Am J Cancer Res 2015;5(1):321-332
www.ajcr.us /ISSN:2156-6976/ajcr0002675

Original Article

Overexpression of CDX2 in gastric cancer cells promotes the development of multidrug resistance

Lin-Hai Yan[1], Wei-Yuan Wei[2], Wen-Long Cao[2], Xiao-Shi Zhang[2], Yu-Bo Xie[3], Qiang Xiao[2]

[1]Department of Gastrointestinal Surgery, Affiliated Tumor Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China; [2]Department of Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China; [3]Department of Anesthesiology, The First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Received September 21, 2014; Accepted November 12, 2014; Epub December 15, 2014; Published January 1, 2015

Abstract: Modulator of multidrug resistance (MDR) gene is a direct transcriptional target of CDX2. However, we still speculate whether CDX2 affects MDR through other ways. In this study, a cisplatin-resistant (SGC7901/DDP) and a 5-fluoro-2, 4(1h,3h)pyrimidinedione-resistant (BGC823/5-FU) gastric cancer cell line with stable overexpression of CDX2 were established. The influence of overexpression of CDX2 on MDR was assessed by measuring IC50 of SGC7901/DDP and BGC823/5-FU cells to cisplatin, doxorubicin, and 5-fluorouracil, rate of doxorubicin efflux, apoptosis, and cell cycle progression detected by flow cytometry. In addition, we determined the *in vivo* effects of CDX2-overexpression lentiviral vector (LV-CDX2-GFP) on tumor size, and apoptotic cells in tumor tissues were detected by deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling and hematoxylin and eosin staining. Results showed that LV-CDX2-GFP led to up-regulation of CDX2 mRNA and protein expression. It significantly inhibited the sensitivity of SGC7901/DDP and BGC823/5-FU cells to cisplatin, doxorubicin, and 5-fluorouracil. Flow cytometry confirmed that the percentage of apoptotic cells decreased after CDX2 up-regulation. This notion was further supported by the observation that up-regulation of CDX2 blocked entry into the M-phase of the cell cycle. Furthermore, up-regulation of CDX2 significantly decreased intracellular accumulation of doxorubicin. In molecular studies, quantitative reverse-transcriptase real-time polymerase chain reaction and western blotting revealed that CDX2 up-regulation could suppress expression of Caspase-3, Caspase-9 and PTEN, and increased the expression of MDR1, MRP, mTOR, HIF-1α.

Keywords: Homeobox gene CDX2, lentiviral vector, gastric cancer, drug resistance, murine model

Introduction

Despite the decline in its incidence in the past few decades, gastric cancer remains the second most common cause of cancer-related deaths worldwide, and about two-thirds of gastric cancer cases occur in developing countries, and 42% in China alone [1]. Most deaths of gastric cancer are due to resistance to chemotherapies [2]. Generally, the molecular genetic basis of resistance to cancer therapeutics is complex, involving multiple processes, such as drug transport, drug metabolism, DNA repair and apoptosis [3]. However, the factors that regulate the chemo-resistance of gastric cancer remain poorly understood.

CDX2 is a member of the caudal-related homeobox gene family. Several investigators have reported that strong and robust expression of CDX2 is found in > 80% of colorectal cancer and non-small cell lung cancer [4, 5], CDX2 enhances proliferation and has tumorigenic potential in human colon cancer cell lines LoVo and SW48 [6]. In addition, CDX2 transgenic mice have been shown to have intestinal metaplasia and a high incidence of gastric carcinoma [7, 8]. Furthermore, Takakura et al. has been reported that inhibition of CDX2 decreases endogenous MDR1 expression [9]. MDR1 was originally identified as an overexpressed and amplified gene in multidrug-resistant cells. Its product, P-glycoprotein (P-gp), appears to

play a critical role in drug resistance [10]. In addition, in our previous study, we have found that reversal of multidrug resistance in gastric cancer cells by CDX2 downregulation. Although these evidence implicated that CDX2 is associated with carcinogenesis and development of multidrug resistance (MDR), the role of CDX2 in multidrug resistant gastric cancer largely remains unexplored.

To define the effects of up-regulation CDX2 in multidrug resistant gastric cancer, we constructed CDX2-overexpression lentiviral vector (LV-CDX2-GFP), transfected them into a cisplatin-resistant gastric cancer cell line SGC7901/DDP and a 5-FU-resistant gastric cancer cell line BGC823/5-FU, selected stable transfectants, and explored changes in IC50, rate of doxorubicin efflux, cell cycle, and apoptosis. We also observed the effects of up-regulation CDX2 on the expression of genes associated with apoptosis and multidrug resistant, including Caspase-3, Caspase-9, PTEN, MDR1, MRP, mTOR, and HIF-1α. Moreover, we investigated the effects of CDX2 up-regulation on the growth and apoptosis of SGC7901/DDP and BGC823/5-FU cells *in vivo*.

Materials and methods

Reagents

5-fluorouracil, cisplatin and doxorubicin were purchased from Sigma-Aldrich (St Louis, MO, United States). Cell culture medium RPMI-1640 was purchased from Invitrogen-Gibco (Carlsbad, CA, United States). Fetal bovine serum (FBS) was from Invitrogen-Gibco. Trypsin, streptomycin and penicillin were obtained from Sunshine Biotechnology (Nanjing, China). CDX2, MDR1, MRP, mTOR, HIF-1α, Caspase-3, Caspase-9, PTEN and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, United States). All other chemicals were of the highest commercial grade available.

Cell culture

The cells were cultured in RPMI 1640 supplemented with 10% FBS (Sijiqing Biotec, Co. Ltd., Hangzhou, China), antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) in a humidified 5% CO_2 atmosphere at 37.8°C. For SGC7901/DDP and BGC823/5-FU cells, 0.6 μg/mL cisplatin was supplemented in the medium to maintain the drug-resistance phenotype [11].

Gene transfection

CDX2-overexpression lentiviral vector (LV-CDX2-GFP) and PLNCX lentiviral vector (LV-GFP) purchased from Shanghai Cancer Institute, China, was used to construct the CDX2-overexpression vector. The methods of LV-CDX2-GFP and transfection of SGC7901/DDP and BGC823/5-FU gastric cancer cells with LV-CDX2-GFP or LV-GFP have been previously described [12]. SGC7901/DDP and BGC823/5-FU cells were seeded in six-well plates with antibiotic-free medium. After 24 h incubation, cells were infected with viral supernatant at a multiplicity of infection of 200 PFU per cell (MOI = 200), and the stable-transfected cell lines were obtained by culturing transfected cells in the presence of 700 mg/mL G418 (Invitrogen, Carlsbad, CA, United States) for 3-4 wk. The different SGC7901/DDP cells were divided into three groups: CDX2 (SGC7901/DDP + CDX2), GFP (SGC7901/DDP + GFP), and NC (SGC7901/DDP), the different BGC823/5-FU cells were divided into three groups: CDX2 (BGC823/5-FU + CDX2), GFP (BGC823/5-FU + GFP), and NC (BGC823/5-FU).

Cytotoxicity assay

The cytotoxicity of gene transfection was determined by Cell Counting Kit-8 (CCK-8) assay [13]. In 96 well plates, cells were seeded in 100 μl PRMI-1640 medium supplemented with 10% FBS at 5×10^4 cells/well. Then chemotherapeutic agents were added in normal growth medium supplemented with FBS. After 48 h incubation, 10 μl Cell Counting Kit-8 (CCK-8) was added and culture was continued for 1 h in humidified atmosphere containing 5% CO_2. Absorbances at 450 nm were measured by Microplate Reader (Bio-Tech Company). The relative drug resistance folds were analyzed by compared with IC50.

Measurement of pump rate of doxorubicin by flow cytometry

Pump rate of doxorubicin by flow cytometry [11]. The cells were inoculated into six-well plates and 4 mg/mL doxorubicin was added, and all wells were placed at 37°C for 30 min. Flow cytometry was used to measure the fluo-

rescent intensity of doxorubicin in cells with an excitation wavelength of 488 nm and emission wavelength of 575 nm. The cells were then washed twice with fresh culture medium and incubated with the new medium at 37°C for 1 h to detect the retained doxorubicin. Subtraction of the fluorescence retained from the total fluorescence was the fluorescent index of doxorubicin. The procedure was repeated three times and an average value was obtained to calculate the pump rate of doxorubicin. The pump rate of the drug from the cells = (accumulated quantity of doxorubicin – retained quantity of doxorubicin)/accumulated quantity of doxorubicin.

Apoptosis analysis by flow cytometry

SGC7901/DDP and BGC823/5-FU cells (1×10^6) were washed twice with ice-cold PBS, treated with trypsin, and fixed in cold 70% ethanol at 4°C for 30 min. The cell pellet was incubated in a solution containing 10 μl/mL Annexin V-FITC, and 10 μl/mL 7-amino-actinomycin D (7-AAD). The cells were analyzed by flow cytometry using an EPICS XL-MCL FACScan (Becton–Dickinson, Mountain View, CA, United States). The data was analyzed with the MultiCycle Software for Windows (Phoenix Flow Systems, San Diego, CA, United States).

Cell cycle analysis by flow cytometry

SGC7901/DDP and BGC823/5-FU cells (1×10^6) were washed twice with ice-cold PBS, treated with trypsin, and fixed in cold 70% ethanol at 4°C for 30 min. The cell pellet was incubated in a solution containing 50 ng/mL propidium iodide, 0.2 mg/mL RNase, and 0.1% Triton X-100 at room temperature for 30 min. The cells were analyzed by flow cytometry using an EPICS XL-MCL FACScan (Becton-Dickinson, Mountain View, CA, United States) [11]. The data was analyzed with the MultiCycle Software for Windows (Phoenix Flow Systems, San Diego, CA, United States).

Quantitative reverse-transcriptase real-time polymerase chain reaction

Total RNA was isolated using the AxyPrep™ Purification Kit (Axygen, USA) according to the manufacturers' instructions. The total RNA concentration and quality were measured with a Nanodrop 2000 micro-volume spectrophotometer (Thermo Scientific, USA) by absorbance measurements. RNA integrity was analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide. First-strand cDNA was synthesized from 3000 ng of total RNA using the RevertAidHMinus First Strand cDNA synthesis kit (Fermentas,USA) as instructed by the manufacturers. Real-time PCR (RT-PCR) reactions were carried out on an Mx3000P real-time PCR system (Stratagene USA). To create the RT-PCR standard, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The PCR primer sequences (CDX2 primers were sense: 5'-CGG CAG CCA AGT GAA AAC-3' and antisense: 5'-GAT GGT GAT GTA GCG ACT GTA-3'. MDR1 primers were sense: 5'-ACC AAG CGG CTC CGA TAC A-3' and antisense: 5'-TCA TTG GCG AGC CTG GTA GTC-3'. MRP primers were sense: 5'-GGA CCT GGA CTT CGT TCT CA-3' and antisense: 5'-CGT CCA GAC TTC ATC CG-3'. PTEN primers were sense: 5'-CTG GAA AGG GAC GAA CTG-3' and antisense: 5'-AGG TAA CGG CTG AGG GA-3'. Caspase-3 primers were sense: 5'-AAG CGA ATC AAT GGA CTC-3' and antisense: 5'-TTC CTG ACT TCA TAT TTC AA-3'. Caspase-9 primers were sense: 5'-GGC TGT CTA CGG CAC AGA TGG A-3' and antisense: 5'-CTG GCT CGG GGT TAC TGC CAG-3'. mTOR primers were sense: 5'-CCC GAG ACA GCC TTG GCA GTT GG-3' and antisense: 5'-CAG GAC TCA GGA CAC AAC TAG CCC-3'. HIF-1α primers were sense: 5'-CTA TGG AGG CCA GAA GAG GGT AT-3' and antisense: 5'-CCC ACA TCA GGT GGC TCA TAA-3'. GAPDH primers were sense: 5'-ACC ACA GTC CAT GCC ATC AC-3' and antisense: 5'-TCA CCA CCC TGT TGC TGT A-3'). All mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method [14]. All RT-PCR procedures were repeated in triplicates. The results were analyzed by Mx3000P real-time PCR software version 2.00.

Western blotting

Cell lysates were prepared in a buffer containing 100 mmol/L NaCl, 10 mmol/L Tris–HCl (pH 7.6), 1 mmol/L EDTA (pH 8.0), 1 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40. After protein quantitation using the Lowery protein assay, equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three-buffer system [11]. The membranes were incubated with a dilution of primary antibody (anti-CDX2: 1:500, anti-Caspase-3: 1:2000, anti-Caspa-

se-9: 1:1000, anti-PTEN: 1:3000, anti-MDR1: 1:3000, anti-MRP: 1:1500, anti-mTOR: 1:2000, anti- HIF-1α: 1:2000), overnight at 4°C. The membrane was washed with TBST and incubated with a peroxidase-conjugated secondary antibody (1:1000) (Santa Cruz Biotechnology) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, Rockford, IL, United States), according to the manufacturer's recommendations. Western blot film was scanned, and the net intensities of the bands were quantified using Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, United States). After development, the membrane was stripped and reprobed with antibody against GAPDH (1:1000) or β-actin (1:1500) to confirm equal sample loading.

Immunoprecipitation and GST pull-down were performed as described previously [15].

Effect of LV-CDX2-GFP on promoting MDR of human gastric cancer in vivo

BALB/c 5-wk-old male nude mice (Guangxi Animal Center, Nanning, China) were kept under specific pathogen-free conditions and tended to in accordance with institutional guidelines [11]. All experimental studies were approved by the Guangxi Medical University Animal Care and Use Committee. SGC7901/DDP and BGC823/5-FU cells were used for tumor implantation. Approximately 2×10^6 tumor cells were harvested, resuspended in 100 μL PBS, implanted subcutaneously into the flanks of the BALB/c nude mice, and resulting tumor was named as SGC7901/DDP and BGC823/5-FU tumors. After 7 d, when the SGC7901/DDP and BGC823/5-FU tumor measured 3-5 mm in diameter, these nude mice were randomly divided into the following six groups. The animals were administered an intratumoral injection of LV-CDX2-GFP or LV-GFP at a titer of 5×10^6 TU in 100 μL PBS, and injection of an equal volume of PBS was used as a blank control. After the first injection, the animals were administered a similar injection every 2 d. DDP was administered by intraperitoneal injection at a dose of 25 mg/kg. After the first injection, the animals were administered a similar injection every 2 d. The tumors were monitored every day and measured every 2 d with a caliper, and the diameters were recorded. The tumor volume (TV) was calculated by the formula: $TV = W^2 \times L/2$, where L is the length and W is the width of the tumor. The relative tumor volume (RTV) was calculated by the formula: $RTV = V_t/V_0$ (V_0 is the TV at the day when the chemicals were given, and V_t is the TV of subsequent measurement). The animals were sacrificed at 34 d after tumor injection and the tumors were analyzed.

Hematoxylin and eosin staining and deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay

For hematoxylin and eosin (HE) staining tumor tissues were fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin wax [11]. Tissue sections were dewaxed and rehydrated according to a standard protocol. Sections were stained with HE. For the deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, apoptotic cells in sections of mouse tumor tissue were detected using an *in situ* apoptosis detection kit (KEYGEN, Nanjing, China) as instructed by the manufacturer. Cells were visualized with a light microscope (Olympus IX70, Tokyo, Japan). The apoptotic index was calculated as follows: the apoptotic index = number of apoptotic cells/total number of cells. The *in vivo* experiments strictly obeyed the ethical principles and guidelines for scientific experiments on animals.

Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined using χ^2 test, Student's t test, or one-way analysis of variance (ANOVA). Statistical analysis were carried out using SPSS version 13.0 (Chicago, IL, United States) or Origin 7.5 software programs (OriginLab, Northampton, MA, United States). A value of $P < 0.05$ was considered as statistically significant.

Results

LV-CDX2-GFP led to up-regulation of CDX2 mRNA and protein expression

Our previous study suggested that recombinant lentiviral vector for CDX2 gene successfully led to up-regulation of CDX2 mRNA and protein expression in MGC-803 cells [11]. In the present study, we further tested the hypothesis that LV-CDX2-GFP could up-regulate CDX2 mRNA and protein expression in SGC7901/DDP and

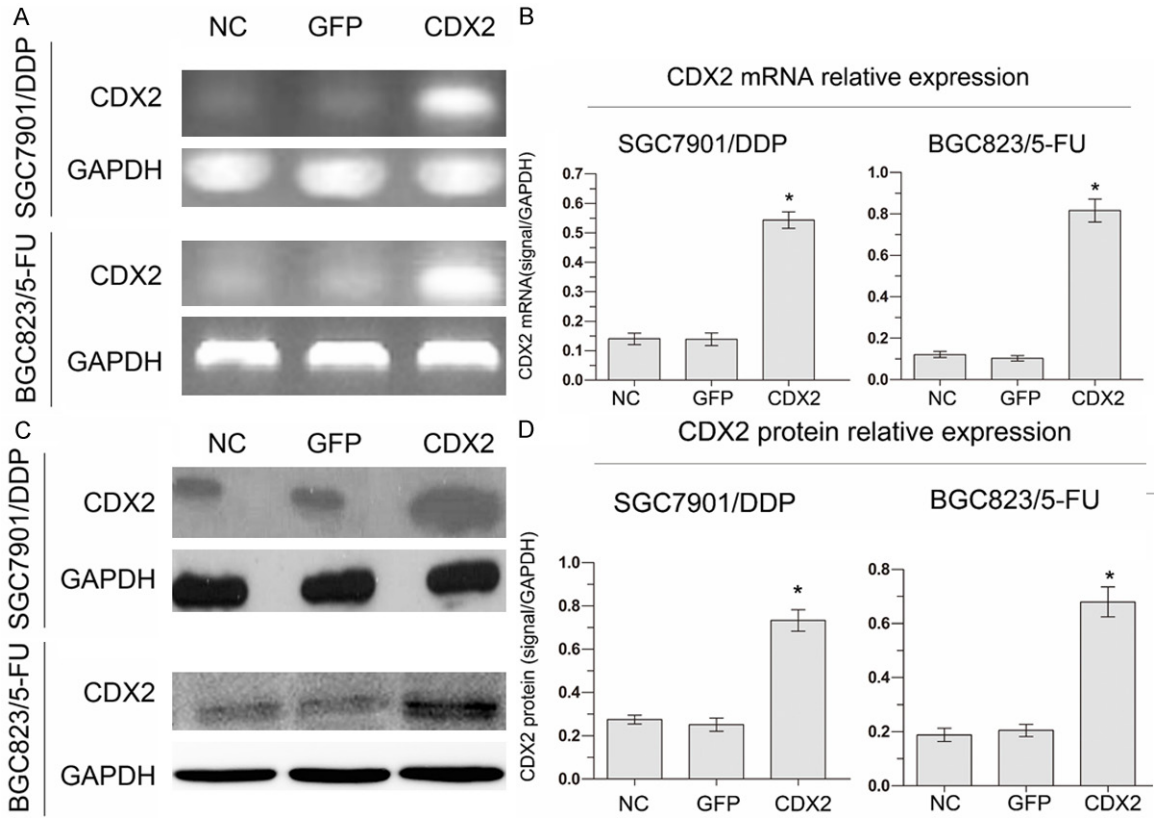

Figure 1. mRNA and protein expressions of CDX2 after gene transfection. A, B: Expression level of CDX2 mRNA was determined by semiquantitative reverse-transcriptase polymerase chain reaction; C, D: Expression level of CDX2 protein was determined by Western blotting. mRNA results were expressed as the ratio of CDX2 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Western blotting results were expressed as the ratio of optical density of CDX2 bands to GAPDH bands. All values are mean ± SE. $^{*}P < 0.05$ for CDX2 groups *vs* GFP groups and NC groups.

Table 1. IC50 values for anticancer drugs in SGC7901/DDP and BGC823/5-FU cells

	Doxorubicin (μg/mL)	5-fluorouracil (μg/mL)	Cisplatin (μg/mL)
SGC7901/DDP			
CDX2	0.82 ± 0.05*	7.52 ± 0.13*	5.82 ± 0.13*
GFP	0.43 ± 0.09	4.29 ± 1.01	3.21 ± 0.38
NC	0.39 ± 0.12	4.52 ± 1.22	2.91 ± 1.03
BGC823/5-FU			
CDX2	1.61 ± 0.05*	9.82 ± 0.95*	6.56± 1.01
GFP	0.91 ± 1.03	2.91 ± 1.03	3.91 ± 1.03
NC	0.73 ± 0.64	3.22 ± 0.73	3.22 ± 1.03

IC50 values were evaluated by CCK-8 assay. Each experiment was conducted in triplicate. Data are expressed as means ± SD of four independent experiments. One-way analysis of varianc followed by Dunnett's multiple comparison tests revealed statistical differences of $^{*}P < 0.05$ *vs* GFP group and NC group.

BGC823/5-FU cells. We treated SGC7901/DDP and BGC823/5-FU cells with LV-CDX2-GFP and LV-GFP. Transfection of LV-CDX2-GFP into SGC7901/DDP and BGC823/5-FU cells led to marked promotion of CDX2 mRNA (**Figure 1A**) and protein expression (**Figure 1C**). Densitometry analysis showed that CDX2 mRNA (**Figure 1B**) and protein (**Figure 1D**) in CDX2 group were higher, respectively, than those in GFP group and NC group ($P < 0.05$). There were no differences between NC groups and GFP groups. These results suggested that LV-CDX2-GFP could up-regulate CDX2 mRNA and protein expression in SGC7901/DDP and BGC823/5-FU cells.

LV-CDX2-GFP promotes MDR

Although SGC7901/DDP cells and BGC823/5-FU cells were selected with the single anticancer drug cisplatin and 5-FU, they also displayed multiple resistances to other anticancer drugs

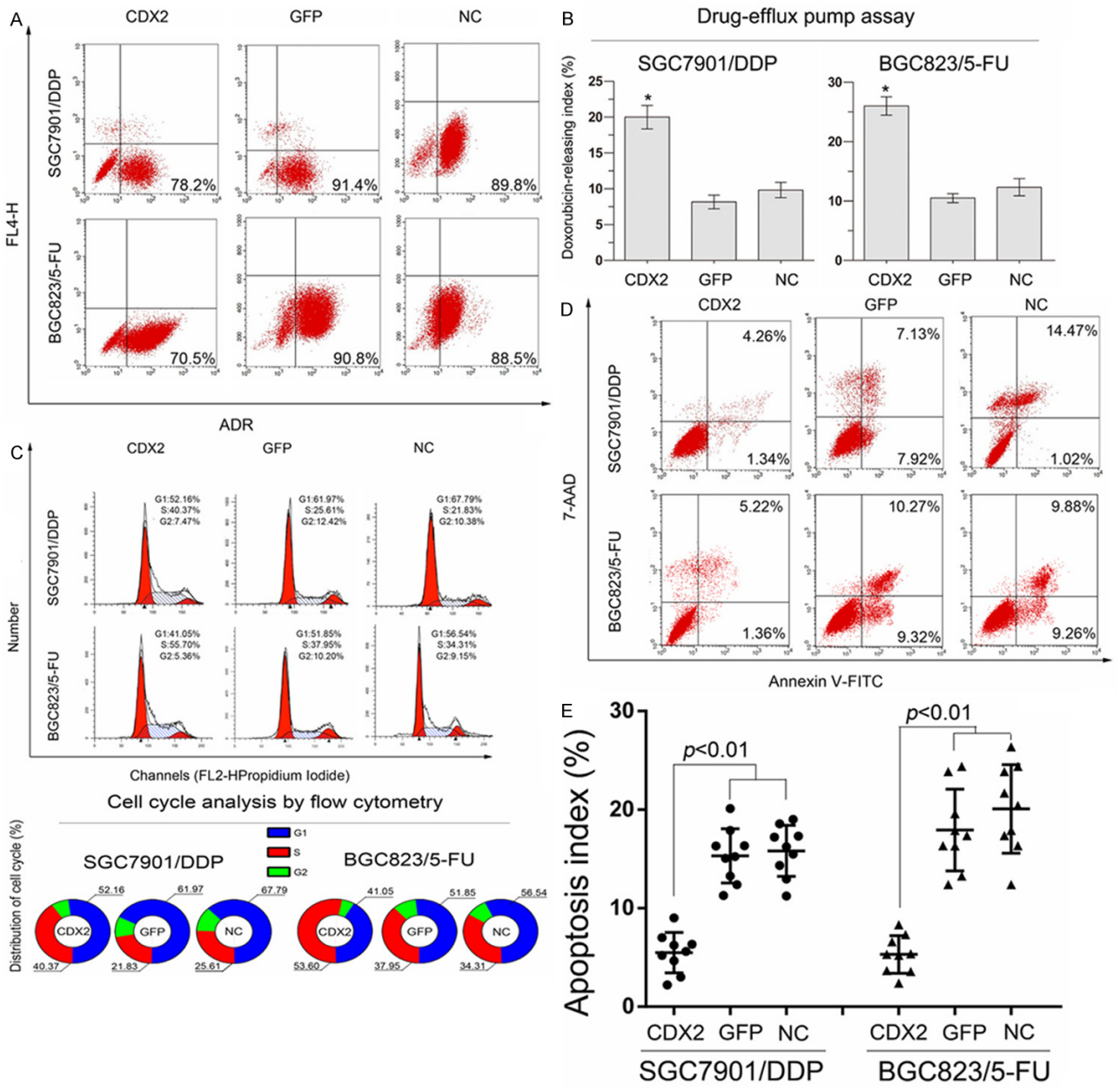
A
CDX2
GFP
NC
SGC7901/DDP
BGC823/5-FU
78.2%
91.4%
89.8%
70.5%
90.8%
88.5%
FL4-H
ADR
B
Drug-efflux pump assay
SGC7901/DDP
BGC823/5-FU
Doxorubicin-releasing index (%)
CDX2
GFP
NC
C
G1:52.16%
S:40.37%
G2:7.47%
G1:61.97%
S:25.61%
G2:12.42%
G1:67.79%
S:21.83%
G2:10.38%
G1:41.05%
S:55.70%
G2:5.36%
G1:51.85%
S:37.95%
G2:10.20%
G1:56.54%
S:34.31%
G2:9.15%
Number
Channels (FL2-HPropidium Iodide)
Cell cycle analysis by flow cytometry
G1
S
G2
Distribution of cell cycle (%)
52.16
61.97
67.79
40.37
21.83
25.61
41.05
51.85
56.54
53.60
37.95
34.31
D
4.26%
7.13%
14.47%
1.34%
7.92%
1.02%
5.22%
10.27%
9.88%
1.36%
9.32%
9.26%
7-AAD
Annexin V-FITC
E
p<0.01
p<0.01
Apoptosis index (%)
SGC7901/DDP
BGC823/5-FU

Figure 2. Effect of up-regulation of CDX2 cell pump rate of doxorubicin, cell cycle, and apoptotic rate in SGC7901/DDP cells after gene transfection. A, B: Pump rate of doxorubicin in SGC7901/DDP and BGC823/5-FU cells after CDX2 gene transfection was analyzed by flow cytometry; C, D: Cell cycle in SGC7901/DDP and BGC823/5-FU cells after CDX2 gene transfection was analyzed by flow cytometry; E, F: Percentage of apoptotic cells was analyzed by flow cytometry. All values are mean ± SE. $^{*}P < 0.05$ for CDX2 groups *vs* GFP groups and NC groups.

[11]. We studied the regulatory effects of LV-CDX2-GFP on the drug sensitivity of gastric cancer cells. CCK-8 assay was used to detect the sensitivity of cells to one P-gp-related drug (doxorubicin) and two P-gp-non-related drugs (5-fluorouracil and cisplatin). As showed in **Table 1**, compared with GFP groups and NC groups, CDX2 group exhibited significantly increased IC50 values for cisplatin, doxorubicin and 5-fluorouracil ($P < 0.05$).

Effects of LV-CDX2-GFP on pump rate of doxorubicin

Pumping out chemotherapeutic agents is the key process in MDR [2]. We proposed that up-regulation of CDX2 promoted drug efflux in gastric cancer groups. To test this hypothesis, intracellular drug accumulation and retention were evaluated using doxorubicin as a probe. As shown in **Figure 2A** and **2B**, compared with GFP groups and NC groups, CDX2 groups exhibited significantly increased accumulation and retention as well as a higher releasing index of doxorubicin (Pump rate of doxorubicin of CDX2 vs GFP and NC groups in SGC7901/DDP cells: 19.71% ± 3.86% vs 8.04% ± 1.18% and 9.65% ± 1.95%, $P < 0.001$; Pump rate of doxorubicin of CDX2 vs GFP and NC groups in BGC823/5-FU cells: 26.71% ± 2.86% vs 12.04% ± 2.18% and 12.65% ± 1.95%, $P < 0.001$).

Effect of LV-CDX2-GFP on cell cycle control

We used flow cytometry to determine whether promotion of MDR by LV-CDX2-GFP in SGC7901/DDP and BGC823/5-FU cells was mediated, at least in part, through an effect on cell cycle progression (**Figure 2C**). We found that the number of cells in S phase markedly increased, while those in G2/M phase decreased in CDX2 groups, compared with GFP groups and NC groups (S phase analysis of CDX2 vs GFP and NC groups in SGC7901/DDP cells: 41.27% ± 3.24% vs 24.21% ± 4.74% and 23.83% ± 3.15%, $P < 0.001$; S phase analysis of CDX2 vs GFP and NC groups in BGC823/5-FU cells: 55.68% ± 2.14% vs 39.74% ± 5.64% and 36.72% ± 4.05%, $P < 0.05$).

LV-CDX2-GFP inhibits apoptosis

Anti-apoptosis is an important mechanism of MDR [2], therefore, we investigated the effect of LV-CDX2-GFP on cisplatin-induced gastric cancer cell apoptosis by calculating apoptosis index. Cells were stained with annexin V PE and 7-AAD, and then subsequently analyzed by flow cytometry [11]. The dual parameter fluorescent dot plots showed that the viable cells were in the lower left quadrant, and the apoptotic cells were in the right quadrant. As shown in **Figure 2D** and **2E**, compared with GFP groups and NC groups, CDX2 groups exhibited significantly decreased apoptosis index (Apoptosis rate analysis of CDX2 vs GFP and NC groups in SGC7901/DDP cells: 5.71% ± 1.86% vs 15.04% ± 2.18% and 15.65% ± 1.95%, $P < 0.05$; Apoptosis rate analysis of CDX2 vs GFP and NC groups in BGC823/5-FU cells: 5.03% ± 1.24% vs 17.04% ± 3.01% and 19.11% ± 3.95%, $P < 0.05$).

LV-CDX2-GFP influenced expression of MDR1, MRP, mTOR, HIF-1α, Caspase-3, Caspase-9 and PTEN

To investigate the mechanism by which LV-CDX2-GFP induces reversal of MDR in SGC7901/DDP groups, we detected expression levels of some well-known regulators of apoptosis (caspase-9, caspase-3, p53, bax, bcl-2, PTEN, Survivin, and c-Myc), and some important multidrug resistant related gene (MDR1, MRP, mTOR and HIF-1α) by qRT-PCR and Western blotting (**Figure 3A** and **3B**). The mRNA and protein expression level of caspase-9, caspase-3 and PTEN in CDX2 groups was lower than that in GFP groups and NC groups. However, the expression of multidrug resistant related gene (MDR1, MRP, mTOR and HIF-1α) increased in CDX2 groups than GFP groups and NC groups. No significant difference in the expression level of p53, bax, bcl-2, Survivin, and c-Myc was found in the cell models (data not shown). To better understand the function of CDX2, we performed a yeast two-hybrid screen using CDX2 as the bait and identified MRP as a MRP-interacting protein. Using

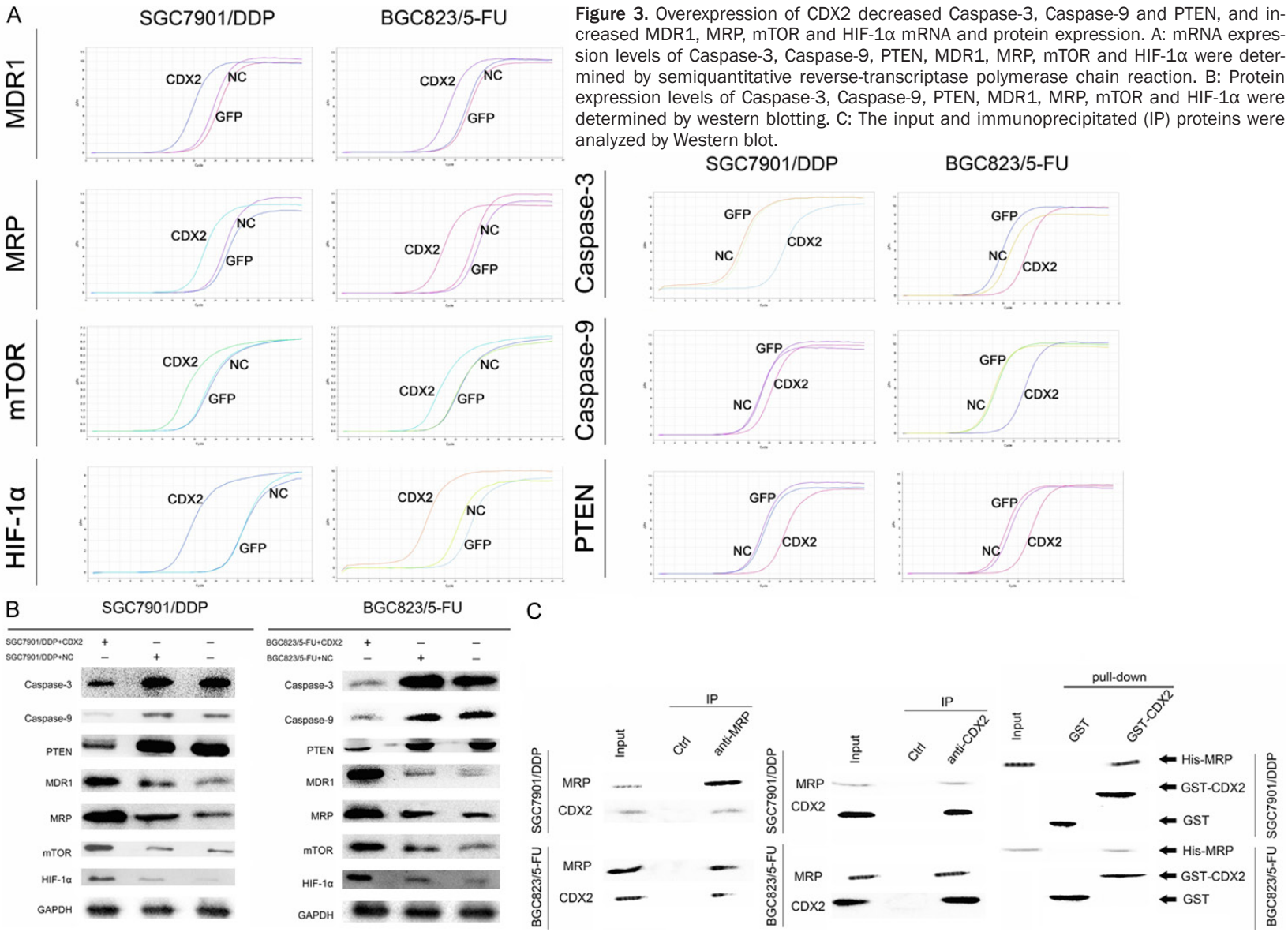

Figure 3. Overexpression of CDX2 decreased Caspase-3, Caspase-9 and PTEN, and increased MDR1, MRP, mTOR and HIF-1α mRNA and protein expression. A: mRNA expression levels of Caspase-3, Caspase-9, PTEN, MDR1, MRP, mTOR and HIF-1α were determined by semiquantitative reverse-transcriptase polymerase chain reaction. B: Protein expression levels of Caspase-3, Caspase-9, PTEN, MDR1, MRP, mTOR and HIF-1α were determined by western blotting. C: The input and immunoprecipitated (IP) proteins were analyzed by Western blot.

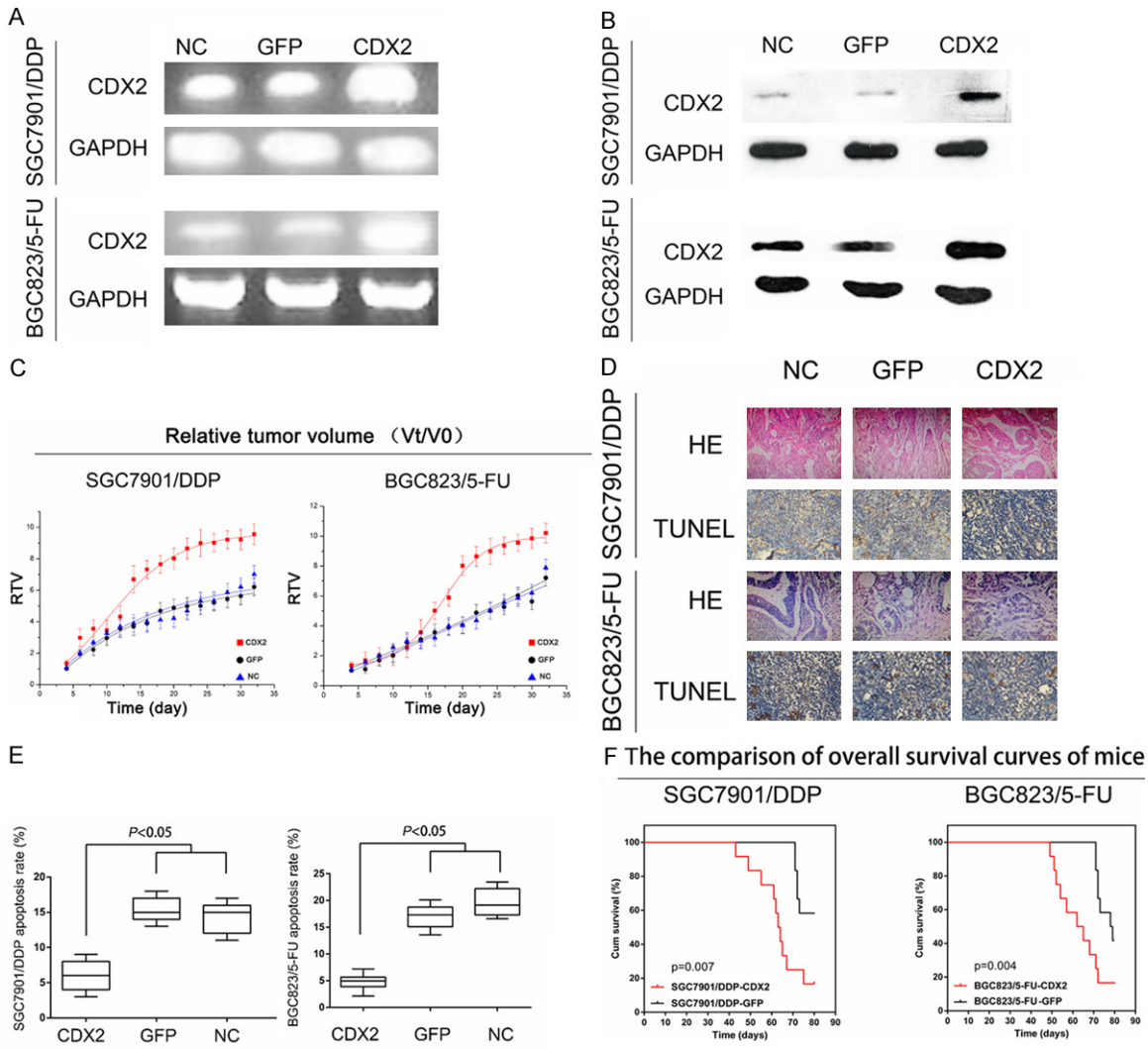

Figure 4. Effect of LV-CDX2-GFP-mediated promotion of CDX2 mRNA and protein expression and up-regulation of CDX2 on apoptosis *in vivo*. A: mRNA expression level of CDX2 was determined by semiquantitative reverse-transcriptase polymerase chain reaction; B: Protein expression level of CDX2 was determined by western blotting; C: RTV of nude mice in each group is presented. Each time point represents the mean RTV for each group. D: Tumor cell was assessed by HE staining and TUNEL assay (× 400); E: Percentage of apoptotic cells was analyzed by TUNEL assay. All values are mean ± SE. $^{*}P < 0.05$ for CDX2 groups *vs* GFP groups and NC groups. F: The combination of CDX2-overexpression lentiviral vector (LV-CDX2-GFP) increased the probability of a poor overall survival.

reciprocal immunoprecipitation assays, endogenous CDX2 and MRP were found to interact with each other in CDX2 group (**Figure 3C**). Furthermore, in an pull-down assay using purified proteins, 6xHis-tagged MRP (His- MRP) bound to a GST fusion of CDX2, but not GST alone, indicating that CDX2 and MRP maybe associate with each other directly.

Effect of LV-CDX2-GFP on promoting MDR of human gastric cancer in vivo

We examined the effect of LV-CDX2-GFP on growth of SGC7901/DDP and BGC823/5-FU groups *in vivo*, by implanting LV-CDX2-GFP and LV-GFP subcutaneously into the flanks of BALB/c nude mice. We detected expression levels of CDX2 *in vivo* by semi-quantitative RT-PCR and Western blotting. The mRNA (**Figure 4A**) and protein (**Figure 4B**) expression level of CDX2 in CDX2 groups were higher than that in GFP groups and NC groups. Five weeks after implantation, RTV in the CDX2 groups were significantly more than in the NC and GFP groups (**Figure 4C**). As determined by the HE staining and TUNEL assay (**Figure 4D**). As shown in **Figure 4E**, the percentage of apoptotic tumor cells in CDX2 groups was less than

that in GFP groups and NC groups (Apoptosis rate analysis of CDX2 vs GFP and NC groups in SGC7901/DDP cells: 7.21% ± 1.86% vs 16.25% ± 3.18% and 14.65% ± 2.85%, $P < 0.001$; Apoptosis rate analysis of CDX2 vs GFP and NC groups in BGC823/5-FU cells: 5.11% ± 1.26% vs 17.34% ± 2.28% and 18.66% ± 2.75%, $P < 0.001$). In addition, mice injected with LV-CDX2-GFP cells had a significantly lower survival rate (survival rate analysis of CDX2 vs GFP mice in SGC7901/DDP cells: 18.9% vs 60.2%; $P = 0.007$; Survival rate analysis of CDX2 vs GFP mice in BGC823/5-FU cells: 17.8% vs 41.2%; $P=0.004$, with 80 days as the cut-off).

Discussion

The development of MDR to cancer chemotherapy is a major obstacle to the effective treatment of gastric cancer [16]. However, the mechanism of MDR remains obscure. Several laboratories have studied the mechanism(s) of MDR in the past decades and many possible mechanism(s) have been identified. These resistance mechanism(s) appears to fall into four major categories. The first category involves DNA damage/repair proteins. The second category involves drug retention (increased influx or decreased uptake). The third category involves increased drug inactivation or prevention of drug to reach the DNA target. The fourth category involves growth signaling via different pathways or increase in antiapoptotic protein(s) [17-23].

In our result, MDR1, MRP, mTOR and HIF-1α expression were all increment when CDX2 was up-regulated. It is generally accepted that after DNA damage by cancer chemotherapy, the surviving cells have to develop the ability to generate repair proteins and/or survival proteins to prepare for the next insult. Both ribosomal proteins and elongation factor are essential for translational process in protein synthesis. Consequently, if ribosomal proteins and/or elongation factor cannot be inhibited, one should be able to promote the development of multidrug resistant. It is well known that mammalian target of rapamycin (mTOR) is important in regulating translation of a set of mRNA which encode ribosomal proteins and elongation factor [24, 25]. Besides mTOR, other genes can also modulate other forms of drug resistance such as P-gp1 or MDR1 mediated drug resistance, MDR1 is a well characterized form of drug resistance which is primarily due to overexpression of an P-gp1 efflux pump [26]. This efflux pump belong to ABC (ATP-binding-cassette) transporter superfamily, and is capable of effluxing many different chemotherapeutic agents, hence the multidrug resistance. The resistance is due to decrease drug accumulation. CDX2 down-regulation has been shown to be able to reverse this form of drug resistance by blocking the efflux pump [11]. Another similar form of multidrug resistance which is due to decrease drug accumulation is the MRP1 mediated drug resistance. MRP1 also belongs to the ABC transporter superfamily, however, this efflux pump most likely transports the glutathione conjugated drug [27]. In addition, Riganti et al. found that increased intracellular calcium concentration ($[Ca^{++}]_i$) have been correlated with P-gp expression, it means that, by increasing $[Ca^{++}]_i$ may regulate the transcription of the MDR1 gene and enhance the expression of P-gp [28]. because increasing $[Ca^{++}]_i$ is known also to activate the transcription factor hypoxia-inducible factor-1 (HIF-1α) [29], which controls several genes involved in cellular growth, glucose and iron metabolism, pH control, angiogenesis and matrix remodelling, and is also involved in P-gp up-regulation [30, 31].

Besides regulation of mTOR/HIF-1α/P-gp and MRP1 Signaling Pathway, apoptosis is also a common pathway that finally mediates the killing effects of anticancer drugs, which is an important cause of MDR, in our result, phosphatase and tensin homolog (PTEN), caspase-9 and caspase-3 expression were all decrement when CDX2 was up-regulated. The PTEN protein product is a lipid phosphatase that antagonizes PI3K function and consequently inhibits downstream signaling transduction through Akt. Caspase-9, a member of the protease family, is intimately associated with the initiation of apoptosis, and is thought to be activated while Akt is inhibited. Activated caspase-9 is able to cleave caspase-3 in vitro, leading to apoptosis [32-34], in our result, CDX2 was up-regulated leading to anti-apoptosis, and then, up-regulation of CDX2 promotes the development of multidrug resistant in gastric cancer via inhibition of apoptosis in gastric cancer cells.

In summary, we demonstrated that up-regulation of CDX2 significantly inhibited sensitivity of SGC7901/DDP cells to anticancer drugs, and decreased the percentage of apoptotic cells. Up-regulation of CDX2 potentiated S phase arrest of the cell cycle. Furthermore, it signifi-

cantly decreased intracellular accumulation of doxorubicin. We conclude that up-regulation of CDX2 promotes the development of multidrug resistant in gastric cancer *via* inhibition of apoptosis related gene expression (Caspase-3, Caspase-9 and PTEN), and increases the expression of multidrug resistant related gene (MDR1, MRP, mTOR and HIF-1α). Our results provide evidence that CDX2 may potentially be used as a predictor of chemotherapy response in gastric cancer, and it is a promising therapeutic target in treatment for MDR-gastric cancer.

Acknowledgements

We acknowledge financial support from the Natural Science Foundation of China, No. 81060201 and No. 81160289; Natural Science Foundation of Guangxi, No. 2013GXNSFAA019163; and the Key Health Science Foundation of Guangxi, No. 1298003-2-6 and No. 14124004-1-9.

Disclosure of conflict of interest

None to declare.

Address correspondence to: Dr. Qiang Xiao or Yu-Bo Xie, Department of Surgery, The First Affiliated Hospital of Guangxi Medical University, No.22 Shuang Yong Street, Nanning 530021, Guangxi Zhuang Autonomous Region, China. Tel: +86-771-5358325; Fax: +86-771-5358325; E-mail: xiaoqiang20050@aliyun.com

References

[1] Amiri M. Stomach cancer mortality in the future: where are we going? Int J Prev Med 2011; 2: 101-102.

[2] Fan D, Liu X. New progresses in researches on multidrug resistance in gastric cancer. Chin J Digest 2000; 20: 77-78.

[3] Dai Z, Huang Y, Sadee W. Growth factor signaling and resistance to cancer chemotherapy. Curr Top Med Chem 2004; 4: 1347-1356.

[4] Grimminger P, Ling FC, Neiss S, Vallbohmer D, Lurje G, Schneider PM, et al. The role of the homeobox genes BFT and CDX2 in the pathogenesis of non-small cell lung cancer. Anticancer Res 2009; 29: 1281-1286.

[5] Witek ME, Nielsen K, Walters R, Hyslop T, Palazzo J, Schulz S, Waldman SA. The putative tumor suppressor Cdx2 is overexpressed by human colorectal adenocarcinomas. Clin Cancer Res 2005; 11: 8549-8556.

[6] Dang LH, Chen F, Ying C, Chun SY, Knock SA, Appelman HD, Dang DT. CDX2 has tumorigenic potential in the human colon cancer cell lines LOVO and SW48. Oncogene 2006; 25: 2264-2272.

[7] Mutoh H, Sakurai S, Satoh K, Tamada K, Kita H, Osawa H, Tomiyama T, Sato Y, Yamamoto H, Isoda N, Yoshida T, Ido K, Sugano K. Development of gastric carcinoma from intestinal metaplasia in Cdx2-transgenic mice. Cancer Res 2004; 64: 7740-7747.

[8] Almeida R, Silva E, Santos-Silva F, Silberg DG, Wang J, De Bolos C, David L. Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. J Pathol 2003; 199: 36-40.

[9] Takakura Y, Hinoi T, Oue N, Sasada T, Kawaguchi Y, Okajima M, Akyol A, Fearon ER, Yasui W, Ohdan H. CDX2 regulates multidrug resistance 1 gene expression in malignant intestinal epithelium. Cancer Res 2010; 70: 6767-6778.

[10] Dupuis ML, Ascione A, Palmisano L, Vella S, Cianfriglia M. Raltegravir does not revert efflux activity of MDR1-P-glycoprotein in human MDR cells. BMC Pharmacol Toxicol 2013; 14: 47.

[11] Yan LH, Wang XT, Yang J, Lian C, Kong FB, Wei WY, Luo W, Xiao Q, Xie YB. Reversal of multidrug resistance in gastric cancer cells by CDX2 downregulation. World J Gastroenterol 2013; 19: 4155-4165.

[12] Xiao Q, Li L, Xie Y, Tan N, Wang C, Xu J, Xia K, Gardner K, Li QQ. Transcription factor E2F-1 is upregulated in human gastric cancer tissues and its overexpression suppresses gastric tumor cell proliferation. Cell Oncol 2007; 29: 335-349.

[13] Song M, Zang W, Zhang B, Cao J, Yang G. GCS overexpression is associated with multidrug resistance of human HCT-8 colon cancer cells. J Exp Clin Cancer Res 2012; 31: 23.

[14] Huang M, Wang HQ, Zhang Q, Yan XD, Hao M, Luo ZJ. Alterations of ADAMTSs and TIMP-3 in human nucleus pulposus cells subjected to compressive load: Implications in the pathogenesis of human intervertebral disc degeneration. J Orthop Res 2012; 30: 267-273.

[15] Du W, Jiang P, Li N, Mei Y, Wang X, Wen L, Yang X, Wu M. Suppression of p53 activity by Siva1. Cell Death Differ 2009; 16: 1493-1504.

[16] Chuman Y, Sumizawa T, Takebayashi Y, Niwa K, Yamada K, Haraguchi M, et al. Expression of the multidrug-resistance-associated protein (MRP) gene in human colorectal, gastric and non-small-cell lung carcinomas. Int J Cancer 1996; 66: 274-279.

[17] Asselin E, Mills GB, Tsang BK. XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. Cancer Res 2001; 61: 1862-1868.

[18] Lee KB, Parker RJ, Bohr V, Cornelison T, Reed E. Cisplatin sensitivity/resistance in UV repair-deficient Chinese hamster ovary cells of complementation groups 1 and 3. Carcinogenesis 1993; 14: 2177-2180.
[19] Samimi G, Katano K, Holzer AK, Safaei R, Howell SB. Modulation of the cellular pharmacology of cisplatin and its analogs by the copper exporters ATP7A and ATP7B. Mol Pharmacol 2004; 66: 25-32.
[20] Murata T, Haisa M, Uetsuka H, Nobuhisa T, Ookawa T, Tabuchi Y, Shirakawa Y, Yamatsuji T, Matsuoka J, Nishiyama M, Tanaka N, Naomoto Y. Molecular mechanism of chemoresistance to cisplatin in ovarian cancer cell lines. Int J Mol Med 2004; 13: 865-868.
[21] Hayakawa J, Depatie C, Ohmichi M, Mercola D. The activation of c-Jun NH2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair. J Biol Chem 2003; 278: 20582-20592.
[22] Yuan ZQ, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance. J Biol Chem 2003; 278: 23432-23440.
[23] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003; 22: 7265-7279.
[24] Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. Nat Rev Cancer 2004; 4: 335-348.
[25] Terada N, Patel HR, Takase K, Kohno K, Nairn AC, Gelfand EW. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. Proc Natl Acad Sci U S A 1994; 91: 11477-11481.
[26] Minchinton AI, Tannock IF. Drug penetration in solid tumours. Nat Rev Cancer 2006; 6: 583-92.
[27] Cole SP. Targeting Multidrug Resistance Protein 1 (MRP1, ABCC1): Past, Present, and Future. Annu Rev Pharmacol Toxicol 2013; 54: 95-117.
[28] Riganti C, Doublier S, Viarisio D, Miraglia E, Pescarmona G, Ghigo D, Bosia A. Artemisinin induces doxorubicin resistance in human colon cancer cells via calcium-dependent activation of HIF-1αlpha and P-glycoprotein overexpression. Br J Pharmacol 2009; 156: 1054-1066.
[29] Yuan G, Nanduri J, Bhasker CR, Semenza GL, Prabhakar NR. Ca2+/calmodulin kinase-dependent activation of hypoxia inducible factor 1 transcriptional activity in cells subjected to intermittent hypoxia. J Biol Chem 2005; 280: 4321-4328.
[30] Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res 2002; 62: 3387-3394.
[31] O'Donnell JL, Joyce MR, Shannon AM, Harmey J, Geraghty J, Bouchier-Hayes D. Oncological implications of hypoxia inducible factor-1alpha (HIF-1αlpha) expression. Cancer Treat Rev 2006; 32: 407-416.
[32] Shultz JC, Goehe RW, Wijesinghe DS, Murudkar C, Hawkins AJ, Shay JW, Minna JD, Chalfant CE. Alternative splicing of caspase 9 is modulated by the phosphoinositide 3-kinase/Akt pathway via phosphorylation of SRp30a. Cancer Res 2010; 70: 9185-9196.
[33] Yamakawa N, Takahashi A, Mori E, Imai Y, Furusawa Y, Ohnishi K, Kirita T, Ohnishi T. High LET radiation enhances apoptosis in mutated p53 cancer cells through Caspase-9 activation. Cancer Sci 2008; 99: 1455-1460.
[34] Chappell WH, Steelman LS, Long JM, Kempf RC, Abrams SL, Franklin RA, Bäsecke J, Stivala F, Donia M, Fagone P, Malaponte G, Mazzarino MC, Nicoletti F, Libra M, Maksimovic-Ivanic D, Mijatovic S, Montalto G, Cervello M, Laidler P, Milella M, Tafuri A, Bonati A, Evangelisti C, Cocco L, Martelli AM, McCubrey JA. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget 2011; 2: 135-164.