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Original Article

MARK2 enhances cisplatin resistance via PI3K/AKT/NF-κB signaling pathway in osteosarcoma cells

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Abstract: Osteosarcoma is the most common primary bone malignancy found in children and young adults. Chemotherapy resistance, especially to cisplatin, presents a major clinical challenge in the treatment and prognosis of osteosarcoma. New biomarkers and mechanisms of cisplatin resistance in osteosarcoma are urgently needed due to poor survival outcomes and currently inadequate treatments. In this study, we investigate the role and potential mechanisms of microtubule-affinity regulating kinase2 (MARK2) during osteosarcoma cisplatin resistance. Gene Expression Omnibus dataset analyses indicated that high MARK2 expression was associated with poor prognosis and may positively correlate with chemoresistance. Moreover, we showed that MARK2 was significantly upregulated in osteosarcoma cells compared with normal cells. The overexpression and inhibition of MARK2 promoted and suppressed, respectively, cisplatin resistance in osteosarcoma cells *in vitro* and *in vivo*. Mechanistically, MARK2 overexpression enhanced P-glycoprotein expression and decreased cell apoptosis through PI3K/AKT/NF-κB signaling pathway activation, resulting in cisplatin resistance. Our results suggest that high MARK2 expression can enhance cisplatin resistance in osteosarcoma cells, supporting the potential of MARK2 as a new therapeutic target and biomarker for predicting cisplatin resistance in osteosarcoma.

Keywords: Osteosarcoma, cisplatin resistance, MARK2, P-glycoprotein, cell apoptosis, PI3K/AKT/NF-κB

Introduction

Osteosarcoma is the most common primary bone malignancy in children and young adults [1]. Standard therapy typically consists of surgery with neoadjuvant and adjuvant chemotherapy, with a 5-year survival rate of about 70% in non-metastatic disease patients [2]. Unfortunately, the survival rate is severely reduced in patients with metastatic disease who respond poorly to initial treatment, with a dismal < 25% 5-year survival rate [3]. Cisplatin-based chemotherapy is the standard regimen for osteosarcoma [4]. However, chemotherapy resistance, especially to cisplatin, is a major challenge in osteosarcoma recurrence and prognosis [5]. Many mechanisms can lead to chemoresistance, including reduced drug influx, increased drug efflux, detoxification system activation, drug target alterations, enhanced DNA repair, impaired apoptosis, and altered oncogene expression [6]. As such, there is an urgent need to identify novel biomarkers that can inform on chemotherapy resistance and prognosis.

Microtubule-affinity regulating kinase 2 (MARK2), a member of the MARK family, is a serine/threonine protein kinase that plays an important role in neurodifferentiation, neurodegeneration, cell polarization, intracellular transport, and cell migration [7]. Recent studies have found that MARK2 is closely associated with malignant biological behavior and drug resistance in tumors, and MARK2 overexpression was observed in cisplatin-resistant cancer cell lines. In cervical cancer, cisplatin sensitivity increased after MARK2 gene silencing; however, there was no significant change in sensitivity to vincristine and paclitaxel [6]. Although MARK2 is connected to the DNA damage response and cisplatin resistance in non-small cell lung cancer [8], the role and mechanism of MARK2 in osteosarcoma chemoresistance remain unclear.

Increased drug efflux and inhibition of apoptosis are two important drivers of chemoresistance [9]. P-glycoprotein (P-gp), a transmembrane glycoprotein, is an energy-dependent drug efflux pump that reduces the intracellular concentration of anticancer drugs [10]. Increasing evidence indicates that P-gp overexpression plays an important role in multidrug resistance of tumors [11, 12]. Additionally, studies showed that high P-gp expression was involved in chemoresistance and associated with poor prognosis in osteosarcoma [10, 13]. Apoptosis is the main mode of chemotherapy-induced cell death, with reports demonstrating that the expression of apoptosis-related genes, including p53, BCL-2, and BAX, are associated with cellular chemoresistance in osteosarcoma [14-17]. Previous studies have shown that in many tumors, P-gp-related chemoresistance is regulated by the PI3K/AKT/NF-κB pathway [18-21] and the AKT/NF-κB pathway is involved in regulating osteosarcoma drug resistance [22]. However, whether MARK2 promotes osteosarcoma cisplatin resistance via regulating P-gp expression and cell apoptosis through the PI3K/AKT/NF-κB signaling pathway is yet to be determined.

In this study, we report on the role and mechanism of MARK2-mediated cisplatin resistance in osteosarcoma. We found that MARK2 expression was significantly upregulated and associated with chemoresistance in osteosarcoma cells. Furthermore, we showed that MARK2 can enhance osteosarcoma cisplatin resistance by regulating P-gp expression and cell apoptosis through the PI3K/AKT/NF-κB signaling pathway both *in vitro* and *in vivo*. Our results suggest that MARK2 could be a novel biomarker for predicting cisplatin resistance in osteosarcoma and a new potential therapeutic target.

Materials and methods

Analysis of public datasets

Gene expression data pertaining to 53 cases of osteosarcoma were obtained from the Gene Expression Omnibus (GEO) database using accession number GSE21157. The corresponding clinical information is publicly available. To determine the overall survival of patients, relative differences in MARK2 expression in the 53 patients were analyzed using X-tile software to obtain thresholds yielding the most significant log-rank test *P* values that enabled the data to be grouped as MARK2-Low and MARK2-High groups. Differences in survival between low and high expression groups were evaluated using the Kaplan-Meier curve. According to the tumor scoring system defined by Huvos [23], those with >90% tumor necrosis and consistent with Huvos grades III and IV were considered to be in the good response group, while those with < 90% tumor necrosis and consistent with Huvos grades I and II were considered to be in the poor response group. Chemotherapy responses were compared between high and low MARK2 groups. Spearman's rank correlation analysis was used to determine the correlation between MARK2 and P-gp gene expression in osteosarcoma patients.

Cell culture

Human osteosarcoma cells, including Saos-2, MG-63, and U-2 OS, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human osteoblast and osteosarcoma cell lines, hFOB1.19 and MNNG/HOS, respectively, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MG-63, MNNG/HOS, and hFOB1.19 cells were cultured in high glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA), U-2 OS cells in RPMI 1640 medium (Gibco), and Saos-2 cells in McCoy's 5A Modified Medium (Gibco). All media were supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Solarbio, Beijing, China). Cells were maintained at 37°C in a 5% CO_2 atmosphere.

Cell transfection

MG-63 and MNNG/HOS cells were transiently transfected with small interfering RNA (siRNA; Genepharma, Shanghai, China) using Endo-Fectin (GeneCopoeia, Rockville, MD, USA) according to manufacturer's instructions. The target sequences of the siRNAs used in this study were: MARK2 siRNA target sequence: GAGGCACUUUAGCAAAUTT, negative control siRNA: UUCUCCGAACGUGUCACGUTT. After 48 h, knockdown efficiency was analyzed by western blotting before cells were further analyzed. For stable transfection, the overexpression or knockdown of MARK2 was achieved using either MARK2 or MARK2 short hairpin RNA (shMARK2) lentiviral particles, respectively. Vector and non-targeting short hairpin RNA (shcontrol) len-

tiviral particles were used as control (Genepharma, Shanghai, China). Following selection with puromycin (2 μg/mL), stably transfected cells were used for subsequent experiments.

Cell viability

The Cell Counting Kit-8 (CCK-8) assay was used to evaluate cell viability and calculate the 50% inhibitory concentration (IC_{50}). IC_{50} values following cisplatin treatment are generally used to determine resistance. MG-63 and MNNG/HOS cells, with or without P-gp inhibitor dofequidar fumarate treatment (5 μM, MedChemExpress, Shanghai, China) and stably transfected osteosarcoma cell lines (vector, MARK2, shcontrol, or shMARK2) were seeded at 1×10^4 cells/well in 96-well plates. After overnight incubation, cells were treated with cisplatin (Qilu Pharmaceutical Co., Ltd., Shandong, China) at the indicated concentrations (0, 5, 10, 20, 40, and 80 μM). After 24 h (for MNNG/HOS cells) or 48 h (for MG-63 cells), CCK-8 (BestBio, Shanghai, China) was added according to manufacturer's instructions, and cells were incubated for an additional 2 h in a humidified incubator. The OD 450 nm value was measured using a SpectraMax Plus 384 Absorbance Microplate Reader (Molecular Devices, USA). Cell viability for all other groups was calculated separately and compared with untreated cells. Each experiment was independently repeated three times.

Western blot analysis

Proteins were extracted from tissue samples and cultured cells using RIPA lysis buffer with 1% phenylmethylsulfonyl fluoride and quantified using a BCA kit (both from Beyotime, Shanghai, China). Equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk (or bovine serum albumin for phosphorylation-specific antibodies) in tris-buffered saline with Tween-20 (TBST) at room temperature for 1 h. Membranes were probed overnight with primary antibodies at 4°C. After washing three times with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Zhong-shan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 2 h. Signals were visualized with enhanced chemiluminescence reagent (Millipore) for 1 min and detected using the Amersham Imager 600 (GE, Fairfield, CT, USA). Each sample was examined three times, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous protein for normalization. Results were analyzed using ImageJ software. Primary antibodies used in the study included: P-gp (1:1,000, ab129450; Abcam, Cambridge, MA, USA); MARK2 (1:1,000, ab133724; Abcam); p-PI3K p85 (1:1,000, 4228; Cell Signaling Technology (CST), Danvers, MA, USA); total PI3K p85 (1:1,000, ab40755; Abcam); p-AKT (S473) (1:1,000, 9271S; CST); total AKT (1:1,000, 9272S; CST); p-NF-κB/p65 (1:1,000, 3033S; CST); total NF-κB/p65 (1:1,000, 8242; CST); p-IκB-α (1:1,000, 9246S; CST); total IκB-α (1:750, AF5002; Affinity Biosciences, Cincinnati, OH, USA); BCL-2 (1:1,000, 2870S; CST); BAX (1:750, AF0120; Affinity Biosciences); caspase 3 (1:750, AF6311; Affinity Biosciences); cleaved caspase 3 (1:750, AF7022; Affinity Biosciences); and GAPDH (1:5,000, AF6311; Affinity Biosciences).

Immunofluorescence

Osteosarcoma cells with MARK2 overexpressed or knocked down were seeded on coverslips and treated with 20 μM cisplatin. The following day, cells were fixed in 4% paraformaldehyde for 15 min. Following three washes with phosphate-buffered saline (PBS), cells were permeabilized in 0.4% Triton X-100 for 20 min at room temperature and incubated with 10% normal goat serum for 30 min. Cells were then incubated with a MARK2 (1:250, DF3329; Affinity Biosciences) or P-gp (1:200, ab129450; Abcam) antibody overnight at 4°C. The next day, cells were incubated with CoraLite594-conjugated goat anti-rabbit IgG (H+L) (1:250, Proteintech, China) for 1 h at room temperature. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 10 min. Stained coverslips were mounted on standard glass slides and examined using an inverted fluorescence microscope (BX53F; Olympus, Tokyo, Japan).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using the RNAfast200 assay kit (*Fastagen* Biotech, Shanghai, China) and quantified using a Nanodrop One C (Thermo Fisher Scientific, Wa-

ltham, MA, USA) according to manufacturer's instructions. Next, the first strand of cDNA was reverse transcribed from total RNA using HIScript II Q RT Supermix for qPCR (Vazyme, Nanjing, China) following manufacturer's protocol. The reaction was performed in a T-Gradient Thermoblock (Biometra GmbH, Gottingen, Germany) with a temperature cycling program of 15 min at 50°C, and then 5 s at 85°C. The resulting cDNAs were used as templates for PCR amplification using ChamQ SYBR Color qPCR Master Mix (Vazyme, Nanjing, China) in a LightCycler 480 II (Roche, Mannheim, Germany) according to manufacturer's instructions. The amplification protocol for all primer sets involved pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. Quantification of the relative expression of the target gene was performed using the comparative cycle threshold (CT) method and calculated by the $2^{-\Delta\Delta CT}$ method. All experiments were performed in triplicate, and GAPDH was used as an internal control. The primer sequences designed and synthesized by Sangon Biotech (Shanghai, China) were: MARK2 forward primer, 5'-AACTGAACTCCTCCAGCCTCCAG-3' and reverse primer, 5'-GCCACTAGCGTACTCCATGACAAG-3; P-gp forward primer, 5'-ACAGAGGGGATGGTCAGTGT-3' and reverse primer, 5'-TCACGGCCATAGCGAATGTT-3; GAPDH forward primer, 5'-CAGGAGGCATTGCTGATGAT-3' and reverse primer, 5'-GAAGGCTGGGGCTCATTT-3.

Cell apoptosis

Cellular apoptosis was evaluated by double staining with Annexin V-APC and 7-AAD. Cells were pretreated with cisplatin for 24 or 48 h, then harvested, washed twice with PBS, resuspended, and stained with an Annexin V-APC and 7-AAD apoptosis kit (BioGems, Westlake Village, CA, USA) according to manufacturer's instructions. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo version 10 software (TreeStar, Ashland, OR, USA). Annexin V(+)/7-AAD(-) and Annexin V(+)/7-AAD(+) represented cells in early apoptosis and late apoptosis, respectively. Experiments were repeated at least thrice.

Tumor xenografts in nude mice

Female Balb/c nude mice (4 weeks old, 10-14 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were approved by the ethical committees of Qilu Hospital, Shandong University (Jinan, China). Mice were maintained in a pathogen-free facility and handled in accordance with standard-use protocols and animal welfare regulations. Mice were randomly divided into eight groups (n = 6 per group). MNNG/HOS cells with stable MARK2 knockdown or overexpression or the respective control cells were harvested and resuspended in PBS. Then, cells (1×10^7 in 0.2 ml PBS) were subcutaneously injected into the right flank of mice as indicated. Tumor size was measured using a caliper every 3 days and volume was calculated using the formula: V = (length × $width^2$)/2. Ten days after injection with the indicated cells, mice were treated with cisplatin (3 mg/kg) or normal saline (NS) intraperitoneally every 3 days. To exclude proliferation changes due to MARK2 protein levels, the cisplatin-induced tumor reduction rate was calculated based on tumor volume in a NS-treated group, using the following formula: reduction rate (%) = (1 -V cisplatin/V NS) × 100%, where V cisplatin is the average tumor volume in the cisplatin-treated group, and V NS is the average tumor volume in the NS-treated group when mice were sacrificed. Mice were sacrificed on day 31, and tumor weight and volume were measured. Following excision, tumors were fixed with formaldehyde or preserved at -80°C for subsequent experiments.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded sections of 4-μm thickness were prepared on glass slides and subjected to IHC analysis using a streptavidin peroxidase IHC assay kit (ZSGB-bio, Beijing, China) according to manufacturer's protocol. Slides were heated at 65°C for 40 min, followed by dewaxing and rehydration. Antigen was retrieved in sodium citrate repair solution (pH 6.0) maintained at a sub-boiling temperature for 13 min. After treating with 3% hydrogen peroxide, sections were blocked with normal goat serum for 30 min at 37°C and incubated with primary antibody against MARK2 (1:100, ET7109-20; HuaBio, Hangzhou, China), P-gp (1:100, AF5185; Affinity Biosciences), and cleaved caspase 3 (1:200, AF7022; Affinity Biosciences) overnight at 4°C. The next day, sections were incubated with biotinylated secondary antibodies followed by 3,3'-Diaminobenzidine (ZSGB-bio, Beijing, China) staining.

IHC staining was analyzed using a microscope (BX53F; Olympus, Tokyo, Japan) , and scored for both the intensity and percentage of positive staining.

Rescue experiments

MARK2 overexpressing cells, including MG63 and MNNG/HOS, were treated with the PI3K inhibitor LY294002 (20 μM; Selleckchem, Houston, TX, USA) for 24 h. Assays for CCK8 cell viability, apoptosis, and western blot analysis were performed.

Statistical analysis

Continuous variables are reported as mean ± standard deviation (SD), and categorical variables are reported in frequency or percentage. χ^2 test was used to analyze statistical differences for categorical data. Mean values between the two groups were compared using Student's t-test. Correlation analyses were performed using Pearson's correlation tests. Overall survival curves were calculated using the Kaplan-Meier method, and differences were evaluated using the log-rank test. Hazard ratios and 95% confidence intervals (95% CI) for death were computed using Cox survival modeling. *P* values < 0.05 (two-sided) were considered statistically significant. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

MARK2 overexpression is correlated with chemoresistance and poor prognosis in osteosarcoma

Studies have reported that MARK2 promotes chemotherapy tolerance in a variety of cancers. However, the clinical correlation between MARK2 expression in osteosarcoma and chemoresistance remains unexplored. To evaluate the role of MARK2 in osteosarcoma chemoresistance, we first analyzed the microarray data of the GEO dataset (GSE21157). Based on the best cut-off values of MARK2 expression obtained using X-tile software, the samples (n = 53) were separated into MARK2-Low and MARK2-High groups. Kaplan-Meier analysis of overall survival showed that upregulation of MARK2 expression in osteosarcoma patients contributes to poor survival (**Figure 1A**). Our analysis also indicated a higher probability of chemoresistance in patients exhibiting high levels of MARK2 mRNA (**Figure 1B**). It was reported that P-gp overexpression is quite frequent in many tumors and is associated with drug resistance [24]. Our CCK-8 assay showed that the dofequidar fumarate treatment group had a lower cisplatin IC_{50} than the vehicle control group in MG-63 and MNNG/HOS cells, further demonstrating that P-gp inhibition can reduce osteosarcoma cisplatin resistance (**Figure 1F**). We also discovered a positive correlation between MARK2 and P-gp mRNA expression in osteosarcoma patients using Spearman's rank correlation analysis (**Figure 1C**). Consistent with these data, we found that both MARK2 and P-gp protein levels were upregulated in the osteosarcoma cell lines U-2 OS, MG-63, MNNG/HOS, and Saos-2 compared to the hFOB1.19 cell line in western blot assays (**Figure 1D**), although P-gp overexpression in U-2 OS and Saos-2 cells was not statistically significant. Therefore, MG-63 and MNNG/HOS cell lines were chosen for further assays. To further explore the relationship between MARK2 and P-gp in osteosarcoma cells, we generated MARK2 knockdown cells for both MG-63 and MNNG/HOS cell lines using siRNA transfection. P-gp expression decreased following MARK2 expression knockdown (**Figure 1E**). Taken together, these results demonstrate that MARK2 overexpression may promote chemoresistance and result in poor prognosis in osteosarcoma.

MARK2 promotes cisplatin resistance in MG-63 and MNNG/HOS cells

To further explore the effects of MARK2 on cisplatin resistance in MG-63 and MNNG/HOS cells, MARK2 knockdown or overexpression was conducted by vector, MARK2, shcontrol, or shMARK2 lentiviral particle transfection and verified by western blot and qRT-PCR assays (**Figure 2B**, **2C**). MG-63 and MNNG/HOS cells were exposed to a series of cisplatin concentrations (0, 5, 10, 20, 40, and 80 μM) for 48 and 24 h, respectively. IC_{50} values following cisplatin treatment were used to determine cisplatin resistance. As demonstrated by the CCK-8 assay (**Figure 2A**), cisplatin IC_{50} for the MARK2 group was significantly higher than the vector control group, and the shMARK2 group IC_{50} was lower than the shcontrol group. Therefore, MARK2 overexpression significantly enhanced

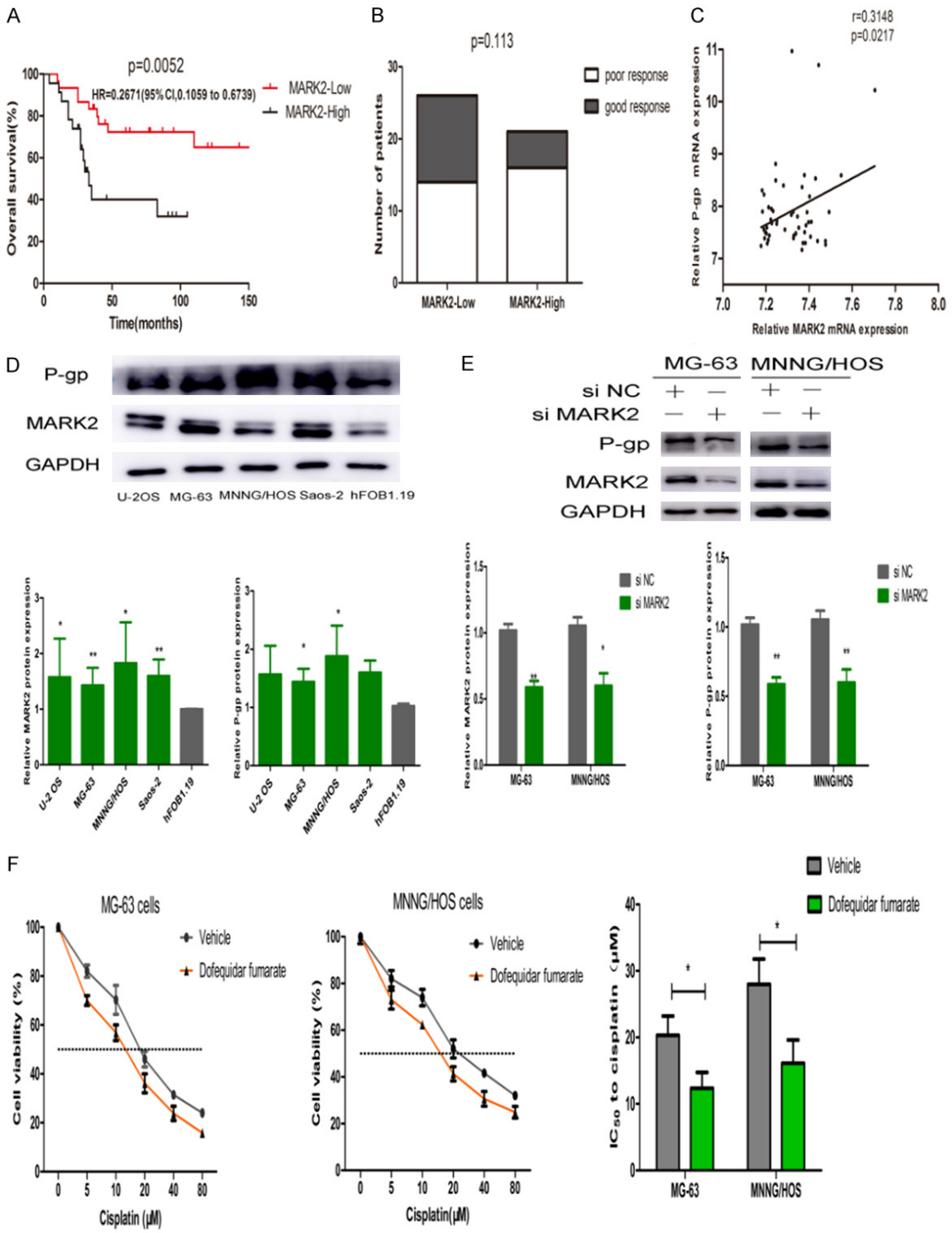


Figure 1. MARK2 overexpression is correlated with chemoresistance and poor prognosis in osteosarcoma. A. Kaplan-Meier analysis of overall survival shows that MARK2 overexpression in osteosarcoma patients contributes to poor survival. Samples (n = 53) using the GEO dataset (GSE21157) were divided into MARK2-Low and MARK2-High groups based on the best cut-off values of MARK2 expression obtained using X-tile software. B. The high probability of poor response to chemotherapy in osteosarcoma patients (from GEO dataset) with MARK2-high groups (Pearson's Chi-squared test). C. The positive correlation between MARK2 and P-gp mRNA expression in the above osteosarcoma patients was analyzed using Spearman's rank correlation analysis. D. Increased protein levels of MARK2 and P-gp in osteosarcoma cell lines U-2 OS, MG-63, MNNG/HOS, and Saos-2, were detected using western blotting, compared to the hFOB1.19 cell line. E. Following MARK2 knockdown in MG-63 and MNNG/HOS cells by

siRNA transfection, P-gp protein expression decreased according to western blot analysis. F. CCK-8 assay showing a significantly lower cisplatin IC_{50} for the P-gp inhibitor dofequidar fumarate (5 μM) treatment group compared with the vehicle control group in MG-63 and MNNG/HOS cells. Results represent the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01. MARK2, microtubule-affinity regulating kinase 2; OS, overall survival; GEO, Gene Expression Omnibus; P-gp, P-glycoprotein; siRNA, small interfering RNA; CCK-8, cell counting kits-8; IC_{50}, 50% inhibitory concentration; SD, standard deviation.

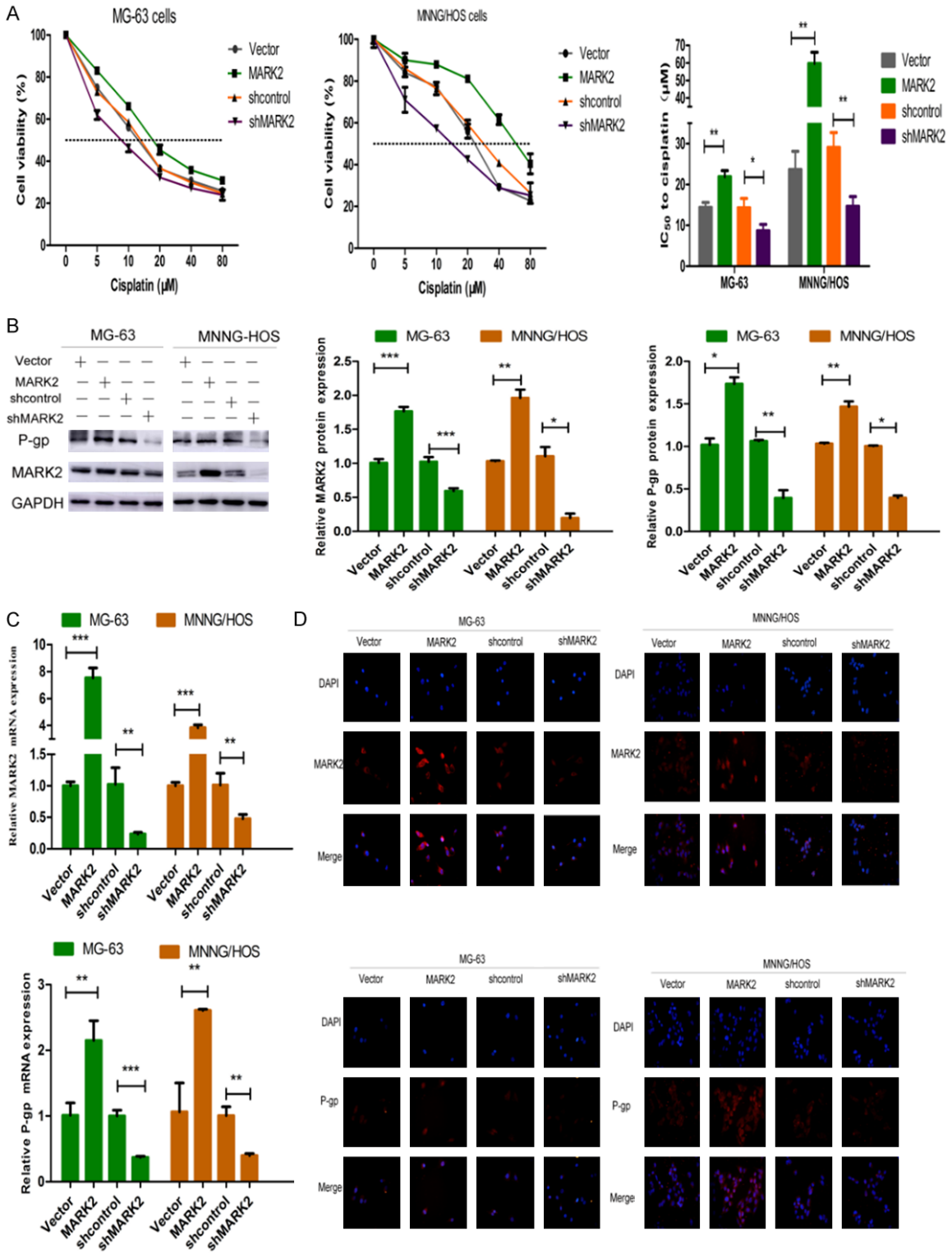


Figure 2. MARK2 promotes cisplatin resistance in MG-63 and MNNG/HOS cells. A. The indicated cells were transfected with vector, MARK2, shcontrol, or shMARK2 lentiviral particles. MG-63 and MNNG/HOS cells were then exposed to a series of cisplatin concentrations (0, 5, 10, 20, 40, and 80 μM) for 48 and 24 h, respectively. IC_{50} values following cisplatin treatment were used to determine cisplatin resistance. B. Protein levels of MARK2 and P-gp in the indicated cells treated with 20 μM cisplatin were detected by western blotting. C. mRNA expression of MARK2 and P-gp in the indicated cells treated with 20 μM cisplatin was determined using qRT-PCR. D. Immunofluorescence shows MARK2 and P-gp expression in the indicated cells treated with 20 μM cisplatin. For each group, representative images were randomly chosen using fluorescent microscopy with 200-fold magnification. All values are mean ± SD of three independent experiments; *P < 0.05, **P < 0.01, ***P < 0.001. shMARK2, MARK2 short hairpin RNAs; shcontrol, control short hairpin RNAs; DAPI, 4', 6-diamidino-2-phenylindole; qRT-PCR, quantitative real-time polymerase chain reaction.

cisplatin resistance in MG-63 and MNNG/HOS cells, while MARK2 knockdown remarkably reduced cisplatin resistance. Additionally, P-gp expression was significantly increased or decreased following the overexpression or knockdown, respectively, of MARK2, as demonstrated by western blot, qRT-PCR, and immunofluorescence (**Figure 2B-D**). These findings consistently support the notion that MARK2 promotes cisplatin resistance by regulating P-gp expression in MG-63 and MNNG/HOS cells.

MARK2 suppresses cisplatin-induced apoptosis in MG-63 and MNNG/HOS cells

Previous studies showed that the sensitivity of osteosarcoma cells to chemotherapy was associated with cell apoptosis [25]. We therefore sought to determine whether MARK2 expression could affect cisplatin resistance via apoptosis in osteosarcoma cells. Cells transfected with vector, MARK2, shcontrol, or shMARK2 lentiviral particles were incubated with 20 μM cisplatin for 24 or 48 h, then analyzed for apoptosis by flow cytometry. Both total and early apoptosis in MARK2-overexpressing cells were significantly lower than in negative control cells following cisplatin treatment (**Figure 3A**). Conversely, MARK2 knockdown cells displayed a higher apoptotic cell ratio than control cells. These results indicate that MARK2 is essential for MG-63 and MNNG/HOS cells during cisplatin treatment. We next studied the expression of apoptosis-related proteins in these cells (**Figure 3B**). Compared with negative control cells, the expression of BCL-2, an anti-apoptotic protein, was increased, while BAX and cleaved caspase 3 expression, two pro-apoptotic proteins, were decreased in cells overexpressing MARK2. The opposite was observed for cells transfected with shMARK2 compared with negative control cells. Together, these results indicate that MARK2 expression may enhance osteosarcoma cellular resistance to cisplatin by reducing apoptosis.

MARK2 regulates P-gp via PI3K/AKT/NF-κB signaling in osteosarcoma cells

Next, we further explored the mechanism of MARK2-mediated cisplatin resistance. It has been reported that the PI3K/AKT/NF-κB pathway is involved in regulating P-gp in other cancers [26]. We hypothesized that MARK2 may enhance osteosarcoma resistance to cisplatin by regulating P-gp expression through the PI3K/AKT/NF-κB signaling pathway. As shown in **Figure 4**, cells treated with 20 μM cisplatin for 24 or 48 h and analyzed by western blot for proteins involved in PI3K/AKT/NF-κB signaling. Levels of P-gp, p-PI3K p85, p-AKT, p-NF-κB/p65, and p-IκB-α increased in MARK2-overexpressing cells and decreased in MARK2-silenced cells. Unlike the phosphorylated forms, we did not observe any significant alterations in total PI3K p85, AKT, NF-κB/p65, and IκB-α. Collectively, our results suggest that the PI3K/AKT/NF-κB signaling pathway may be involved in MARK2-mediated cisplatin resistance of osteosarcoma cells by increasing P-gp expression.

Inhibition of PI3K/AKT/NF-κB signaling rescues MARK2-mediated cisplatin resistance by inhibiting P-gp expression and promoting cell apoptosis

To further clarify whether MARK2 enhances cisplatin resistance in osteosarcoma cells through P-gp expression and cell apoptosis through PI3K/AKT/NF-κB signaling, and to study the potential therapeutic effects of a PI3K inhibitor, we performed rescue experiments. MARK2-overexpressing cells, treated with the PI3K inhibitor LY294002 (20 μM) for 24 h, were subjected to CCK8 cell viability and cell apoptosis assays. CCK8 assays (**Figure 5A**) showed that cisplatin IC_{50} was partially impaired in MARK2-overexpressing cells following LY294002 treatment, suggesting that the PI3K inhibitor rescued the effects of MARK2 overexpression on

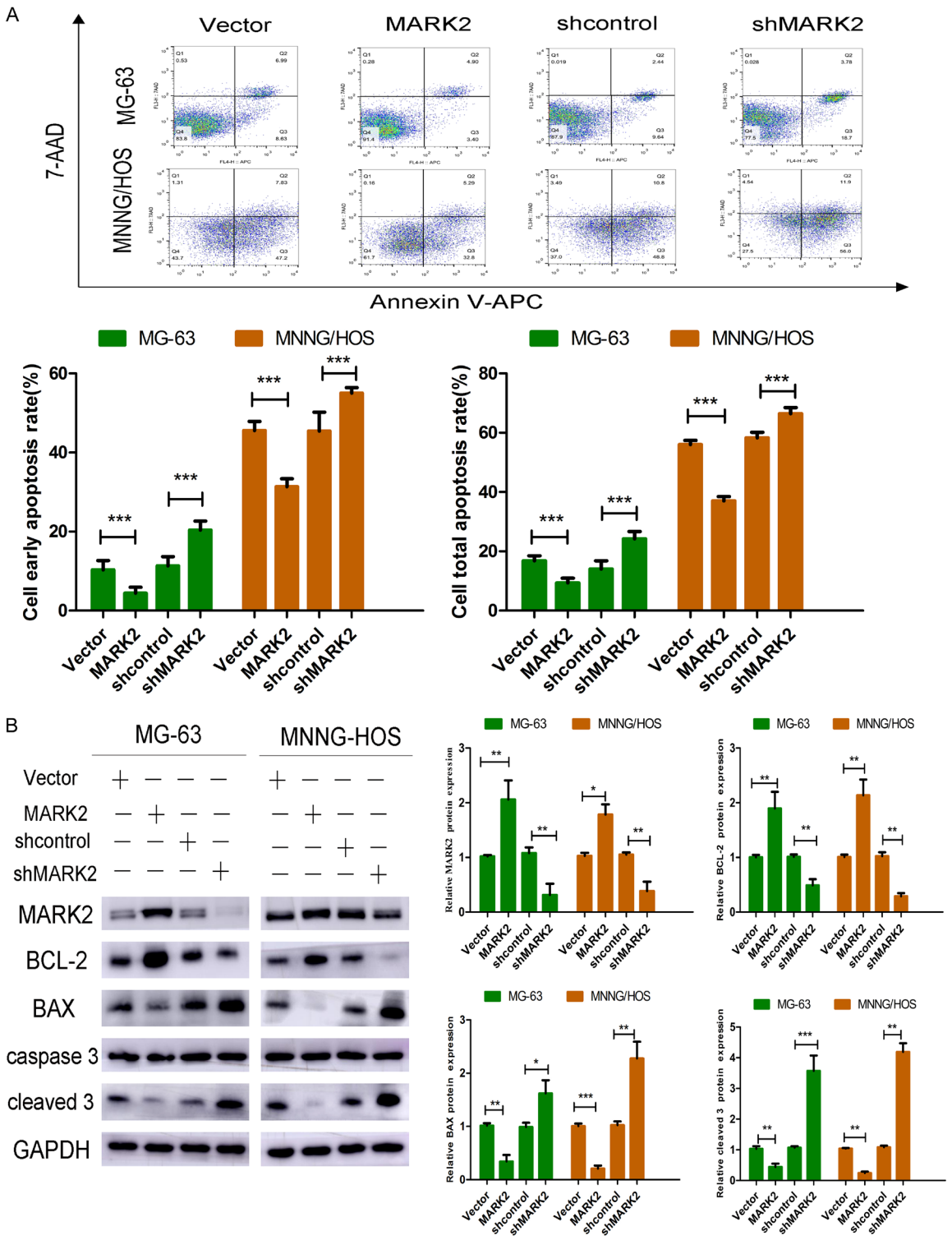


Figure 3. MARK2 suppress cell apoptosis in MG-63 and MNNG/HOS cells treated with cisplatin. A. Cells transfected with vector, MARK2, shcontrol, or shMARK2 lentiviral particles were incubated with 20 μM cisplatin for 24 or 48 h. Early and total cell apoptosis were determined by flow cytometry using Annexin V-APC and 7-AAD double staining. B. Western blot of the expression of apoptosis-related proteins in the indicated cells. Data were expressed as mean ± SD of three independent measurements. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group.

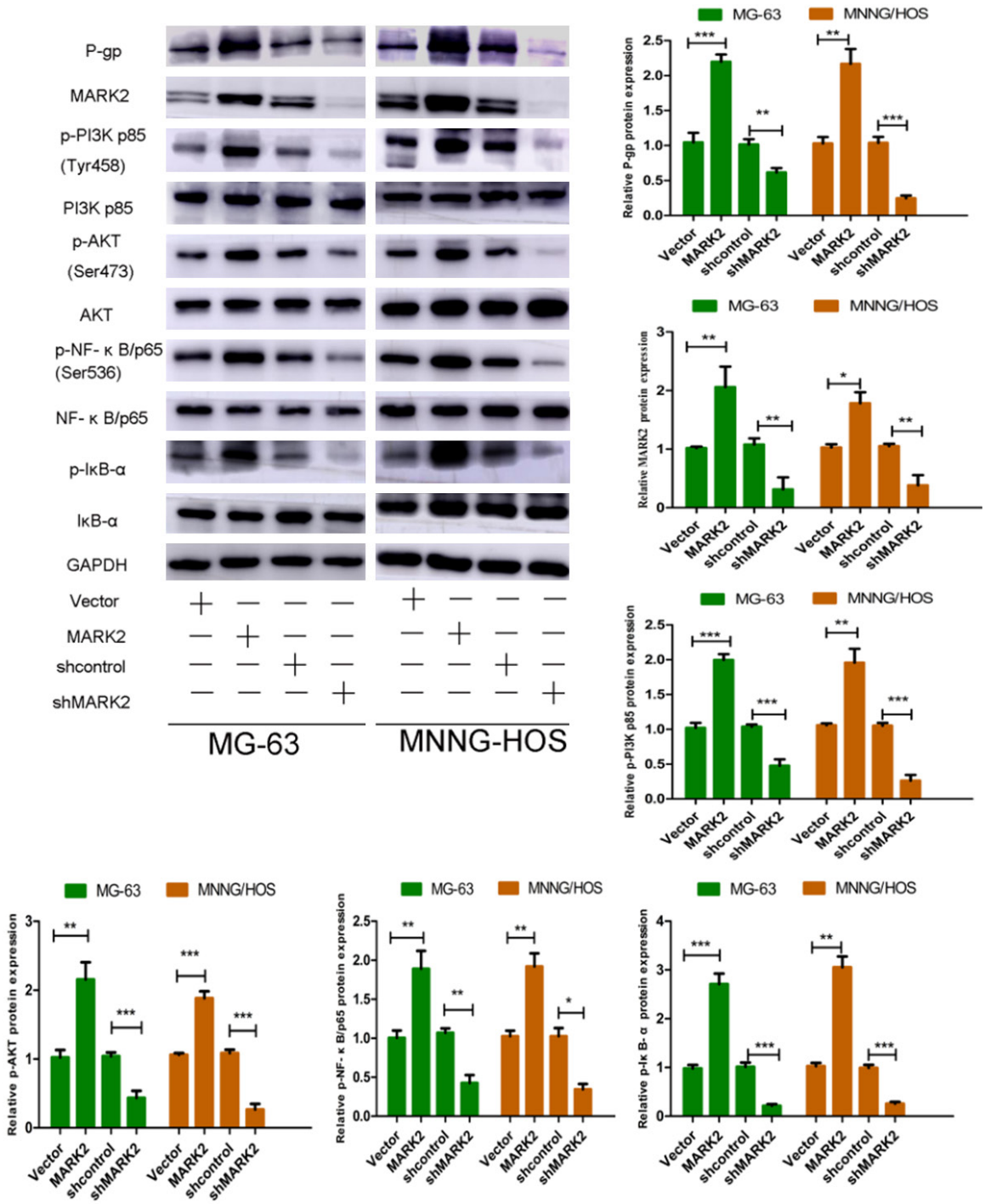


Figure 4. PI3K/AKT/NF-κB signaling is involved in the MARK2 regulation of P-gp. MARK2-transduced or MARK2 shRNA-infected cells, including MG-63 and MNNG/HOS cells, were treated with 20 μM cisplatin for 24 or 48 h. Western blot of P-gp, MARK2, p-PI3K p85, total PI3K p85, p-AKT, total AKT, p-NF-κB/p65, total NF-κB/p65, p-IκB-α, and total IκB-α in the indicated cells. GAPDH was used as a loading control. Values are mean ± SD of three independent experiments; *P < 0.05, **P < 0.01, ***P < 0.001. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

cisplatin resistance. Apoptosis assays (**Figure 5B**) indicated that in MARK2-overexpressing cells treated with LY294002, the apoptotic cell ratio was partially elevated. Western blotting further confirmed this rescuing effect (**Figure 5C**). In cells overexpressing MARK2 and treated with LY294002, the expression of P-gp, p-AKT, p-NF-κB/p65, and BCL-2 were suppressed,

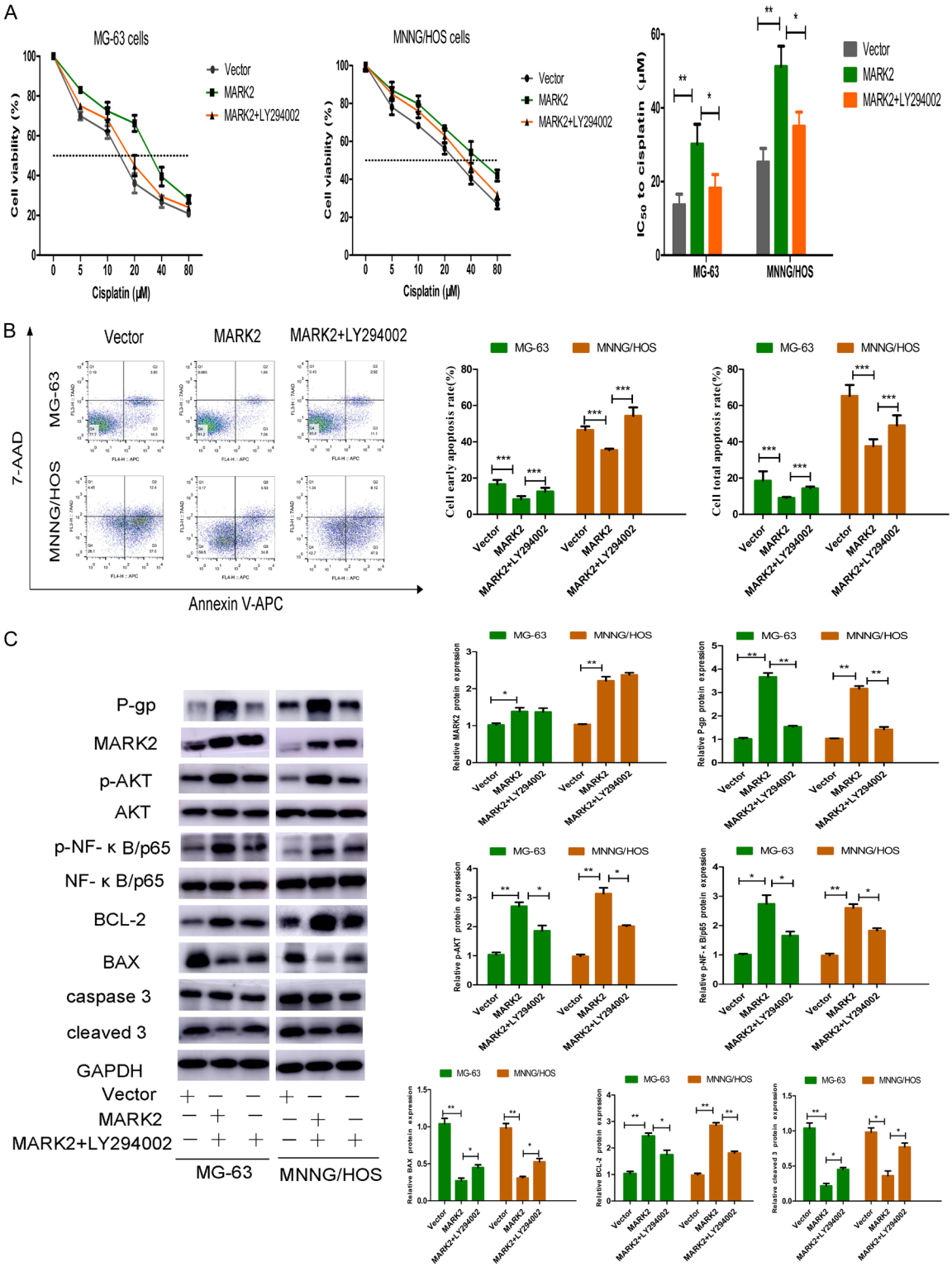


Figure 5. Inhibition of PI3K/AKT/NF-κB signaling rescues MARK2-induced effects on cisplatin resistance by inhibiting P-gp expression and promoting cell apoptosis. A, B. MARK2-overexpressing cells, treated with the PI3K inhibitor LY294002 (20 μM) for 24 h, were subjected to the CCK-8 cell viability and cell apoptosis assays. C. LY294002 (20 μM) suppressed expression of P-gp, p-AKT, and p-NF-κB/p65 and downstream cell apoptosis-associated factors including BCL-2 and elevated the expression of BAX and cleaved caspase 3 in MARK2-overexpressing cells. The expression of MARK2 was not affected. GAPDH was used as the internal control. Data are presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group.

while BAX and cleaved caspase 3 were elevated. The expression of MARK2 was not affected. Together, these data indicate that MARK2 promotes cisplatin resistance in osteosarcoma cells by regulating P-gp expression and cell apoptosis, at least partially by activating the PI3K/AKT/NF-κB signaling pathway.

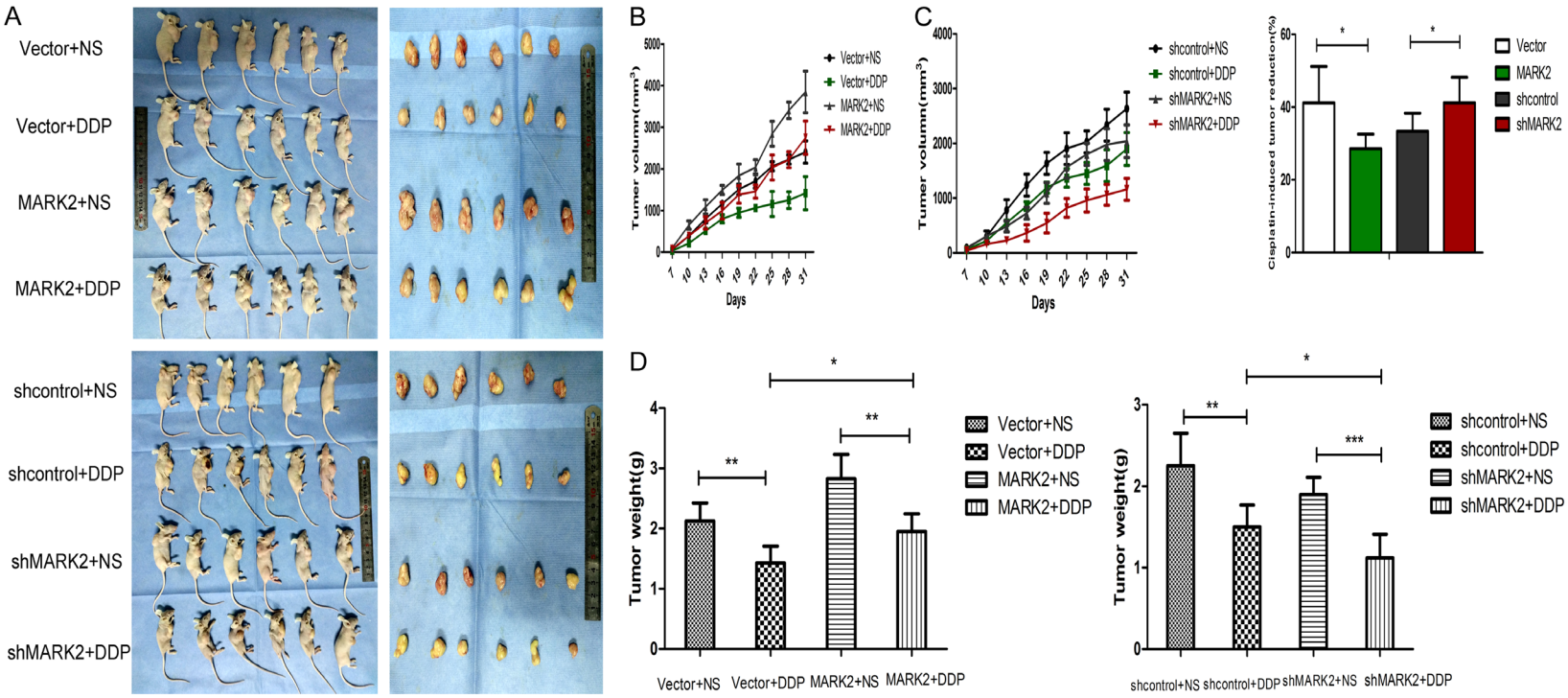
MARK2 promotes cisplatin resistance of osteosarcoma cells in vivo

We next evaluated whether MARK2 promotes the cisplatin resistance of osteosarcoma cells *in vivo*. We established a xenograft tumor model via subcutaneous injection of MNNG/HOS cells with stable knockdown or overexpression of MARK2 and their respective control cells in nude mice. Ten days after injection with the indicated cells, mice were treated with cisplatin (3 mg/kg) or NS intraperitoneally every 3 days (n = 6 per group). Tumor size in each group was measured every 3 days once tumors formed, and tumor volumes were calculated. Tumor weights were measured on day 31 when the mice were sacrificed. In MNNG/HOS cells overexpressing MARK2 and treated with cisplatin, tumor volume and final weight were significantly increased, and decreased when MARK2 expression was knocked down (**Figure 6A**, **6B**, **6D**). To exclude proliferation changes due to MARK2 protein levels, the cisplatin-induced tumor reduction rate was calculated based on tumor volume in a NS-treated group at the final time point. Our results (**Figure 6C**) consistently showed that the rate of tumor reduction decreased when MARK2 was overexpressed and increased when MARK2 was knocked down in MNNG/HOS cells. Consistent with *in vitro* results, western blotting (**Figure 6E**) showed that protein levels of P-gp, p-AKT, p-NF-κB/p65, and BCL-2 increased, while BAX and cleaved caspase 3 decreased in MARK2-overexpressing mouse tumor specimens. The opposite results were observed in MARK2-silenced mouse tumor specimens. Furthermore, IHC staining (**Figure 6F**) exhibited stronger MARK2 and P-gp and lower cleaved caspase 3 signals in MARK2-overexpressing xenograft tumor sections than in the control group. These results were reversed in the shMARK2 group compared to the control group. Combined with data from the *in vitro* study, these data demonstrate that MARK2 promotes cisplatin resistance in osteosarcoma cells by regulating P-gp expression and cell apoptosis.

Discussion

It is well known that patients with recurrent or metastatic osteosarcoma have a poor prognosis and are often resistant to traditional chemotherapeutic drugs [27-31]. Discovering new biomarkers that reduce chemotherapy resistance is important for improving prognosis. Previous studies implicated a role for MARK2 in cisplatin resistance in cervical and non-small cell lung cancers [6, 8]. However, the role of MARK2 in osteosarcoma has not yet been investigated. This study demonstrates a significant correlation between MARK2 overexpression and cisplatin resistance in osteosarcoma cells, which may be associated with poor prognosis in osteosarcoma. We provide the first evidence that MARK2 is upregulated in osteosarcoma cell lines compared to normal osteoblasts and found that high MARK2 expression was associated with cisplatin tolerance. In fact, our Kaplan-Meier survival analysis revealed that osteosarcoma patients with high expression of MARK2 had shorter overall survival.

Notably, our novel findings demonstrate molecular mechanisms for the role of MARK2 in osteosarcoma cisplatin resistance. Through a series of *in vitro* and *in vivo* experiments, we provide evidence that MARK2 overexpression significantly contributes to cisplatin resistance by regulating P-gp expression and cell apoptosis via the PI3K/AKT/NF-κB signaling pathway. Mounting evidence has established that P-gp overexpression is an important factor affecting chemotherapeutic resistance [32-34]. In addition, studies have shown that inhibiting P-gp reverses drug resistance in osteosarcoma patients by reducing intracellular drug concentrations [35-38]. In this study, we found that P-gp inhibition can reduce osteosarcoma cisplatin resistance, and osteosarcoma cells overexpressing MARK2 exhibited increased P-gp expression and cisplatin resistance. Studies show that apoptosis induction can markedly suppress tumor growth, chemoresistance, and metastasis in various human cancers, including osteosarcoma [31, 39, 40]. Thus, promoting apoptosis is one strategy for cancer treatment. Our results indicate that MARK2 overexpression can significantly decrease cisplatin-induced apoptosis in osteosarcoma cells. Furthermore, activation of the PI3K/AKT pathway is associated with chemotherapy resistance in a number of malignancies, including osteosarco-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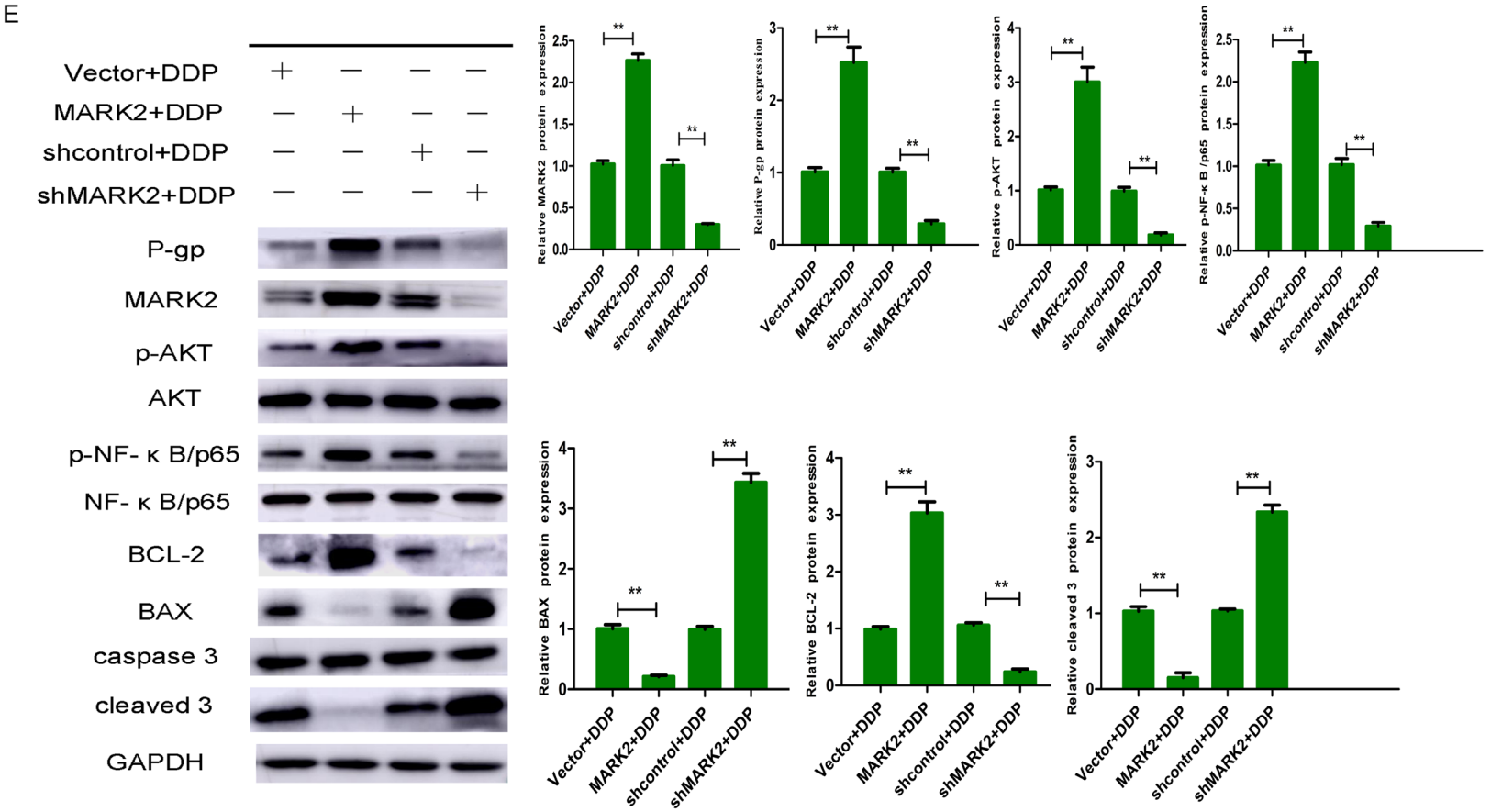
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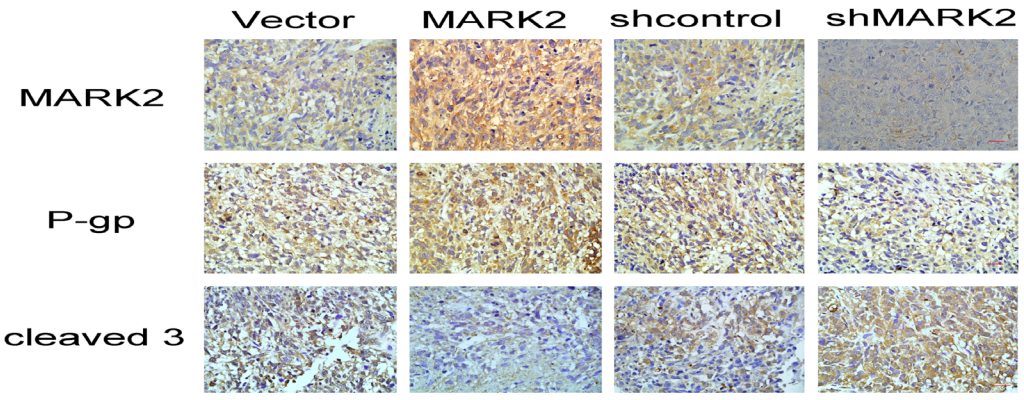
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Figure 6. MARK2 promotes cisplatin resistance of osteosarcoma cells *in vivo*. MNNG/HOS cells with stable knockdown or overexpression of MARK2 and their respective control cells were subcutaneously injected in nude mice. Ten days after injection with the indicated cells, mice were treated with cisplatin (3 mg/kg) or NS intraperitoneally every 3 days (n = 6 per group). A. The morphology of tumor xenografts in each nude mouse from different groups. B. Tumor size in mice from each group was measured every 3 days once tumors formed, and tumor volumes were calculated. C. The cisplatin-induced tumor reduction rate was calculated as described in Methods using the following formula: (1-V cisplatin/V NS) × 100%. D. Tumor weights were measured on day 31 when the mice were sacrificed. E. Western blot of MARK2, P-gp, p-AKT, p-NF-κB/p65, BCL-2, BAX, and cleaved caspase 3 in the indicated groups of mouse tumor specimens. GAPDH was used as a loading control. Values are mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. F. IHC of MARK2, P-gp, and cleaved caspase 3 protein levels in xenograft tumor sections collected from mice treated with cisplatin. Magnification: 400 ×, scale bars: 100 μm. NS, normal saline; IHC, immunohistochemistry.

ma [21, 22, 41-45]. Activated AKT stimulates phosphorylation and could impact various downstream targets, including BAD, IKK, and MDM2, involved in apoptotic and NF-κB signaling [46, 47]. Here, we show that levels of phospho-PI3K, phospho-AKT, and phospho-NF-κB p65 increased in MARK2-overexpressing cells and decreased in MARK2-silenced cells. Our rescue experiments further clarified that PI3K inhibitors could attenuate the effect of MARK2 on cisplatin resistance. Together, our data clearly demonstrate that MARK2 overexpression can upregulate P-gp expression and suppress cell apoptosis by partially activating PI3K/AKT/NF-κB signaling and promoting cisplatin resistance in osteosarcoma cells. This investigation suggests that MARK2 restoration to normal levels may be a useful strategy for overcoming chemoresistance in osteosarcoma patients.

In addition to our mechanistic study, we found that osteosarcoma patients with high MARK2 expression experience poor prognoses. Whether MARK2 is an independent risk factor in the prognosis of osteosarcoma requires further investigation. In our *in vivo* experiments, we found that MARK2 promoted proliferation. More *in vitro* experiments are needed to identify a role for MARK2 in tumorigenesis, which could provide new diagnostic and prognostic methods. For patients with metastatic osteosarcoma that have a poor prognosis, MARK2 could also contribute to osteosarcoma cell migration and metastasis, supporting the need for further exploration as a potential therapeutic target.

In summary, our results suggest that MARK2 plays an important role in osteosarcoma chemoresistance. By activating the PI3K/AKT/NF-κB signaling pathway, MARK2 regulates P-gp expression and cell apoptosis, promoting cisplatin resistance in osteosarcoma cells. These findings support the clinical development of MARK2 as a therapeutic target and potential biomarker for cisplatin-resistant osteosarcoma.

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Disclosure of conflict of interest

None.

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