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Emodin enhances sensitivity of gallbladder cancer cells to platinum drugs via glutathion depletion and MRP1 downregulation

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ABSTRACT

Glutathione conjugation and transportation of glutathione conjugates of anticancer drugs out of cells are important for detoxification of many anticancer drugs. Inhibition of this detoxification system has recently been proposed as a strategy to treat drug-resistant solid tumors. Gallbladder carcinoma is resistant to many anticancer drugs, therefore, it is needed to develop a novel strategy for cancer therapy. In the present study, we tested the effect of emodin (1,3,8-trihydroxy-6-methylanthraquinone), a reactive oxygen species (ROS) generator reported by our group previously, in combination with cisplatin (CDDP), carboplatin (CBP) or oxaliplatin in treating the gallbladder carcinoma cell line SGC996. Our results showed that co-treatment with emodin could remarkably enhance chemosensitivity of SGC996 cells in comparison with cisplatin, carboplatin or oxaliplatin treatment alone. We found that the mechanisms may be attributed to reduction of glutathione level, and downregulation of multidrug resistance-related protein 1 (MRP1) expression in SGC996 cells. The experiments on tumor-bearing mice showed that emodin/cisplatin co-treatment inhibited the tumor growth in vivo via increasing tumor cell apoptosis and downregulating MRP1 expression.

In conclusion, emodin can work as an adjunct to enhance the anticancer effect of platinum drugs in gallbladder cancer cells via ROS-related mechanisms.

1. Introduction

Gallbladder carcinomas are highly malignant human cancers [1,2]. For patients with unresectable tumors, the expected survival is dismal. Generally, the role of systemic chemotherapy in the palliation of gallbladder carcinomas is undefined [3]. But recently, a retrospective review suggests that adjuvant chemoradiotherapy using 5-fluorouracil concurrently with radiotherapy in patents who underwent surgical resection of gallbladder carcinoma may improve overall survival [4]. And the combination of gemcitabine and oxaliplatin has promising results in patients with advanced biliary tract carcinomas [5,6]. Therefore, it is necessary to search effective chemotherapeutic agents or alternative chemotherapies for these gallbladder cancers, either alone or in combination.

The major platinum-containing drugs are cisplatin, carboplatin and oxaliplatin. They are widely used anticancer agents in the treatment of various solid tumors, but have not been proven effective for gallbladder cancers [7]. Insufficient sensitivity or resistance to platinum agents is usually the major obstacle for their effective application in cancers including gallbladder cancers. The mechanisms for platinum drug resistance have been referred to decrease the intracellular drug accumulation, increase the levels of cellular thiols and increase the nucleotide excision-repair activity [8,9]. Reverse of these mechanisms may show benefits for enhancing platinum cytotoxicity in gallbladder cancers, however, effective synergistic approaches have not been studied adequately.

We have previously demonstrated that emodin (1,3,8-trihydroxy-6-methylanthraquinone), a kind of natural anthraquinone enriched in the traditional Chinese herbal medicines, facilitates arsenic trioxide-induced apoptosis in various cancer cell types,

Abbreviations: Emodin, 1,3,8-trihydroxy-6-methylanthraquinone; ROS, reactive oxygen species; MDR, multidrug resistance; MRP, multidrug resistance-related protein; HDF, human dermal fibroblasts; CDDP, cisplatin; CBP, carboplatin; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DCF, 2,7-dichlorofluorescein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AnnexinV-FITC, AnnexinV-fluorescein isothiocyarate; PI, propidium iodide; GSH, glutathione; GS-Pt, glutathione S–platinum; GS-X pump, ATP-dependent glutathione S-conjugate export.

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both in vitro and in vivo via the mechanism of generation of reactive oxygen species (ROS) [10–12]. We have also found that emodin sensitizes Du-145, a cell line derived from prostate carcinoma, to cisplatin in ROS-dependent manner [13]. In addition, we found that the multidrug resistance protein MDR1 is downregulated and its transcription factor, hypoxia inducible factor-1 (HIF-1), is inhibited. In the present study we investigated whether emodin could synergize with cisplatin and other two platinum drugs in gallbladder cancer cells. We found that emodin exerted enhancing action to anticancer efficacy of cisplatin, carboplatin and oxaliplatin via decrease of reduced glutathione, and inhibition of expression of MRP1, a drug efflux pump other than MDR1.

Glutathione (GSH) conjugation and transportation of GSH conjugates of anticancer drugs out of cells have been proposed for long time as a system in the detoxification of many anticancer drugs [14]. The major components of this system include GSH, GSH-related enzymes and GSH conjugate export pump (GS-X pump), and all of them are found increased or overexpressed in many drug-resistant cancer cells [14]. MRP1 has been reported to be a member of the GS-X pump based on evidence that MRP1 transports Leukotriene C4 [15]. Inhibition of this detoxification system is a reasonable strategy for modulation of drug resistance. Recently, some small molecule inhibitors of GSH have been shown to result in a significant enhancement in cisplatin cytotoxicity [16]. Our present study proposed that the small natural compound can target to MRP1, in addition to the depletion of GSH, to enhance cisplatin, carboplatin or oxaliplatin cytotoxicity in gallbladder cancer cells.

2. Materials and methods

2.1. Cells and reagents

The human gallbladder cancer cell line SGC996 was provided by Academy of Life Sciences, Tongji University (Shanghai, China). The prostate carcinoma-derived DU-145 cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA). SGC996 and DU-145 cells were maintained in RPMI-1640 medium (GibcoBRL, Gaitherburg, MD, USA). Normal human dermal fibroblasts (HDF), preserved in our laboratory, were maintained in DMEM medium (GibcoBRL, Gaitherburg, MD, USA). These media were supplemented with antibiotics and 10% newborn calf serum. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. Cisplatin (CDDP) and carboplatin (CBP) were obtained from Qilu Pharmaceutical Co., Ltd. (Ji Nan, China). Oxaliplatin was obtained from Jiangsu Hengrui Medicine Co., Ltd. (Lian Yungang, China). Emodin was purchased from Sigma (St. Louis, MO, USA).

2.2. Cell viability assay

Cells were seeded at 1.5×10^4/ml cells per well in 96-microculture-well plates. After exposed to the agents as indicated for 24 h, cell viability was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) assay as previously described [11,12,17].

2.3. Cell apoptosis analysis

Cells were treated with drugs for 24 h and apoptotic rates were assessed with flow cytometry using AnnexinV-fluorescein isothiocyarate (AnnexinV-FITC)/propidium iodide (PI) kit (BD Pharmingen, San Diego, CA, USA). Samples were prepared according to the manufacturer's instruction and analyzed by flow cytometry on FACS Calibur (Becton Dickson, San Diego, CA, USA) [11,12,17].

2.4. ROS measurement

2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, St. Louis, MO, USA) was used as ROS capture in the cells. The average fluorescent intensity of 2,7-dichlorofluorescein (DCF) stands for intracellular ROS levels [11,12,17]. Cultured cells were exposed to various drugs and 10 μM of DCFH-DA at 37 °C for 15 min. After washed once with ice-cold PBS, cells were harvested and kept on ice for an immediate detection by flow cytometer FACS Calibur.

2.5. GSH measurement

Cells were treated with drugs for 12 h and prepared according to the instructions of the GSH assay kit (Jiancheng Bioengineering Institute, Nan Jing, China). Briefly, cells were sonicated on ice and the homogenates were mixed with the kit provided reagents and then centrifuged at $4000 \times g$ for 10 min. Five hundred microliters of supernatant was mixed subsequently with the kit provided reagents and incubated at room temperature for 5 min before being read at OD_{420}, thus the values of OD_{420} reflect the level of GSH. The GSH content of the samples was determined by comparison with an external standard which was prepared according to the instructions. All the results obtained were normalized according to the cellular protein content, which was measured using the BCA protein assay kit (Pierce Biotechnology, Rockford, USA).

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of the *MRP1*, *MRP2* and *MDR1* were monitored by RT-PCR. SGC996 and DU-145 cells were lysed with 1 ml of RNAse-clean Trizol reagent (Invitrogen, Carlsbad, CA, USA) after treatment or no, and then the samples were processed according to the manufacturer's protocol to obtain total cellular RNA. One microgram of the isolated total RNA was reverse-transcribed using random primers and AMV reverse transcriptase (Promega, Madison, WI, USA) for 5 min at 70 °C, 5 min on ice and 60 min at 37 °C. The single-stranded cDNA was amplified by polymerase chain reaction using GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR of *MRP1*, *MRP2* gene was performed under the following conditions: 30 s, 94 °C; 30 s, 58 °C; 30 s, 72 °C; 34 cycles. The sequences for *MRP1* sense and antisense primers were 5′-TGGTGGGCCTCTCAGTGTCTTA-3′ and 5′-TCGGTAGCGCAGGCAGTAGTTC-3′. The sequences for *MRP2* sense and antisense primers were 5′-ATGCTTCCTGGGGATAAT-3′ and 5′-TCAAAGGCACGGATAACT-3′. PCR of *MDR1* gene was performed under the following conditions: 20 s, 94 °C; 30 s, 55 °C; 60 s, 72 °C, 35 cycles. The sequences for *MDR1* sense and antisense primers were 5′-CCCATCATTGCAATAGCAGG-3′ and 5′-GTTCAAACTTCTGCTCCTGA-3′. Equal amounts of RT-PCR products were loaded on 1.5% agarose gels respectively. GAPDH was used as an internal control. The sequences for GAPDH sense and antisense primers were 5′-TGGGGAAGGTGAAGGTCGG-3′ and 5′-CTGGAAGATGGTGATGGGA-3′.

2.7. MRP1 cDNA and siRNA transfection

To determine the role of MRP1 in cellular sensitivity to cisplatin, MRP1 cDNA plasmid (kindly provided by Susan P.C. Cole, Queen's University at Kingston, Canada) or siRNA oligonucleotides were transiently transfected, using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A nonsense plasmid or non-specific siRNA were also transfected as mock. Forty-eight hours later, SGC996 cells were lysed for RT-PCR or exposed to CDDP for an additional 24 h before apoptosis assay. The sequences of siRNA for MRP1 were 5′-CGAUGAAGACCAAGACGUAUU-3′ and 3′-UUGCUACUUCUGGUUCUGCAU-5′.

2.8. In vivo study in tumor-bearing mice

SGC996 cells were harvested, washed, and resuspended in serum-free optimum medium and then injected subcutaneously into 6-week old BALB/c-nu/nu mice, with 6×10^6 cells per mouse ($n = 8$ mice per group, purchased from Shanghai Experimental Animal Center, Shanghai, China). Three days after inoculation, the tumor-bearing mice were intraperitoneally administered with dissolvent, emodin (50 mg/kg), CDDP (1 mg/kg), emodin/CDDP every day. The mice were sacrificed after 18 days, and body weight and tumor weight were measured. Hearts, kidneys and livers of the mice were histologically examined to determine the systemic toxicity. TUNEL [terminal deoxynucleotidyl transferase(TdT)-mediated dUTP nick end label] assay (ApopTag Peroxidase Kit, Intergen, New York, NY, USA) was performed on paraformaldehyde-fixed and paraffin-embedded tumor sections, using the methods described previously [11].

2.9. In situ hybridization for MRP1 expression in tumors

The sequence of digoxiginin-labeled single-stranded RNA probe for MRP1 was: 5′-TAAGACACTGAGAGGCCCACCA-3′. Sections of the tumor tissue were de-paraffined and re-hydrated before incubation with Protease K at 37 °C for 15 min. Sections were then washed in 0.1 M Tris-buffered saline/diethyl pyrocarbonat (TBS/DEPC) for 5 min ×3, incubated with 5× standard sodium citrate (SSC) solution at RT for 15 min and incubated with RNA probe sequentially. After 48 h hybridization at 37 °C, the sections were washed with graded diluted SSC solutions, all at 37 °C for 15 min. Then the sections were incubated with anti-digoxiginin antibody (Roche Diagnostics GmbH, Mannheim, Germany) at RT for 3 h, washed with 0.5 M TBS and 0.01 M TBS (pH 9.5). Hybridization signal was visualized by 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) (Sigma, St. Louis, MO, USA). Sections were finally counterstained by nuclear fast red.

2.10. Statistical analysis

Data were shown as mean values ± S.E. SPSS11.5 software was used for statistical analysis. ANOVA (analysis of variance) was applied for comparison of the means of two or multiple groups, in which SNK (Student–Newman–Kewls) was further used for comparison of each two group. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Emodin enhances CDDP-induced inhibition of cell viability in tumor cells but not in normal cells

To examine the synergistic effect of emodin on cell viability, human gallbladder cancer cells SGC996 were treated with CDDP or

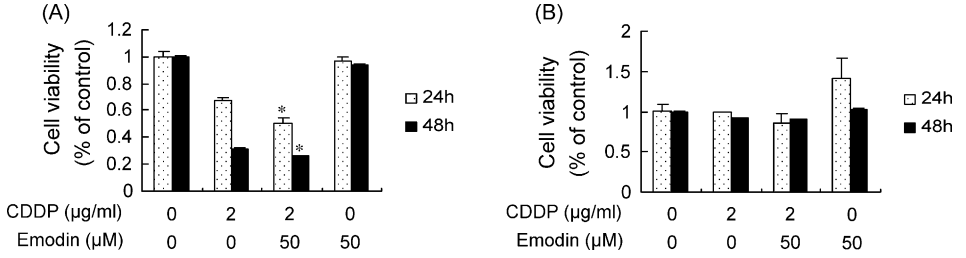

Fig. 1. Cell viability in SGC996 cells and human skin fibroblasts (HSF). Cells were exposed to CDDP alone, emodin alone or CDDP/emodin co-treatment. (A) Cell viability in SGC996 cells (MTT, drug treatments for 24 h or 48 h). Columns, mean of nine replicates from three experiments; bars, S.E. *$p < 0.05$, combination treatment group compared with CDDP-alone group. (B) Cell viability in HSF (MTT, drug treatments for 24 h or 48 h).

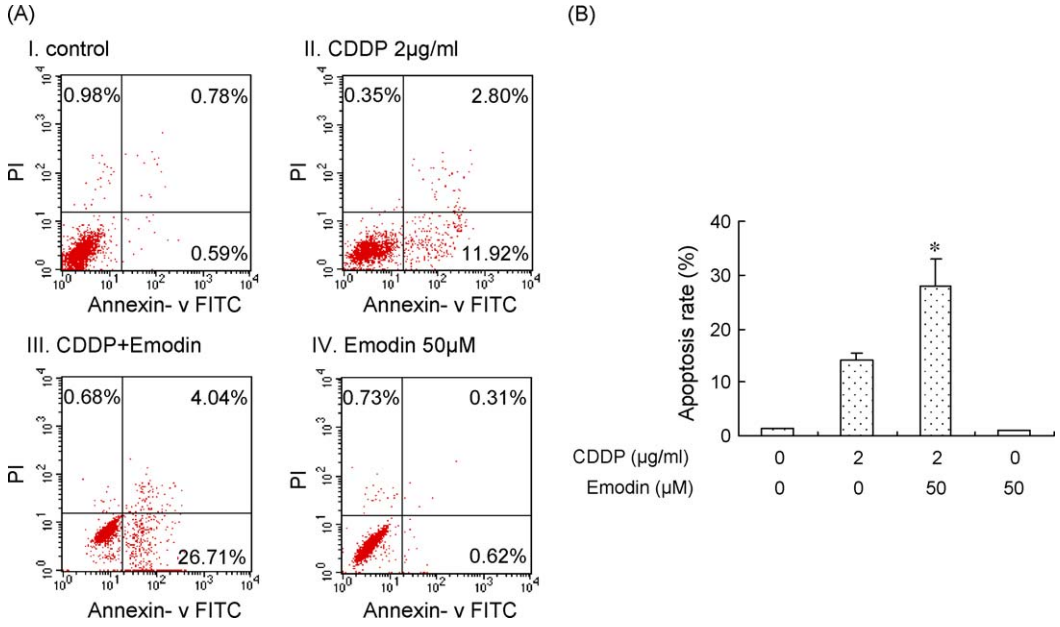

Fig. 2. Apoptosis in SGC996 cells. Apoptosis rate analysis using Annexin V/propidium iodide flow cytometry in SGC996 cells treated with CDDP alone, emodin alone and CDDP/emodin co-treatment for 24 h. Density plots (A); bar chart (B). Columns, mean of three experiments; bars, S.E. *$p < 0.05$, combination treatment group compared with CDDP-alone group.

emodin alone, or co-treated. Although no obvious reduction in viable cell number was observed in emodin treated alone group, co-treatment with CDDP led to a significant reduction of cell viability at both 24 h and 48 h, compared with CDDP treatment alone (Fig. 1A).

The normal human dermal fibroblasts were selected as representative for nontumor cells. Notably, we do not observe cell viability repression in CDDP/emodin co-treatment group in comparison with SGC996 cells under the same conditions (Fig. 1B). These results indicated that the enhancement of the toxicity by emodin co-treatment was relatively selective for tumor cells.

3.2. Emodin enhances CDDP-induced apoptosis of SGC996 cells

To determine whether the viability repression was attributed to the increase of apoptosis, Annexin V-FITC/PI double labeling flow cytometry was conducted. As shown in Fig. 2, CDDP really caused cell apoptosis, and emodin used at indicated dose could markedly enhance CDDP-induced apoptosis at 24 h.

3.3. Emodin/CDDP co-treatment downregulates expression of multidrug resistance gene MRP1 that is responsible for blockade of cisplatin cytotoxicity

It has been reported that overexpression of multidrug resistance genes such as MDR1, MRP1 and MRP2 often render cancer cells a constitutive characteristic of multidrug resistance [9,18], therefore inhibition of their expression may lead to the increase of sensitivity to anticancer drugs. We then questioned whether the observed effects of CDDP/emodin could be correlated to regulating expression of these multidrug resistance genes. Results from RT-PCR showed that SGC996 cells expressed both MRP1 and MRP2 at higher levels, but the MDR1 expression was low to an undetectable level (Fig. 3A). CDDP alone downregulated the expression of MRP1 and MRP2, while CDDP/emodin co-treatment resulted in an additive effect on downregulating expression of MRP1, but not MRP2 (Fig. 3B).

To determine whether MRP1 expression is responsible for cytotoxic sensitivity of SGC996 cells to CDDP, cells were transfected with a plasmid encoding whole MRP1 gene or siRNA oligonucleotide for silencing MRP1 expression, and then were treated with CDDP for 24 h. Results showed that exogenous overexpression of MRP1 in SGC996 cells could impede CDDP-induced apoptosis (Fig. 3C), while knockdown of MRP1 could increase cell apoptosis (Fig. 3D). These data suggested that MRP1 was responsible for CDDP resistance in SGC996 cells, and emodin might facilitate cytotoxicity of CDDP through suppressing MRP1 expression.

3.4. Emodin elicits an immediate elevation of cellular ROS level and leads to a sustained decrease of cellular GSH level

Emodin is able to generate ROS in a variety of tumor cells [10–13]. We found that exposure of SGC996 cells to emodin or emodin/CDDP resulted in an immediate elevation of cellular ROS level, while CDDP treatment alone did not have this effect (Fig. 4A). To study oxidative impact of emodin on cellular redox state, we measured the cellular GSH level after exposing SGC996 cells to CDDP, emodin or CDDP/emodin respectively for 12 h. As shown in Fig. 4B, emodin alone or in combination with CDDP could remarkably reduce the cellular GSH level, whereas CDDP caused a slight elevation of cellular GSH level. These data indicated that the synergistic effect of emodin on enhancement of CDDP-induced cytotoxicity was related to its depletion of GSH in cells.

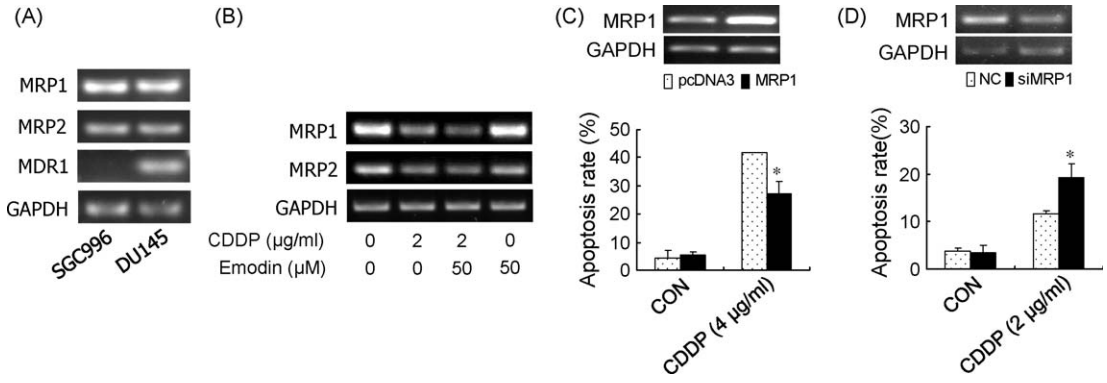

Fig. 3. Multidrug resistance in SGC996 cells. (A) Expression of MRP1, MRP2 and MDR1. (B) Expression of MRP1 and MRP2. Cells were exposed to CDDP alone, emodin alone and CDDP/emodin co-treatment for 24 h before harvested for RT-PCR. (C) Apoptosis in SGC996 cells. Cells were transfected with pcDNA3 or MRP1 plasmid for 48 h. Apoptosis rate analysis using Annexin V/propidium iodide flow cytometry in SGC996 cells transfected with MRP1 plasmid after treated with CDDP for 24 h. Columns, mean of three experiments; bars, S.E. $^*p < 0.05$, MRP1/CDDP group compared with pcDNA3/CDDP group. (D) Apoptosis in SGC996 cells. Cells were transfected with non-specific siRNA (NC) or MRP1 siRNA for 48 h. Apoptosis rate analysis using Annexin V/propidium iodide flow cytometry in SGC996 cells transfected with MRP1 siRNA after treated with CDDP for 24 h. Columns, mean of three experiments; bars, S.E. $^*p < 0.05$, siMRP1/CDDP group compared with NC/CDDP group.

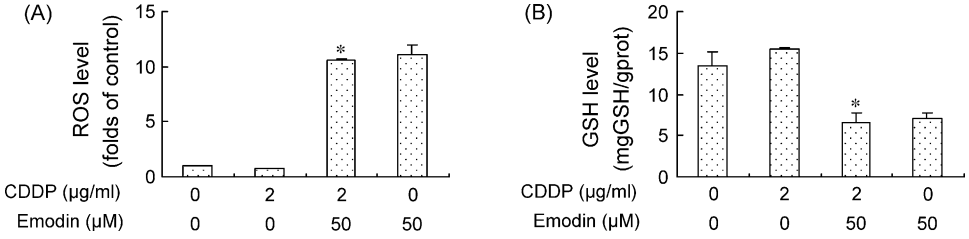

Fig. 4. ROS and GSH in SGC996 cells. Cells were exposed to CDDP alone, emodin alone and CDDP/emodin co-treatment. (A) ROS level in SGC996 cells (DCF flow cytometry, drug treatments for 15 min). Columns, mean of three experiments; bars, S.E. $^*p < 0.05$, combination treatment group compared with CDDP-alone group. (B) GSH level in SGC996 cells (GSH analysis kit, drug treatments for 12 h). Columns, mean of three experiments; bars, S.E. $^*p < 0.05$, combination treatment group compared with CDDP-alone group.

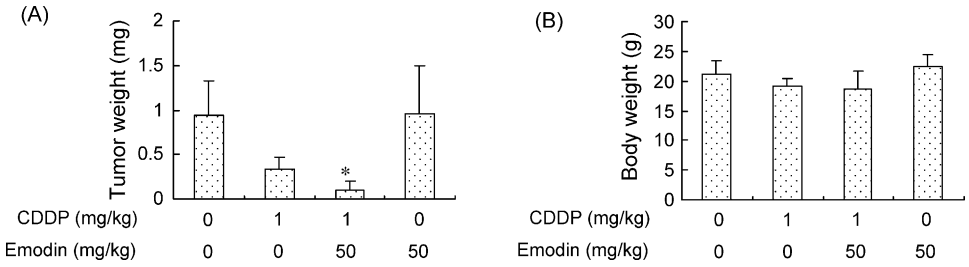

Fig. 5. Growth capability of transplanted tumors formed by SGC996 cells. The tumor-bearing mice were injected intraperitoneally with dissolvent, 50 mg/kg emodin alone, 1 mg/kg CDDP alone and CDDP/emodin coadministration. (A) Average weight of transplanted tumors after the mice were exposed to treatments. Columns, mean; bars, S.E. $^*p < 0.05$, combination treatment group compared with CDDP-alone group ($n = 8$). (B) Average body weight of tumor-bearing mice.

3.5. Emodin markedly sensitizes the tumor xenografts to CDDP cytotoxicity without displaying obvious systemic toxicity in vivo

The above in vitro experiments showed that multidrug-resistant phenotype of SGC996 could be partially overcome by using emodin in combination with chemotherapeutic drug CDDP. To verify this effect in vivo and evaluate its systemic efficacy and side effects, SGC996 cells were transplanted into nude mice and the mice were synchronously administered with emodin in combination with CDDP for 18 days. Our results showed that mice exposed to the combinative therapy had significantly smaller tumors than mice in other groups (Fig. 5A). While tumor cytotoxicity was strikingly enhanced by the combinative treatment, the systemic toxic effects were evaluated by examining the pathological changes of the major organs and the body weight loss of mice. No notable differences were observed among these groups (Fig. 5B and data not shown), demonstrating that emodin/CDDP co-treatment had no obvious toxic effects on normal tissues in vivo.

3.6. Emodin/CDDP co-treatment represses the expression of MRP1 and promoted cell apoptosis in tumors

To ascertain the action of emodin/CDDP co-treatment on MRP1 expression in vivo, in situ hybridization for MRP1 mRNA was performed on paraffin-embedded tissue sections of tumors. As shown in Fig. 6A, the expression of MRP1 in tumors was downregulated by CDDP, and, in particular, more significantly by emodin/CDDP combinative treatment. Meanwhile, the in situ TUNEL assay revealed that combinative therapy obviously promoted tumor cell apoptosis (Fig. 6B), which explained why these tumors in the co-treatment group had much smaller volume than those in the group of single CDDP treatment.

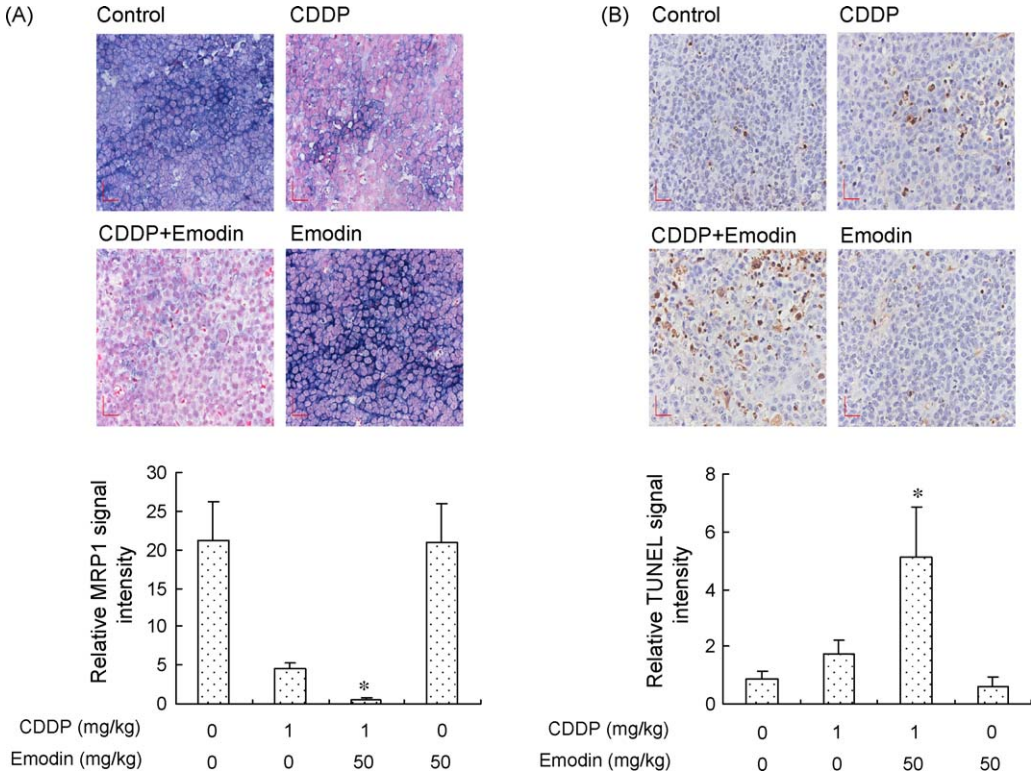

Fig. 6. Expression of MRP1 mRNA in transplanted tumor tissues and the toxic effects of therapy to normal tissues in vivo. (A) Expression of MRP1 mRNA in the tumor tissues. The cytoplasmic blue–purple staining represented positive hybridization signal for MRP1 mRNA, and the nucleus were stained by fast red. Columns, mean of three experiments; bars, S.E. $^*p < 0.05$, combination treatment group compared with CDDP-alone group. Scale bar: 30 μm. (B) Apoptosis in the tumor tissues. The nucleus brown staining represented positive labeling for TUNEL, and the cytoplasm was stained by hematoxylin. Two bottom charts were the quantification. Columns, mean of three experiments; bars, S.E. $^*p < 0.05$, combination treatment group compared with CDDP-alone group. Scale bar: 30 μm.

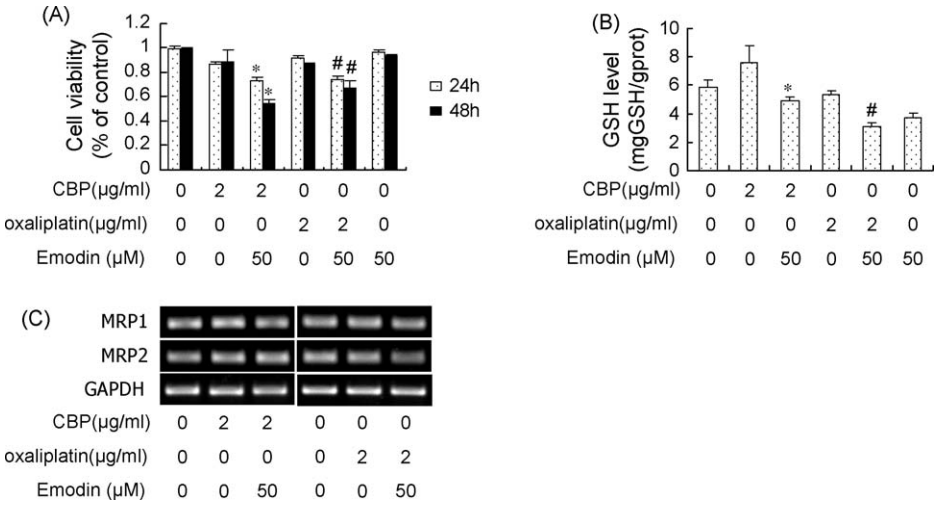

Fig. 7. Emodin enhances sensitivity of SGC996 cells to CBP and oxaliplatin. Cells were exposed to CBP alone, oxaliplatin alone, emodin alone, CBP/emodin co-treatment or oxaliplatin/emodin co-treatment. (A) Cell viability in SGC996 cells (MTT, drug treatments for 24 h or 48 h). Columns, mean of nine replicates from three experiments; bars, S.E. $^{*}p < 0.05$, combination treatment group compared with CBP-alone group. $^{\#}p < 0.05$, combination treatment group compared with oxaliplatin-alone group. (B) GSH level in SGC996 cells (GSH analysis kit, drug treatments for 12 h). Columns, mean of experiments; bars, S.E. $^{*}p < 0.05$, combination treatment group compared with CBP-alone group. $^{\#}p < 0.05$, combination treatment group compared with oxaliplatin-alone group. (C) Expression of MRP1 and MRP2 (RT-PCR, drug treatments for 24 h).

3.7. Emodin enhances sensitivity of gallbladder cancer cells to CBP and oxaliplatin

To further test whether emodin had the synergistic effect to other platinum agents on gallbladder cancer cells, and thus to evaluate the generality of above findings, we also assessed the cell viability, MRPs expression and GSH level of SGC996 cells after cells were treated with CBP, oxaliplatin and emodin alone or in combination. As shown in Fig. 7A, both platinum agents led to a more significant reduction of cell viability when they were combined with emodin. Co-treatment of CBP or oxaliplatin with emodin also obviously reduced the cellular GSH level (Fig. 7B), although co-treatment only slightly downregulated MRP1 expression (Fig. 7C). These results suggested emodin possessed general effect on enhancing the sensitivity of gallbladder cancer cells to platinum agents.

4. Discussion

As gallbladder carcinomas are highly resistant to drug treatment at the onset of therapy (intrinsic multidrug resistance), there is a real need to better understand the mechanism of drug resistance and to develop novel therapeutic strategies.

One of the mechanisms of drug resistance in cancer cells is associated with altered anticancer drug transport, mediated by members of the ABC superfamily of transport proteins [18] such as MDR1 [19], MRP1 [20] and MRP2 [21]. These ABC membrane transport proteins are identified as a drug efflux pump and can actively decrease the intracellular concentration of anticancer drugs. In the present study we have examined the potential role of the multidrug resistance proteins in the chemoresistance phenotype of gallbladder carcinoma and found that the expression of MDR1 in SGC996 cells is undetectable, while both MRP1 and MRP2 express at higher levels. Moreover, exogenous reconstitution of the MRP1 expression in SGC996 cells rescues the apoptotic resistance to CDDP, and silencing of MRP1 abolishes it. These data suggest for the first time that MRP1 may play a critical role in the multidrug resistance phenotype of gallbladder cancers, although overexpression of MRP1 has been documented for some other drug-resistant cancer cells [22]. Whether MRP2 contributes to the drug resistance of biliary tract cancers needs to be clarified in the future work.

The therapeutic effect of platinum drugs is believed to result from the formation of covalent adducts with DNA. DNA damage signals then induce apoptosis in various solid tumor cells. However, only a small fraction of the intracellular cisplatin can bind to genomic DNA. A major fraction, about 60% of the intracellular cisplatin, is conjugated with GSH to form GS–platinum complexes, which show inactivated cytotoxicity and are discharged from cancer cells via the glutathione conjugate export pump [23]. And similar to cisplatin, this resistance mechanism also hampers the effectiveness of other two platinum drugs carboplatin and oxaliplatin [9]. As a type of GS-X pump, MRP1 is mainly responsible for exporting cellular glutathione conjugation. Thus, downregulation of MRP1 expression may consequently contribute to accumulation of cellular cisplatin and enhancement of its cytotoxicity. In this study, we found that the expression of MRP1 in SGC996 cells was additively downregulated by co-treatment of emodin with CDDP/CBP/oxaliplatin in vitro, indicating that emodin might exert synergistic anticancer actions via suppressing MRP1 expression. We have previously demonstrated that emodin can inhibit the transcriptional activity of HIF-1α that controls the expression of MDR1 gene [13]. Differently with MDR1, the transcription of MRP1 is regulated by other transcription factors, such as p53, Sp1 and so on [24–28]. In this study, we also tried to identify the upstream regulators that might serve as the target of emodin to mediate downregulation of MRP1, but failed to find it via overexpression approach (data not shown). Thus this important mechanistic details remains to be further investigated. Different with cisplatin (2 μg/ml) alone, CBP or oxaliplatin (2 μg/ml) alone failed to induce downregulation of MRPs, which might explain the fact that CBP or oxaliplatin had less strong effect on cell viability, especially at 48 h, and that co-treatment of CBP or oxaliplatin with emodin induced less significant downregulation of MRPs than co-treatment of cisplatin with emodin.

Increased activity of intracellular pathways of thiol production, including GSH, metallothionein and thioredoxin, can also contribute to the detoxification of cisplatin [29]. GSH is a redox-sensitive molecule and acts as an antioxidant of the cell. It has for long been known a chemoresistance factor in cancer cells [30]. Depletion of reduced GSH level sensitizes HT29 and HepG2 cells towards combined treatment with apoptosis-inducing ligand and antican-

cer drugs [31]. Meanwhile, GSH can be combined with anticancer drugs to form less toxic and more water-soluble GSH conjugates which then be exported from cells by GS-X pump. For example, several ovarian cell lines known to be resistant to cisplatin showed a correlation between the degree of resistance and the intracellular levels of GSH [32]. In the present study we found a remarkable reduction of intracellular GSH via emodin/CDDP, emodin/CBP and emodin/oxaliplatin co-treatments. Thus, observed increase of platinum-induced cytotoxicity by the combinative treatments could also be attributed to a decrease of GSH availability to form platinum conjugates and thereby subsequent reduction of cellular efflux of the drug.

It is known that GSH, GSH-related enzymes and GS-X pump, like MRPs, constitute a key detoxification system for cancer cell's escape of killing by drugs [14]. Apparently, emodin in combination with CDDP, CBP or oxaliplatin exerts both of above actions concomitantly, which orchestrates an overriding on this system. However, enhancement of apoptosis cannot be achieved by simple augmentation of ROS or decrease of GSH, because single administration of emodin has no effect in vitro and in vivo. It is noted that synergistic effect of CBP and oxaliplatin depend mainly on depletion of GSH, rather than downregulation of MRP1. This conforms predominant contribution of GSH depletion to enhancement of platinum cytotoxicity.

Interestingly and promisingly, our in vivo data have shown that emodin can effectively enhance the anticancer effect of CDDP through downregulation of MRP1, and with little systemic toxic effects. The present study implies potential application of emodin as sensitizer for cytotoxic therapies in gallbladder cancers. This line of evidence strongly supports the concept that ROS manipulation strategy could be selective between cancerous and normal cells, as indicated by an increasing body of documents [33,34]. Thereby, the combinative therapeutic strategy using emodin or other ROS-producing agents may develop a clinical promising approach to treat cancers.

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