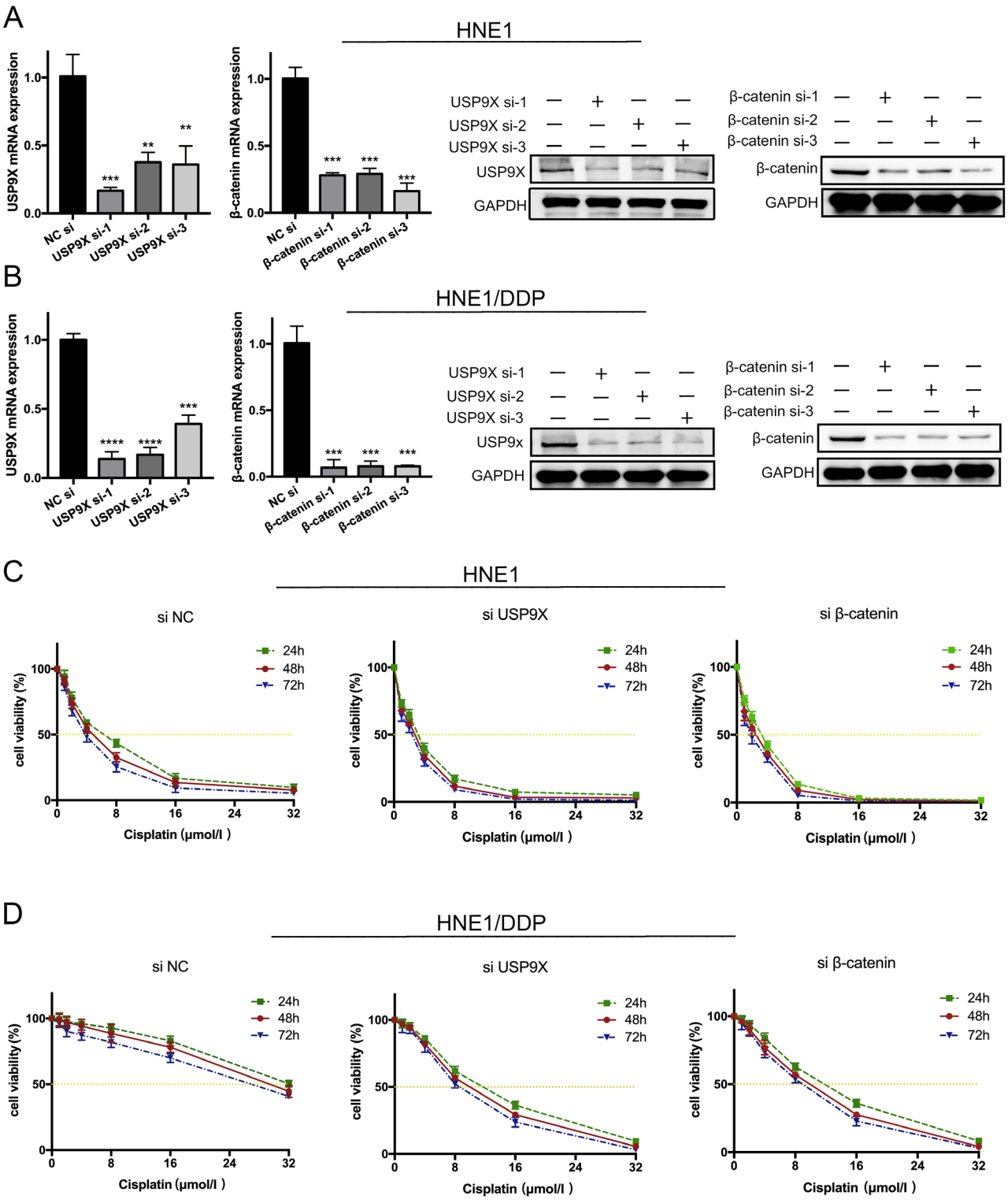

Figure 2. Knockdown of USP9X and β-catenin was able to partially reverse cisplatin resistance. A, B) qRT-PCR analysis of USP9X and β-catenin mRNA expressions after Negative Control (NC), USP9X, or β-catenin siRNAs were transfected into HNE1 and HNE1/DDP cells, respectively for 48 h. Western blot analysis of USP9X and β-catenin protein expressions after USP9X or β-catenin siRNAs were transfected into HNE1 and HNE1/DDP cells for 72 h respectively. **p<0.01, ***p<0.001, ****p<0.0001 compared to NC. The mRNA and protein expressions were normalized by GAPDH. C, D) CCK-8 was used to determine cell viability after NC, USP9X, or β-catenin siRNAs were transfected into HNE1 and HNE1/DDP cells respectively. USP9X or β-catenin knockdown led to increased inhibition of cell viability after cisplatin administration.

NC (12.62 vs. 6.11 μmol/l for 24 h, 9.92 vs. 4.77 μmol/l for 48 h, 7.82 vs. 3.96 μmol/l for 72 h) (Figure 3C), and IC50 of HNE1/DDP cells compared with NC (54.4 vs. 31.08 μmol/l for 24 h, 48.88 vs. 27.45 μmol/l for 48 h, 43.09 vs. 19.98 μmol/l for 72 h) (Figure 3D) as well. These results indicated that USP9X and β-catenin occupied a vital position in the modulation of cisplatin resistance.

USP9X interacts with β-catenin and is significantly correlated with MDR1 and MRP2. In our previous work, we demonstrated that USP9X enhances the stability of β-catenin and counteracts its ubiquitination [20]. To further determine the endogenous interaction between USP9X and β-catenin in NPC cells, HNE1 and HNE1/DDP cells were subjected to the co-IP assay using either an anti-USP9X or

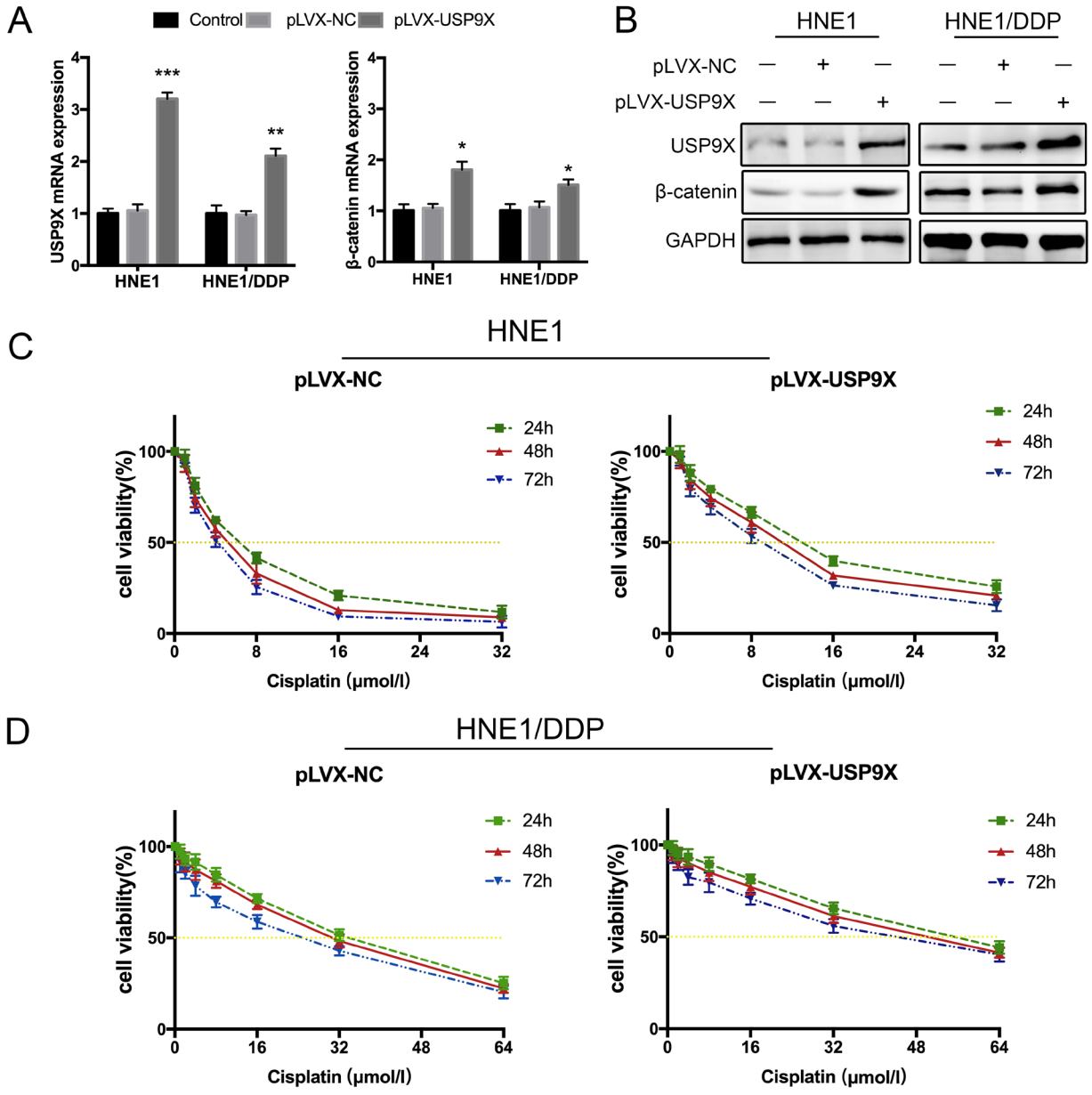

Figure 3. Overexpression of USP9X can increase cisplatin resistance in NPC cells. A, B) qRT-PCR analysis of USP9X and β-catenin mRNA expressions after pLVX-NC and pLVX-USP9X vectors were transfected into HNE1 and HNE1/DDP cells. Western blot analysis of USP9X and β-catenin protein expressions after pLVX-NC and pLVX-USP9X were transfected into HNE1 and HNE1/DDP cells. **p<0.01, ***p<0.001 compared to NC. The mRNA and protein expressions were normalized by GAPDH. C, D) CCK-8 was used to determine cell viability after pLVX-NC and pLVX-USP9X vectors were transfected into HNE1 and HNE1/DDP cells, respectively. USP9X overexpression resulted in reduced cell viability inhibition after cisplatin administration.

an anti-β-catenin antibody. As shown in Figure 3A, USP9X was immunoprecipitated with β-catenin in the HNE1 and HNE1/DDP cell lines. qRT-PCR (Figure 4B) and western blot (Figure 4D) results revealed that knockdown of USP9X was able to partially reduce β-catenin mRNA and protein levels in HNE1 and HNE1/DDP cells. Similarly, knockdown of β-catenin was able to partially reduce USP9X mRNA and protein levels. To further investigate whether the expression of USP9X was associated with multidrug resistance-associated proteins, levels of MDR1 and MRP2 were analyzed using qRT-PCR and western blot (Figures 4C, 4D). Results showed that USP9X depletion significantly reduced MDR1 and MRP2 protein levels but did not affect their mRNA expressions. However, β-catenin depletion significantly reduced

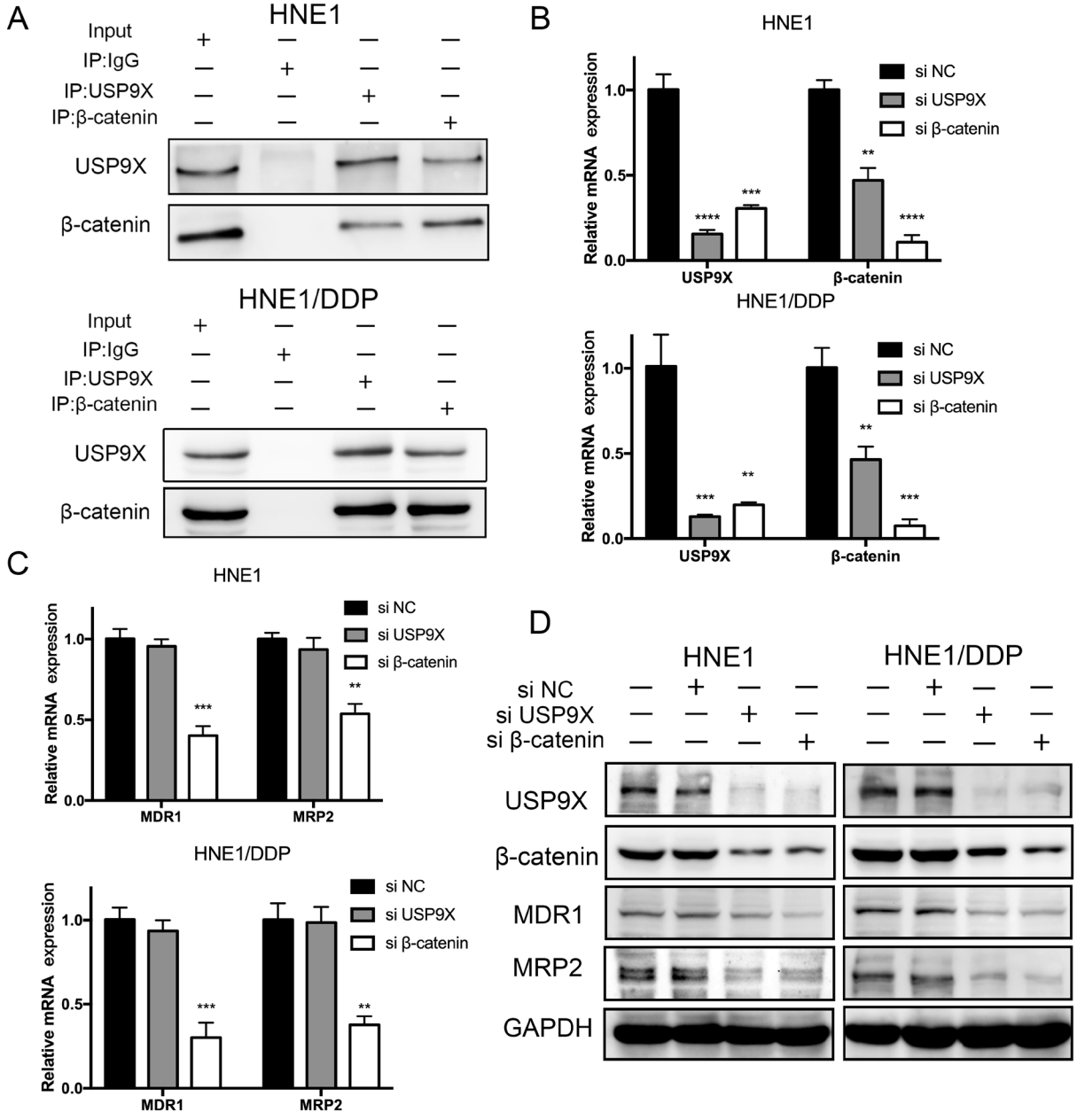

Figure 4. USP9X interacts with β-catenin and is significantly correlated with MDR1 and MRP2. A) Lysates from HNE1 or HNE1/DDP cells were immunoprecipitated with control IgG, anti-USP9X, or anti-β-catenin antibody, followed by western blot analysis with the indicated antibodies. B, C) qRT-PCR analysis of USP9X, β-catenin, MDR1, and MRP2 mRNA expressions after NC, USP9X, or β-catenin siRNAs were transfected into HNE1 and HNE1/DDP cells, respectively for 48 h. **p<0.01, ***p<0.001, ****p<0.0001 compared to NC. D) Western blot analysis of USP9X, β-catenin, MDR1, and MRP2 protein expressions after NC, USP9X, or β-catenin siRNAs were transfected into HNE1 and HNE1/DDP cells for 72 h respectively. The mRNA and protein expressions were normalized by GAPDH.

both protein and mRNA expressions. Together, these results suggest that USP9X interacts with β-catenin, and is significantly correlated with MDR1 and MRP2.

Knockdown of USP9X or β-catenin decreased cell proliferation and increased cisplatin-induced apoptosis in NPC cells. To confirm that USP9X or β-catenin decreases cell proliferation and enhances cisplatin-mediated apoptosis, CCK-8 and flow cytometry were performed to detect cell proliferation and apoptosis in NPC cells treated with DMEM medium (as a blank control group), NC siRNA, USP9X siRNA, or β-catenin siRNA. In the absence of cisplatin treatment, USP9X or β-catenin knockdown significantly reduced cell proliferation (Figures 5A, 5B). Moreover, HNE1/DDP cells showed more dependence on USP9X or beta-catenin than parental HNE1 cells. According to IC50 measured in previous experiments, HNE1 and HNE1/DDP cells were both exposed to a complete medium with 6 μmol/l of cisplatin for 48 h. The results revealed that total apoptotic rates in the HNE1 control group (24.16±1.05%) were higher compared with the HNE1/DDP control group (8.95±1.27%) (Figures 5C, 5D). Furthermore, knockdown the expression of USP9X and β-catenin was able to increase the apoptosis rate of the HNE1 and HNE1/DDP cells compared with the si-NC group (38.81±1.47%, 38.55±0.93% vs. 28.44±0.79% in HNE1, $^{**}p<0.01$, $^{***}p<0.001$; 14.57±0.92%, 15.53±0.97% vs. 9.8±0.94% in HNE1/DDP, $^{**}p<0.01$, $^{***}p<0.001$). Simultaneously, levels of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax were analyzed using qRT-PCR and western blot (Figures 5E, 5F). Deletion of USP9X did not affect the mRNA expressions of Bcl-2 and Bax, while deletion of β-catenin did. In both HNE1 and HNE1/DDP cells, the protein levels of Bcl-2 were lower in the USP9X siRNA and β-catenin siRNA groups compared with the control groups. Additionally, the protein levels of Bax were higher. Moreover, the Bcl-2/Bax ratio was lower compared with the control. These results suggest that knockdown of USP9X and β-catenin sensitizes HNE1 and HNE1/DDP cells to cisplatin might be restraining cell proliferation and promoting apoptosis.

Knockdown of USP9X and β-catenin decreased the migration and invasion capacities in HNE1 and HNE1/DDP cells. Since chemoresistance of tumor cells generally correlated with an increased migration and invasion capacities, we then measured these capacities in HNE1 and HNE1/DDP cells by wound-healing and Transwell Boyden chamber assays (Figures 6A, 6B). Furthermore, we divided them into four groups as before (control, si-NC, si-USP9X, and si-β-catenin groups). The obtained data showed that HNE1 and HNE1/DDP cells transfected with USP9X siRNA and β-catenin siRNA exhibited weaker migratory and invasive abilities than the control cells (Figures 6C, 6D). Since the matrix metalloproteinases (MMPs) family plays essential roles in tumor invasion and metastasis, we also examined whether USP9X and β-catenin knockdown affected the expressions of MMP2 and MMP9 (Figures 6E, 6F). Results of qRT-PCR indicated that USP9X depletion did not affect the mRNA expressions of MMP2 and MMP9, while β-catenin depletion reduced its mRNA expressions. Results of western blot indicated that the protein levels of MMP2 and MMP9 were dramatically reduced when USP9X and β-catenin were knocked down compared with the control groups. These data suggested that USP9X and β-catenin contributed to NPC cells' migration and invasion.

Discussion

With the progress of imaging diagnosis technology, intensity-modulated radiotherapy technology, the application of chemotherapy, and targeted therapy, NPC's therapeutic effect has been significantly improved. Compared with radiotherapy alone, concurrent chemoradiotherapy can improve OS in patients with locally recurrent NPC [25]. In the past decades, PF regimen (5-fluorouracil + cisplatin) has been the first-line treatment for recurrent and metastatic NPC. In recent years, studies have found that GP regimen (gemcitabine + cisplatin) is superior to PF regimen [26]. Hence, cisplatin resistance and metastasis become the main reason for the failure of the treatment of NPC.

The deubiquitination and stabilization of USP9X on different substrates lead to different clinicopathological and prognostic significance [27, 28]. Recently, multiple studies have reported that overexpression of USP9X can make cancer cells resistant to cisplatin. For example, USP9X stabilizes PBX1, a prostate cancer cell, and inhibiting USP9X reduces chemotherapeutic resistance [29]. The loss of USP9X increases the polyubiquitination of YAP1 in breast cancer cells, thereby increasing the conversion rate of YAP1 and its cell sensitivity to chemotherapy [30]. USP9X knockdown eliminated the synergistic effect of WP1130 (a selective USP9X inhibitor) plus cisplatin in NSCLC cells [31]. However, the role of USP9X in NPC cells has not been reported. In our previous work, we demonstrated that USP9X enhances the stability of β-catenin and counteracts its ubiquitination in glioma cells [20]. A recent study showed an increase in nuclear β-catenin in CNE2/DDP and 5-8F/DDP cells (two cisplatin-resistant NPC cells) [16]. Therefore, we hypothesized that USP9X plays an oncogenic role in NPC cells. In the present study, the levels of USP9X and β-catenin were significantly higher in cisplatin-resistant HNE1/DDP cells compared with the parental HNE1 cells, which indicated that the overexpression of USP9X and β-catenin reduced the cisplatin sensitivity of NPC cells. Conversely, we used the siRNAs transfection technique to verify further the decreased expressions of USP9X and β-catenin in NPC cells suppressed cell proliferation and making the cells more sensitive to cisplatin. Overexpression of USP9X can increase cisplatin resistance in NPC cells. However, the molecular mechanisms of cisplatin resistance and the relationship with abnormal expression of USP9X in NPC have not been clearly defined.

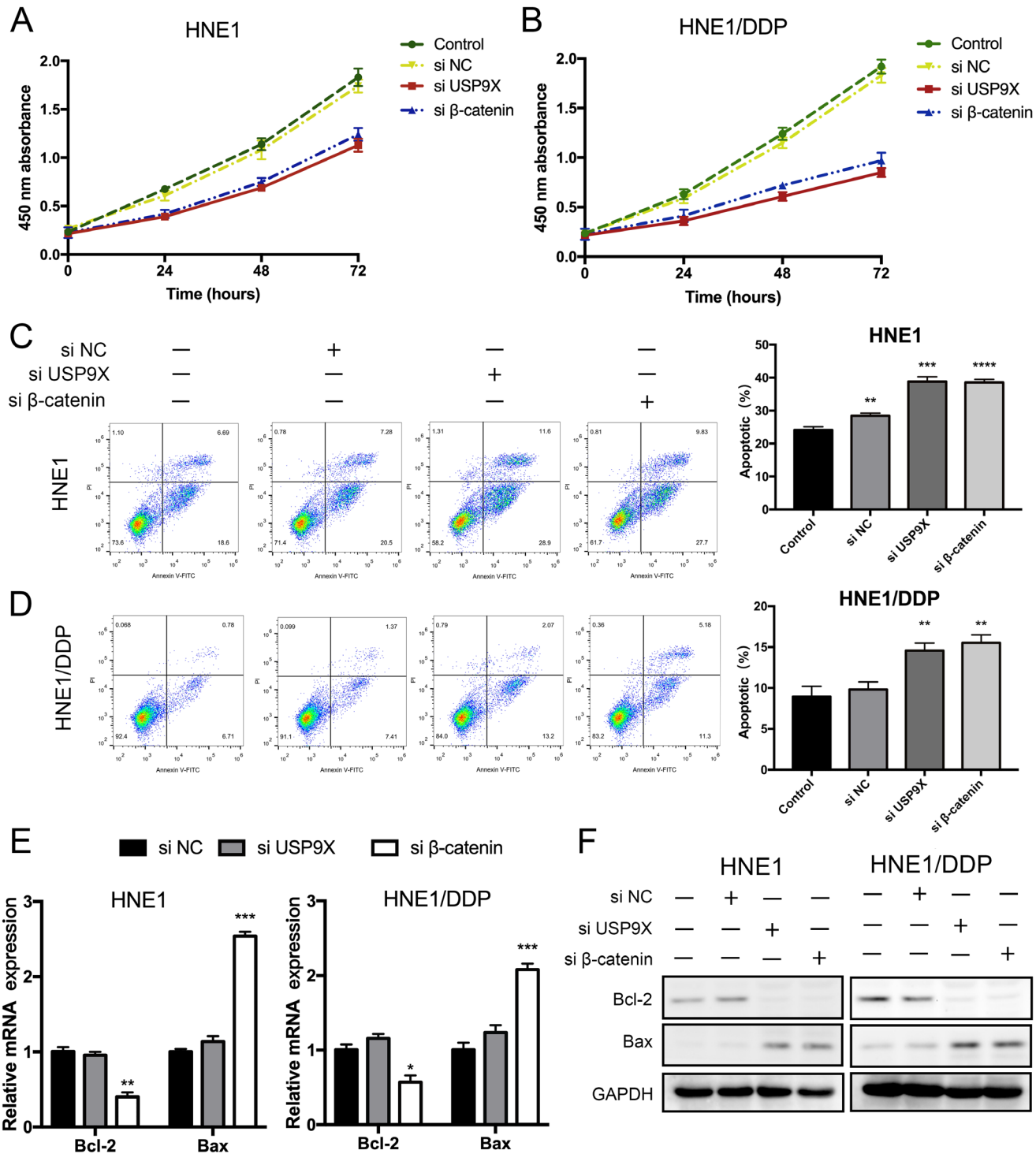

Figure 5. Knockdown of USP9X or β-catenin decreased cell proliferation and increased cisplatin-induced apoptosis in NPC cells. A, B) The proliferation of HNE1 and HNE1/DDP cells was detected by the CCK-8 assay. C, D) Apoptotic HNE1 and HNE1/DDP cells were determined with flow cytometry after transfected with NC, USP9X, or β-catenin siRNAs for 72 h, respectively. USP9X or β-catenin knockdown increased the apoptosis rate of HNE1 and HNE1/DDP cells. **p<0.01, ***p<0.001, ****p<0.0001 compared to control. E) qRT-PCR analysis of Bcl-2 and Bax mRNA expressions after NC, USP9X, or β-catenin siRNAs were transfected into HNE1 and HNE1/DDP cells for 48 h, respectively. **p<0.01, ***p<0.001. F) Western blot analysis of Bcl-2 and Bax protein expressions after NC, USP9X, or β-catenin siRNAs were transfected into HNE1 or HNE1/DDP cells for 72 h, respectively. The protein expression was normalized by GAPDH.

Wnt/β-catenin signaling pathway has been shown to be involved in cisplatin resistance through the regulation of β-catenin. Resistance to cisplatin is attributed to three molecular mechanisms: increased DNA repair, altered cellular accumulation, and increased drug inactivation [10]. Several mechanisms are thought to mediate the resistance of cancer cells to chemotherapy drugs. One of the most studied mechanisms is the high expression of the multidrug resistance-1 (MDR1) gene and the P-glycoprotein (P-GP) transporter encoded by MDR1 [32]. MRP2 is also an ATP Binding Box (ABC) transporter and belongs to the multi-drug resistant (MRP) family [33], which is overexpressed in many cisplatin-resistant cell lines [34]. In the current study, co-IP results showed that USP9X co-precipitation with β-catenin in NPC cells. qRT-PCR and western blot results also showed that the expression of USP9X was positively correlated with β-catenin. Deletion of USP9X did not affect the mRNA expressions of MDR1 and MRP2, while deletion of β-catenin did. Moreover, MDR1 and MRP2 protein levels were significantly reduced after the knockdown of USP9X and β-catenin. These results suggest that USP9X interacts with β-catenin in NPC cells and affects cisplatin resistance by regulating the expressions of MDR1 and MRP2, which are related to drug resistance.

Upregulation of Bax protein expression or downregulation of Bcl-2 protein expression leads to an increase in the ratio of Bax to Bcl-2 and promotes the occurrence of apoptosis [35]. The regulation of Bcl-2 and Bax expression is a complex network system, among which β-catenin is one of the important regulatory factors [36]. MMP2 and MMP9 are traditional prognostic indicators, and their elevated expression is associated with breast cancer cell metastasis [37, 38]. There is evidence that the sequential formation of β-catenin and TCF/β-catenin complexes transferred to the nucleus induces the expression of MMP2 and MMP9 in cancer cells [39, 40]. In the present study, we used flow cytometry and Transwell chamber assay, whether knockdown of USP9X expression on apoptosis and the influence of the migration and invasion. The results showed that knockdown of USP9X or β-catenin obviously induced apoptosis, and significantly reduced the migration and invasive ability of NPC cells, suggesting that USP9X may play an essential role in apoptosis, migration, and invasion of NPC cells. On this basis, we further analyzed the expressions of apoptosis-related proteins Bax and Bcl-2 involved in invasion-related proteins MMP2 and MMP9 after different treatment of NPC cells. We found that knockdown of USP9X can significantly reduce the protein levels of Bcl-2, MMP2, and MMP9 but significantly increased the expression of Bax. Interestingly, knockdown of USP9X had a similar effect on the levels of these proteins as β-catenin. However, deletion of USP9X did not affect the mRNA expressions of Bcl-2, Bax, MMP2, and MMP9, while deletion of β-catenin did. Therefore, it is reasonable to think that knockdown of USP9X induces apoptosis and reduces the migration and invasive ability by decreasing β-catenin expression in NPC cells.

In addition, evidence suggests that Noxa promotes Mcl-1 ubiquitination by reducing USP9X availability to Mcl-1 [41]. USP9X is downregulated in prostate cancer, and may inhibit tumor cell invasion and migration by regulating ERK/MMP9 and ERK/DRP1 signaling pathways [42]. Besides, USP9X is a new binding partner for BRCA1 and stabilizes BRCA1. The knockout of USP9X enhances human cancer cells' sensitivity to PARP inhibitor Olaparib and MMS [43]. There was a study showing that the USP4 suppressed the MyoD activity in a catalytic activity independent manner [44]. Moreover, overexpression of USP9X promotes nuclear translocation of NF-κBp65, while NF-κB inactivation by the well-known NF-κB inhibitor PDTC, in turn, inhibits USP9X transcription [45]. In most biological processes, crosstalk of Wnt/β-catenin and NF-κB signaling is bidirectional [46]. The above shows USP9X can act on a variety of proteins through ubiquitination pathway or noncanonical, deubiquitination-independent mechanism. Therefore, we have reason to believe that in addition to the deubiquitination of β-catenin in NPC cells, USP9X may indirectly act on β-catenin through other pathways (such as DNA damage repair) or other proteins, to reduce the expression of β-catenin and affect downstream target genes. The specific mechanism remains to be further studied. These studies indicate that further elucidating its detailed molecular regulatory mechanism in the future is expected to provide a theoretical basis for molecular targeted therapy of cancer-targeting USP9X.

In summary, our study identifies that the expression level of USP9X was higher in the cisplatin-resistant HNE1/DDP cell line compared with the parental HNE1 cell line. Due to the interaction between USP9X and β-catenin in NPC cells, the knockdown of USP9X helped partially reverse cisplatin resistance. Moreover, knockdown of USP9X increased cisplatin-induced apoptosis and decreased the cell proliferation, migration, and invasion capacities. These results may provide clues for the therapeutic feasibility of USP9X in NPC chemoresistance and metastasis.

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