European Review for Medical and Pharmacological Sciences 2020; 24: 7753-7763

Effect of verapamil in the reversal of doxorubicin chemotherapy resistance in advanced gastric cancer

X. WANG[1,2], Y. LI[2], G.-F. FAN[3], T.-Y. ZHANG[3], B. SUN[2], P.-S. FAN[1,3]

[1]Cheeloo College of Medicine, Shandong University, Jinan, China
[2]Department of Gastroenterology, The First Hospital of Anhui Medical University, Hefei, China
[3]Department of Oncology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China

Abstract. – OBJECTIVE: Gastric carcinoma is one of the most common malignant tumors and one of the most common cancer-related fatal diseases. Chemotherapy is considered as the major therapy for advanced gastric cancer, and the curative effect of chemotherapy directly affects the treatment of advanced gastric cancer. Drug resistance of tumor cells is one of the important causes that induces failure of chemotherapy. Previous studies have demonstrated that verapamil (VER) can reverse drug resistance by inhibiting the P-glycoprotein (P-gp), which is one of the main targets of VER. The present study aimed at investigating the function of glucosylceramide synthase (GCS) in the VER-induced reversal of doxorubicin (ADM) chemotherapy resistance in gastric carcinoma.

PATIENTS AND METHODS: In the current study, the 4 GC cell line was selected for investigation. The IC50 values of gastric cancer cells were measured using CCK-8 assay. The expression levels of candidate genes in gastric carcinoma cells were measured by RT-qPCR. The expression levels of candidate protein in gastric carcinoma cells were measured by Western blot. The expression of *GCS* protein in clinical specimens of GC receiving VER+TACE therapy was measured by immunohistochemistry. The test of gastric carcinoma cell apoptosis was measured by Annexin V-FITC/PI double-staining.

RESULTS: We found that the expression levels changes of the *GCS* gene can influence the effects of ADM+VER on cell apoptosis. The role and mechanism of *GCS* gene in reversing the chemotherapy resistance of gastric carcinoma cells to ADM were explored.

CONCLUSIONS: In future research, we will explore the mechanism of how GCS affects drug resistance in gastric carcinoma and related signal transduction pathway.

Key Words:
Verapamil, Gastric cancer, Apoptosis, Drug resistance, Reversal ability.

Abbreviations

GC, gastric carcinoma; 5-Fu, 5-fluorouracil; VER, verapamil; ADM, doxorubicin; TACE, transarterial chemoembolization; GCS, glucosylceramide synthase, RT-qPCR, reverse transcription-quantitative PCR; P-gp, P-glucoprotein.

Introduction

Gastric cancer is one of the most common malignant tumors of the digestive system, and it is the second leading cause of cancer-associated mortality[1]. Only less than 50% of patients with early gastric cancer are diagnosed, and the majority of the patients are usually treated at a late stage[2,3]. The main treatment methods of advanced gastric carcinoma are neoadjuvant radiotherapy and chemotherapy, molecular targeted therapy and immunotherapy[4]. Chemotherapy plays an important role in treating gastric carcinoma. The 5-year survival rate of patients treated with adjuvant chemotherapy after resection is increased by an estimated 20%, compared with surgery alone. Chemotherapy is considered as the major therapy for advanced gastric cancer, and its curative effect of chemotherapy directly affects the treatment of advanced gastric cancer[5]. Acquired drug resistance is the main cause of chemotherapy failure. Multidrug resistance of gastric cancer cells leads to ineffective chemotherapy[6]. Drugs resistance of tumor cells is one of the most notable causes of chemotherapy failure[7]. According to present studies, overcoming drug resistance could be a key breakthrough point for improving gastric cancer treatment. Researches have shown that P-glycoprotein (P-gp) hydrolyzes adenosine triphosphate (ATP) to generate adenosine diphosphate (ADP) and releases energy.

Corresponding Author: Pingsheng Fan, MD; e-mail: fanpingsheng@csco.ac.cn

The target artery infusion of verapamil (VER) can increase the tissue drug concentration, reverse the multidrug resistance of malignant cells, and increase the sensitivity of tumor cells to chemotherapeutic drugs[6,8,9]. Verapamil, a calcium channel blocker, can significantly reverse multidrug resistance (MDR) in tumor cells. It inhibits the expression of MDR-1 gene and inhibits the synthesis of P-gp. Then the concentration of chemotherapeutic drugs in the tumor cells could be increased, the drug resistance of the tumor cells could be suppressed[10]. VER can reverse drug resistance by inhibiting P-gp, which is the main target of VER[11,12]. Ning et al[13] has shown that VER can reverse drug resistance in P-gp-negative gastric cancer cells. VER can induce the movement of drugs into cells by antagonizing P-gp, inhibiting the delivery pump and increasing the concentration of the drug in the cell. VER can increase cell sensitivity to drugs via non-classical pathways and it can reverse multidrug resistance for a variety of tumor cells *in vitro*. However, the effective concentration of VER to reverse multidrug resistance is 6.0-10.0 umol/L, which was higher than the safe concentration (1.0-2.0 umol/L)[14]. VER can lead to serious cardiovascular side effects, such as sinus bradycardia and atrioventricular block[9]. The present study aimed at identifying the effect of transcatheter arterial chemoembolization (TACE) and *GCS* expression, to improve the effectiveness of VER in reversing chemotherapy resistance.

Patients and Methods

Cell Culture

Human gastric carcinoma cells SGC-7901, BGC-823, AGS were purchased from the Cell Bank of the Chinese Academy of Sciences, and cultured in Roswell Park Memorial Insitute-1640 (RPMI-1640, Gibco; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 U/mL), streptomycin (100 µg/mL), and 600 µg/mL glutamine. Human gastric cancer drug resistant cell line SGC-7910/5-Fu was purchased from Shanghai Meixuan Biological Science and Technology Co., Ltd. and cultured in Roswell Park Memorial Insitute-1640 (RPMI-1640, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 2 µg/mL 5-Fu, penicillin (100 U/mL), streptomycin (100 µg/mL), and 600 µg/mL glutamine. All cells were grown in a 37°C humidified incubator with 5% CO_2.

Cell Transfection

SGC-7901 and SGC-7910/5-Fu cells were seeded into 24-well pastes at a density of $5x10^4$ for 24h at 37°C. When the cells reached 70-80% confluence, they were transfected with 120nM small interfering (si)RNAs, with the following sequences: Sense, 5'-GATCCCCCCTGGAAACATTCTTTGAATTCAAGAGATTCAAAGAATGTTTCCAGGTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAACCTGGAAACATTCTTTGAATCTCTTGAATTCAAAGAATGTTTCCAGGGG-3') and NC Sense, 5'-GTAGGCGTGTACGGTGGGAG-3' and antisense, 5'-AACGCACACCGGCCTTATTC-3'. For the overexpression the expression of *GCS*, the oligonucleotide pEGFP-GCS was generated with the following sequence: Sense, 5'-TCTCGGTCTTCTGCCTTCGC-3'; and antisense, 5'-CCTTAATCAATTTCTGGCTCACT-3') and NC Sense, 5'-TGGGAGGTCTATATAAGCAGAG-3' and antisense, 5'-CGTCGCCGTCCAGCTCGACCAG-3'. Recombinant plasmids were purchased from General biological system (Anhui) Co., Ltd., which were cloned into the pEGFP plasmid and the corresponding empty pEGFP plasmid These plasmids were used as negative control. Cell transfection was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 72h, cells were harvested.

CCK-8 Assay to Determine the IC_{50} V alues of Gastric Cancer Cells

The concentration of 5-fluorouracil (5-Fu) used to treat cells were 80, 40, 20, 10, 5, 2.5 ug/mL. The concentrations of doxorubicin (ADM) were 8, 4, 2, 1, 0.5, 0.25 ug/mL. The concentrations of cisplatin (DDP) were 32, 16, 8, 4, 2, 1 ug/mL, and 4.91 µg/ml VER was used[9].

CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was used to evaluate the OD_{450} value of each cell group. The drug concentration and the OD_{450} values were used to plot a concentration effect curve, from which the IC_{50} value was determined[10]. The experiment was repeated a minimum of three times. IC_{50}-1/IC_{50}-2 was used to evaluate the resistance efficiency index (antagonizing drug-resistance index) of chemotherapy drug resistance reversal.

Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was isolated using the Qiagen RNeasy Kit (Qiagen, Valencia, CA, USA). RevertAid M-MuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) was then used to synthesize cDNA from the RNA. According to the manufacturer's protocol, qPCR was performed using SYBR Green Master Mix (Qiagen, Valencia, CA, USA) and the ABI 7500 Prism Sequence Detection System (Thermo Fisher Scientific, Waltham, MA, USA). The thermocycling conditions were as follows: 95˚C for 15 min, 40 cycles of 95˚C for 15 sec, annealing at 60˚C for 31sec, and a final elongation step at 95˚C for 15 sec, 60˚C for 30 sec, 95˚C for 15sec. The $2^{-\Delta\Delta Ct}$ method was employed to measure the relative expression levels of target genes, and GAPDH was used as a loading control. The experiment was repeated a minimum of three times. The primers used for qPCR were designed as follows:

human multidrug resistance protein 1(hMDR-1):
F: 5'-TTGCTGCTTACATTCAGGTTTCA-3'
R: 5'-AGCCTATCTCCTGTCGCATTA-3'.

low density lipoprotein receptor-related protein (LRP):
F: 5'-AGTCAGAAGCCGAGAAG-3'
R: 5'-CCCAGCCACAGCAAGGT-3'.

glutathione S-transferase-π (GST-π):
F: 5'-CAGGAGGGCTCACTCAAAG-3'
R: 5'-GATCAGCAGCAAGTCCAGCAG-3'.

glucosylcemmide synthase (GCS):
F: 5'-CACCCGATTACACCTCAA-3'
R: 5'-CCGTGAACCAAGCCTACT-3'.

type II topoisomerase (TOPO II):
F: 5'-GCTGTCGATGACAACCTCCT-3'
R: 5'-GCCATCTAGCATTCGTCTGAC-3'.

cellular prion protein (PrPc):
F: 5'-CGCGGATGGCGAACCTTGGCTGCTG-3'
R: 5'-CCGGAATTCTCCCACTATCAGGAAGATG-3'.

MGr1-antigen (MGr1-Ag):
F: 5'-GCGGTACCTTCCCGTCGTAACTTAAAGGGAAACT-3'
R: 5'-CGGAATTCTGCTGCTTAAGAGCCTATGCAAGAAC-3'.

cytokine induced apoptosis inhibitor 1 (CIAPIN1):
F: 5'-CGGAATTCATGGCAGATTTTGGGATCTC-3'
R: 5'-GGTCGACCTAGGCATCAAGATTGCTATC-3'.

GAPDH:
F:5'-AGAAGGCTGGGGCTCATTTG-3'
R:5'-AGGGGCCATCCACAGTCTTC-3'.

Western Blot Analysis

Cells were lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Biotechnology, Jiangsu, China). Equal amounts of protein (30 ug) were loaded into each lane of a 10% gel, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). The membranes were then blocked with 5% non-fat milk at room temperature for 2h and incubated overnight at 4°C with primary antibodies (dilution: 1:1000) against hMDR1 (cat. no. 13978; Cell Signaling Technology, Danvers, MA, USA), Lung resistance protein (LRP) (cat. no. DF2935; Affinity Biosciences, Cincinnati, OH, USA), GCS (cat. no. DF8551; Affinity Biosciences, Cincinnati, OH, USA), GST-π (cat. no. DF7462; Affinity Biosciences, Cincinnati, OH, USA), TOPO II (cat. no. AF0283; Affinity Biosciences, Cincinnati, OH, USA) and β-tubulin (cat. no. AF7011; Affinity Biosciences, Cincinnati, OH, USA). The membranes were washed three times with Tris Buffered Saline Tween-20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse (cat. no. S0002; Affinity Biosciences, Cincinnati, OH, USA) or goat anti-rabbit (cat. no. S0001; Affinity Biosciences, Cincinnati, OH, USA) IgG secondary antibody (dilution: 1:5000; Affinity Biosciences, Cincinnati, OH, USA) at room temperature for 1h. β-tubulin was used as a loading control. Protein expression levels were quantified using ChemImager 5500 software (version 2.03; ProteinSimple, San Jose, CA, USA) with β-tubulin as the loading control. Bound antibodies were detected using BeyoECL Plus reagent kit (Advansta Inc, San Jose, CA, USA) according to the manufacturer's instructions and quantitated using ImageQuant software. The experiment was repeated a minimum of three times.

Cell Apoptosis Assay

A flow-based Annexin V assay was used to measure cell apoptosis after transfection. The cells were washed in PBS, resuspended in 400 μl of Annexin-V binding buffer and then stained with 5 μl of Annexin-V-fluorescein isothiocyanate (FITC) for 15 min on ice in the dark, according to the manufacturer's instructions. Subsequent to staining, the cells were incubated with 10 µl of propidium iodide (PI) for 5 min on ice in the dark. Samples were analyzed using a fluorescence activated cell sorting (FACS)can system (BD Biosciences, San Jose, CA, USA). The experiment was repeated a minimum of three times.

Analysis of GCS Protein Expression in Cancer Tissue Samples of Patients with GC by the Immunohistochemical Method

Clinical data and grouping

A total of 14 patients with advanced GC were included in the present study. They were treated in our hospital (the First Affiliated Hospital of USTC) from March 2012 to December 2015. All cases were confirmed as gastric carcinoma. Their age was from 38 to 75 years with an average age of 62 years (10 males and 4 females). All patients received intervention once per month or a total of two to four interventions. Those patients treated by surgery will be excluded. The exclusion criteria also included women in pregnancy and lactation, patients with psychiatric and mental hypoplasia, acute infection and central nervous system symptoms, and patients with allergies. Additional exclusion criteria were white blood cell count (WBC) $<4.0*10^9$ g/L, blood platelet count (BPC) $< 10.0*10^9$ g/L, hemoglobin (Hb) < 60 g/L, and patients with coagulopathy. The therapeutic effects of these interventions were assessed to divide the patients into the following groups: i) Effective VER anti-resistance treatment group (high cure rate; complete remission (CR)=7); and II) ineffective VER anti-resistance treatment group (progression of disease; disease progression (PD)=7). There were no significant differences in gender and age between the two groups. Both groups provided written informed consent for the VER+TACE treatment. All patients signed the informed consent before operation. The study was approved by the research Ethics Committee of the First Affiliated Hospital of USTC[9].

Immunohistochemical method

The paraffin-embedded GC tissues were collected and sectioned to a thickness of 4 mm for dewaxing and hydration. All tissues sections were heated in a microwave for 15 min for antigen retrieval. The primary antibody was used at a dilution of 1:100. The immunohistochemistry was performed as follows: sodium citrate buffer was used for antigen retrieval, followed by conventional streptavidin-peroxidase (SP) immunohistochemical staining, diaminobenzidine (DAB) staining, and a final step of counterstaining with hematoxylin. Known GC-positive sections were used as a positive control, and phosphate-buffered solution (PBS) instead of primary antibody was used as a negative control[9]. The experiment was repeated a minimum of three times.

Statistical Analysis

All data were analyzed by one-way ANOVA or Mann-Whitney U test using Excel (Microsoft, Redmond, WA) or Prism (Prism6.0, GraphPad Inc, La Jolla, CA, USA) and expressed as mean ± standard deviation(x±S). Each experiment was repeated at least 3 times, and $p<0.05$ was considered statistically significant.

Ethics

All methods were performed in accordance with the relevant guidelines and regulations.

Results

Determination of the Ability of VER to Reverse Drug Resistance in Gastric Cancer Cells

Different concentrations of DDP, ADM, and 5-Fu were used to treat gastric carcinoma cell lines with or without VER (4.91 mg/mL) treatment. The results are listed in Table I. Following treatment with VER, the IC_{50} values for DDP, ADM, and 5-Fu in the three cell lines decreased by varying extents, which suggests that VER could improve the sensitivity of three chemotherapy drugs to different degrees.

The results of relative $IC_{50}=IC_{50}\text{-}1/IC_{50}\text{-}2$ were used to determine the ability of VER to reverse chemoresistance. IC_{50}-1 represents the sensitivity of the gastric carcinoma cells to the three chemotherapeutic drugs. IC_{50}-2 represents the sensitivity of the gastric carcinoma cells to the three chemotherapeutic drugs with VER. In SGC-7901 cells, VER exhibited the strongest ability to reverse ADM chemotherapy resistance (Relative IC_{50} = 6.77) and it was significantly different from BGC-823 (Relative IC_{50} =1.66, Table II, Figure 1A).

It was also identified that the resistance of SGC-7910/5-Fu to VER + ADM was strongest (Relative IC_{50} = 15.26). It was significantly different compared with that of SGC-7901 (Relative IC_{50} =6.77, Table II, Figure 1B).

Table I. Cytotoxic activity of the examined drugs against GC cells.

A: Cytotoxic activity of the examined drugs against SGC-7901, BGC-823, AGS and SGC-7910/5-Fu cells[a]				
	IC_{50} (µg/ml)[b]			
Drug	**SGC-7901**	**BGC-823**	**AGS**	**SGC-7910/5-Fu**
5-Fu	143.6±8.9	98.8±4.2	126.7±10.2	176.5±11.2
5-Fu+VER (4.91 µg/ml)	24.2±1.6	40.3±2.7	42.9±3.1	52.1±3.8
DDP	106.2±7.9	125.4±9.6	120.1±9.6	220.1±18.4
DDP+VER (4.91 µg/ml)	66.9±4.5	97.3±7.7	78.6±8.2	108.6±9.8
ADM	50.1±2.3	11.3±1.9	46.4±3.4	186.2±13.8
ADM+VER (4.91 µg/ml)	7.4±0.9	6.8±0.8	8.9±1.7	12.1±1.9
B: Evaluation of VER reversal of drugs resistance, Relative IC_{50} = IC_{50}-1/IC_{50}-2				
	Relative IC_{50}			
Drug	**SGC-7901**	**BGC-823**	**AGS**	**SGC-7910/5-Fu**
5-Fu	5.93	2.45	2.95	7.38
DDP	1.59	1.29	1.53	2.02
ADM	6.77	1.66	5.21	15.26

Negative control 0.1%DMSO, no activity
[a]The data represent the mean of three experiments in triplicate and are expressed as means±SD; only descriptive statistics were done in the text.
[b]The IC_{50} value was defined as the concentration at which 50% survival of cells was observed.
(A) Cytotoxic activity of the examined drugs against SGC-7901, BGC-823, AGS and SGC-7910/5-Fu cells. **(B)** Evaluation of VER reversal of drugs resistance, Relative IC_{50} = IC_{50}-1/IC_{50}-2.

Association Between the Expression of P-gp and the Effectiveness of VER in Reversing ADM Chemotherapy Resistance in Gastric Carcinoma Cells

RT-qPCR was used to detect the expression level of *MDR1*, a P-gp encoding gene, in gastric carcinoma cells. Among the three gastric carcinoma cells, the expression level of *MDR1*/P-gp was the lowest in SGC-7901. However, it exhibited a significantly stronger association with the effects of VER on the reversal of ADM resistance (Relative IC_{50} =6.77) in these cells compared with the

Table II. Changes of VER reversal of chemotherapy resistance after changing expression level of *GCS* genes.

Cytotoxic activity of the examined drugs against SGC-7901 and SGC-7910/5-Fu cells[a]		
	IC_{50} (µg/ml)[b]	
Drug	**SGC-7901**	**SGC-7910/5-Fu**
ADM	50.1±2.3	186.2±13.8
ADM+VER (4.91 µg/ml)	7.4±0.9	12.1±1.9
ADM+GCS	65.3±3.9	202.6±14.7
ADM+VER (4.91 µg/ml)+GCS	5.9±0.87	11.6±1.73
ADM+siR-GCS	45.91±2.07	154.3±11.7
ADM+VER (4.91 µg/ml)+siR-GCS	10.23±1.6	16.3±2.13

Negative control 0.1%DMSO, no activity
[a]The data represent the mean of three experiments in triplicate and are expressed as means±SD; only descriptive statistics were done in the text.
[b]The IC50 value was defined as the concentration at which 50% survival of cells was observed.
Resistance evaluation to VER reversal ADM, (Relative IC_{50} = IC_{50}-1/IC_{50}-2). The IC_{50} values of SGC-7901 and SGC-7910/5-Fu cell lines, which were treated by chemotherapeutics in the absence (IC_{50}-1) or in the presence (IC_{50}-2) of VER.

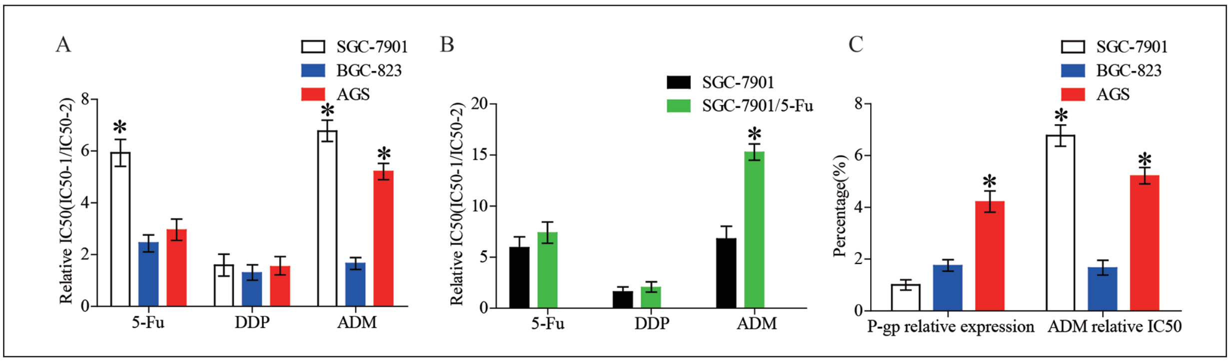

Figure 1. Evaluation of VER-induced reversal of resistance to three chemotherapeutic agents in GC cell lines. **A**, Evaluation of resistance reversal (Relative IC_{50} = IC_{50}-1/IC_{50}-2). The IC_{50} values in three GC cell lines (SGC-7901, BGC-823, and AGS) were used for chemotherapy in the absence (IC_{50}-1) or in the presence (IC_{50}-2) of VER. **B**, Evaluation of reversal (Relative IC_{50} = IC_{50}-1/IC_{50}-2). The IC_{50} values of SGC-7901 and SGC-7910/5-Fu were used for chemotherapy in the absence (IC_{50}-1) or in the presence (IC_{50}-2) of VER. **C**, Relationship between *MDR1*/P-gp expression and VER+ADM resistance (Relative IC_{50}).

other two cell lines (Figure 1C). This indicated that there was no consistent association between the expression level of *MDR1*/P-gp and the effects of VER on ADM resistance.

Expression Levels of Candidate Genes in Gastric Carcinoma Cells Determined by RT-qPCR

Through the preliminary experiments, it was identified that VER exhibited a significantly strong reversal effect to ADM resistance in SGC-7901 cells. Furthermore, SGC-7910/5-Fu cells have a stronger resistance to VER reversal of ADM resistance compared with SGC-7901 cells. Therefore, SGC-7901 and SGC-7910/5-Fu cell lines were selected to screen possible candidate genes that may mediate resistance to VER reversal of ADM resistance. A total of eight candidate genes were selected according to a literature search, namely, *MDR1, LRP, GCS, GST-π, TOPO II, PrPc, MGr1-Ag, CIAPIN1* (Figure 2A). RT-qPCR demonstrated that the expression levels of *GCS, LRP* and *TOPO II* were significantly altered following VER treatment.

Western Blot Analysis of the Expression Levels of Candidate Proteins in Gastric Carcinoma Cells

The protein expression levels of MDR1, LRP, GCS, GST-π, TOPO II, PrPc, MGr1-Ag, CIAPIN1 in SGC-7901 and SGC-7910/5-Fu cell lines were detected. As presented in Figure 2B and C, the protein expression levels of GCS, LRP and TOPO II protein were increased following VER treatment.

Immunohistochemical Detection of the Expression of GCS Protein in Clinical Specimens of GC Receiving VER+TACE Therapy

Immunohistochemical was performed to detect the protein expression of GCS in tumor tissue samples of the VER responsive (CR; n=7) and unresponsive (PD; n=7) groups. Image Pro Plus 6.0 (IPP) software was used to analyze the mean density (IOD/area) of the positive expression region (Figure 3). GCS protein was mainly expressed in the nucleus and cytoplasm of gastric carcinoma cells. In tumor tissue, the IOD/area of GCS in the VER responsive group was significantly higher compared with that in unresponsive group.

Changes in VER Reversal of Chemotherapy Resistance Following GCS Upregulation or Downregulation

As presented in Figure 4A, the expression level of GCS was decreased in the siR-GCS group compared with that in the control group, indicating that the transfection was successful. Figure 4B demonstrates the successful overexpression of GCS, as validation by Western blotting analysis.

The IC50 values of ADM and ADM+VER in GC cells were detected by CCK-8 assay before or after GCS upregulation and downregulation. In SGC-7901 cells, silencing of *GCS* (siR-GCS) significantly reduced the ability of VER to reverse ADM chemotherapy resistance. Whereas, overexpression of *GCS* gene (pEGFP-GCS) significantly enhanced the ability of VER to reverse ADM chemotherapy resistance (Table II).

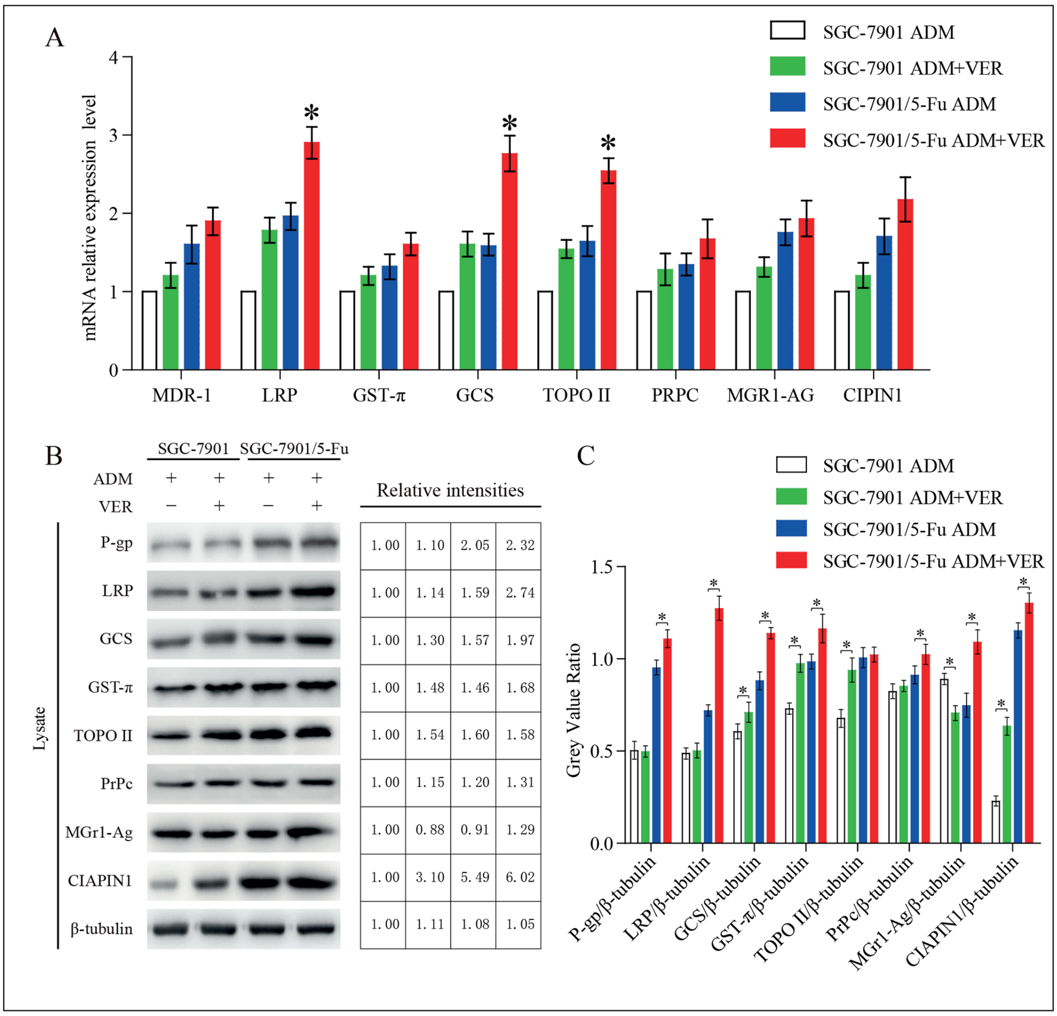

Figure 2. Evaluation of candidate gene/protein expression in GC cells. **A**, RT-qPCR detection of candidate gene expression in GC cells. Independent experiments were performed during the whole *in vitro* study. **B**, Western blot analysis of candidate protein expression levels in GC cells. **C**, Quantitative of the Western blot results of candidate protein expression levels in GC cells. Independent experiments were performed throughout the *in vitro* studies in triplicate. *p<0.05 *vs.* the ADM group.

Annexin V-FITC/PI Double-Staining Test of Gastric Carcinoma Cell Apoptosis

To investigate the effect of VER on the apoptosis of gastric carcinoma cells, Annexin V-PI double staining was performed to evaluate cell apoptosis. As presented in Figure 4C, the cell apoptosis rates in the SGC-7901(ADM) and SGC-7901(ADM+VER) groups were significantly different at 20.36% and 33.63%, respectively.

Following overexpressing of *GCS*, the apoptosis rate changed to 31.21% and 65.5% in the SGC-7901 (ADM) and SGC-7901 (ADM+VER) groups, respectively. After silencing of *GCS*, the apoptosis rate was 23.53% and 26.2% in the SGC-7901 (ADM) and SGC-7901 (ADM+VER) groups, respectively (*p<0.05). The results demonstrated that VER significantly promoted the apoptosis of GC cells over expression *GCS* in the presence of ADM. (*p<0.05).

Discussion

According to statistics, there are about 679000 new cases of gastric cancer in China each year, and about 498000 gastric cancer-associated deaths. Gas-

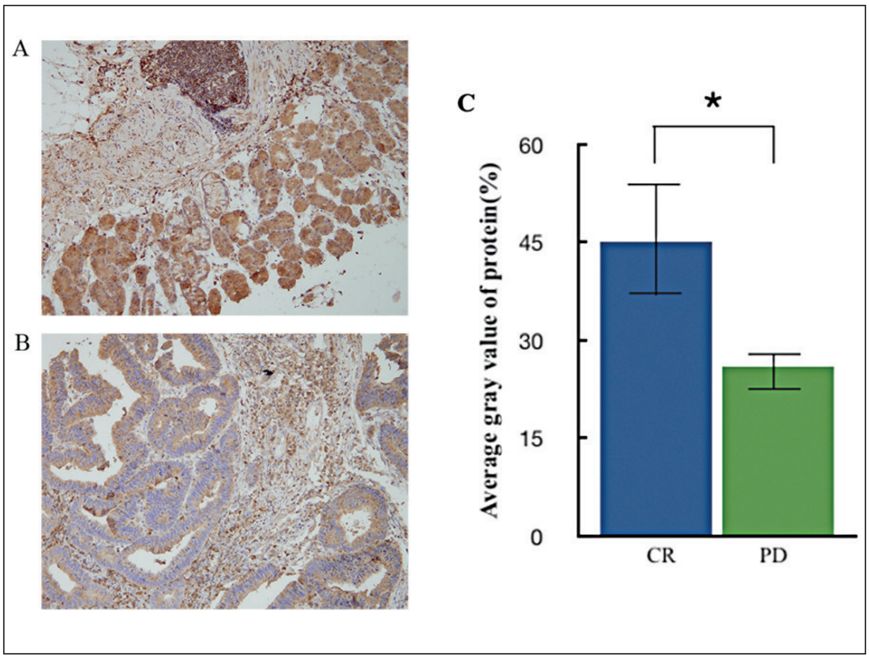

Figure 3. Immunohistochemical analysis of the expression of GCS protein in patients treated with VER+TACE therapy. **A**, Representative image from the CD group (magnification, ×200); **B**, Representative image from the PD group (magnification, ×200). **C**, Mean expression level of protein. *p<0.05 *vs.* PD group.CR, effective; PD, ineffective.

tric cancer has the second highest morbidity and mortality rates, and it is the second most common type of cancer[1]. Due to the low rate of early gastric cancer screening, about 40% of new cases are diagnosed as advanced gastric cancer[15]. In clinic, the majority of advanced gastric cancer cases are administered chemotherapy. However, multidrug resistance of gastric cancer cells limits the curative effect of chemotherapy and leads to the failure of gastric cancer treatment. It not only affects patient survival, but also causes a lot of waste of medical resources[11].

VER is a calcium channel inhibitor. P-gp, also known as multidrug resistance protein 1 (MDR1), is an important protein for the reversal of drug resistance[6,12]. P-gp can transfer the drug from inside to outside of the cell and induce tumor cells drug resistance. However, it can effectively reverse the drug resistance concentration at 6.0-10.0 mmol/L, which is higher than the safe vein concentration (1.0-2.0 umol/L)[14]. The MDR phenotype is associated with the overexpression of P-gp. MDR drugs can inhibit cell growth inhibitory drugs, which is unrelated structurally and functionally from the intracellular to the extracellular environment. It can also reduce the cell concentration, leading to a failure of chemotherapy[16]. P-gp is expressed in most of human tumor cells, in particular, tumor cells with chemotherapy drug resistance have a higher expression level[17]. However, the expression of MDR is lower in paclitaxel-resistant ovarian cancer cells and ADM-resistant gastric carcinoma cells[18,19]. It has been observed that the reversal of multidrug resistance in lung carcinoma cells is not associated with the expression of P-gp[20].

Our early studies[9] demonstrated that the drug concentration in local tissue was 3-10 times higher compared with that in venous blood, and 4-20 times higher than that needed to reverse drug resistance. Following informed consent from the patients, target artery perfusion combined with chemotherapy drugs was used to improve the clinical efficacy of chemotherapy in patients with primary liver carcinoma, colorectal carcinoma, lung carcinoma, gastric carcinoma and malignant ascites[21-27]. In addition, we demonstrated that[14] target artery infusion chemotherapy combined with VER can improve the curative effect of advanced gastric cancer. The total effective rate could reach 75%; however, there were still some poor cases of curative effects. Previous researches on VER-induced reversal of drug resistance of gastric cancer cells may focus on P-gp. However, according to previously published clinical data, VER may improve the sensitivity of gastric cancer cells to chemotherapy drugs via genes other than P-gp protein. This is rarely referred in the literature.

The present study identified that VER can promote ADM-induced apoptosis of gastric cancer cells. To find a new target that might mediate VER-induced reversal of drug resistance in gastric carcinoma cells, the current work investigated the VER-induced reversal of resistances to DDP, ADM, and 5-Fu in four gastric carcinoma cell lines (SGC-7901, SGC-7910/5-Fu, BGC-823, and AGS).

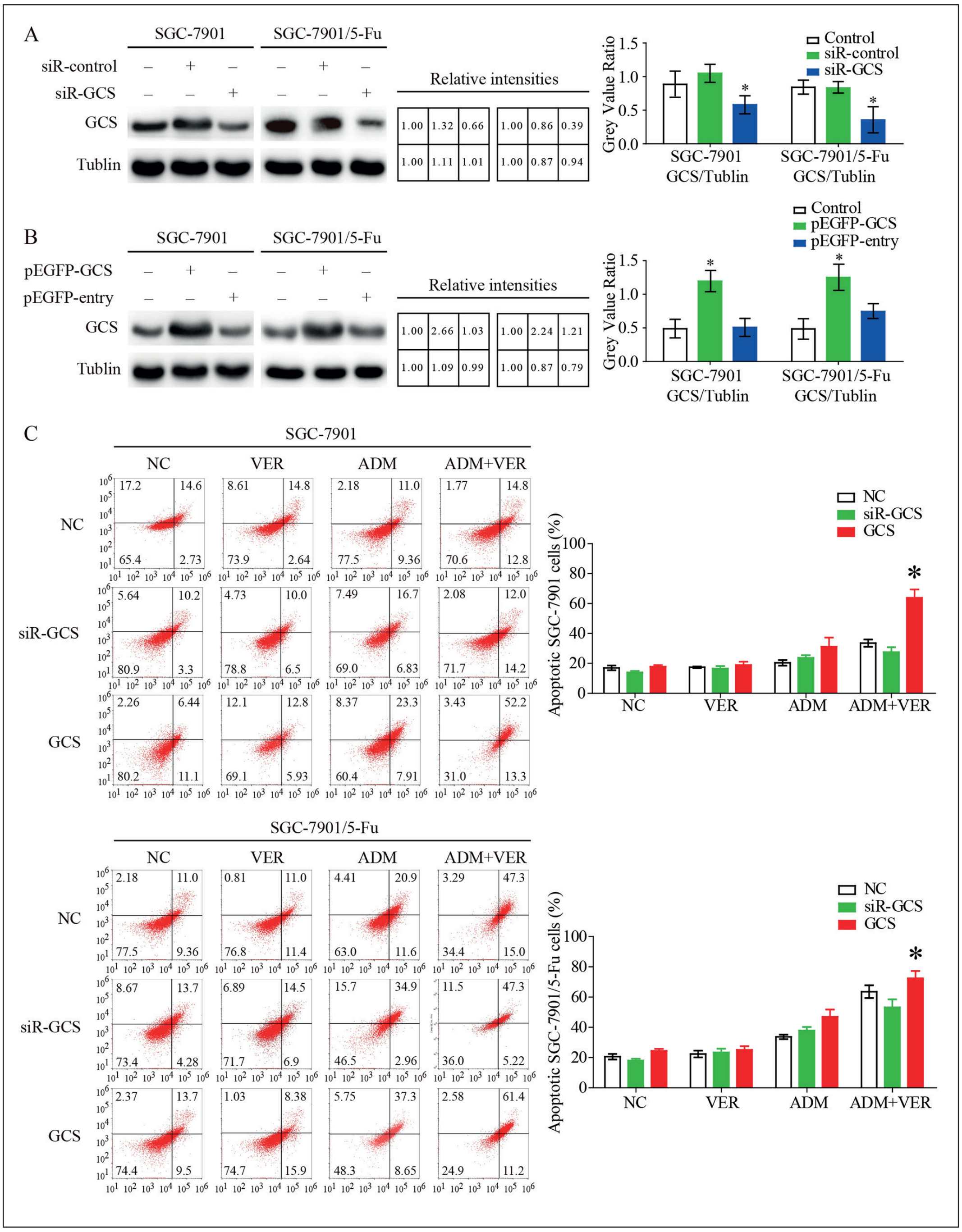

Figure 4. Changes in VER-induced reversal of chemotherapy resistance following loss of expression or overexpression of GCS. **A**, Verification of *GCS* gene expression knockdown in SGC-7901 and SGC-7910/5-Fu cells. **B**, Verification of *GCS* gene overexpression in SGC-7901 and SGC-7910/5-Fu cells. **C**, The effect of VER+ADM on apoptosis was detected by flow cytometry.

The results demonstrated that in SGC-7901 cells, VER exhibited strongest ability to reverse ADM chemotherapy resistance (Relative IC_{50}=6.77), and it was significantly stronger in these cells compared with that of the other gastric carcinoma cells. According to RT-qPCR and Western blot assays, upregulating the expression of GCS can improve the VER-induced reversal of ADM chemotherapy resistance. The expression of GCS protein in cases of VER effective/ineffective treatment was detected by immunohistochemistry. It was identified that the protein expression level of GCS in VER-sensitive group was significantly higher compared with that in the insensitive group.

Glycosylceramide synthase (GCS) is a common tumor multi-resistance gene. Of note, GCS is closely associated with tumor MDR, and ceramide is the second messenger in the process of cell apoptosis, which can lead to cell differentiation, growth retardation and apoptosis[28]. Intracellular GCS is a key enzyme of ceramide metabolism pathway. It can catalyze the combination of the glucose group and ceramide on UDP-glucose[29]. GCS converts ceramics via glycosylation into glucosylceramide, so as to reduce the content of intracellular ceramide, and helps cells escape the apoptosis effect of ceramide, playing an important role in the occurrence of multidrug resistance[30]. To further confirm the association between GCS and the reversal of drug resistance in gastric carcinoma, we overexpressed and silenced *GCS*, and a CCK-8 assay was performed to detect the change in IC_{50} values in the ADM and ADM+VER groups before and after *GCS* expression was altered. The results demonstrated that when GCS expression was silenced, the effect of VER on ADM chemotherapy resistance was significantly reduced. When *GCS* was overexpressed, the effect of VER on ADM chemotherapy resistance was significantly enhanced. This suggests that *GCS* is involved in the mechanism of VER-induced reversal of chemoresistance in gastric carcinoma. The present study identified that by altering the expression level of *GCS*, the ability of VER to promote ADM cell apoptosis could be significantly changed, and the expression level of the gene can be increased. The ability of VER to promote ADM-induced cell apoptosis can be significantly enhanced, otherwise, the expression level of the gene can be decreased. This indicated that VER combined with ADM could promote the apoptosis of gastric carcinoma cells. GCS participates in the ability of VER to promote tumor cell apoptosis.

Conclusions

The aforementioned data demonstrated that the ability of VER to promote ADM cell apoptosis can be significantly enhanced, otherwise, the expression level of the gene can be decreased. This indicated that VER combined ADM could promote the apoptosis of gastric carcinoma cells, and *GCS* gene participates in the ability of VER to promote tumor cell apoptosis.

Acknowledgments and Funding

This project was supported by the National Nature Science Foundation of China (81350005).

Conflict of Interests

The authors declare that they have no conflicts of interest.

References

1) Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.
2) Zhang XY, Mu JH, Liu LY, Zhang HZ. Upregulation of miR-802 suppresses gastric cancer oncogenicity via targeting RAB23 expression. Eur Rev Med Pharmacol Sci 2017; 21: 4071-4078.
3) Wang L, Chunyan Q, Zhou Y, He Q, Ma Y, Ga Y, Wang X. BCAR4 increase cisplatin resistance and predicted poor survival in gastric cancer patients. Eur Rev Med Pharmacol Sci 2017; 21: 4064-4070.
4) Okada K, Oka Y, Nagata H, Taniguchi S, Yoshioka S, Ueshima S, Higaki N, Hayashida H, Nezu R. [A Case of Surgical Treatment of Pulmonary Metastasis from Gastric Cancer]. Gan To Kagaku Ryoho 2017; 44: 1574-1576.
5) Li L, Guo LY, Mao J. A network meta-analysis protocol of adjuvant chemotherapy for unresectable patients with advanced gastric cancer. Medicine (Baltimore) 2019; 98: e16108.
6) Jin MS, Oldham ML, Zhang Q, Chen J. Crystal structure of the multidrug transporter P-glycoprotein from Caenorhabditis elegans. Nature 2012; 490: 566-569.
7) Zhang JT. Use of arrays to investigate the contribution of ATP-binding cassette transporters to drug resistance in cancer chemotherapy and prediction of chemosensitivity. Cell Res 2007; 17: 311-323.
8) Liu N, Huang H, Liu S, Li X, Yang C, Dou QP, Liu J. Calcium channel blocker verapamil accelerates gambogic acid-induced cytotoxicity via enhancing proteasome inhibition and ROS generation. Toxicol In Vitro 2014; 28: 419- 425.
9) Sun X, Yin Q, Chen D, Dong X, Zhou L, Zhang H, Fan P. [Determination of verapamil in dog serum and tissues by reversed-phase high performance liquid chromatography]. Se Pu 2004; 22: 255-257.
10) Dönmez Y, Akhmetova L, İşeri ÖD, Kars MD, Gündüz U. Effect of MDR modulators verapamil and promethazine on gene expression levels of MDR1 and MRP1 in doxorubicin-resistant MCF-7 cells. Cancer Chemother Pharmacol 2011; 67: 823-828.
11) Sydiuk A. Current practice for gastric cancer treatment in Ukraine. Transl Gastroenterol Hepatol 2017; 2: 456-461.

12) Wang F, Zhang D, Zhang Q, Chen Y, Zheng D, Hao L, Duan C, Jia L, Liu G, Liu Y. Synergistic effect of folate-mediated targeting and verapamil-mediated P-gp inhibition with paclitaxel -polymer micelles to overcome multi-drug resistance. Biomaterials 2011; 32: 9444-9456.

13) Ning Z, Chen D, Liu A, Fan P, Duan Q, Zhang T, Fan G. Efficacy of chemotherapy combined with targeted arterial infusion of verapamil in patients with advanced gastric cancer. Cell Biochem Biophys 2014; 68: 195-200.

14) Zhang T, Ma K, Huang J, Wang S, Liu Y, Fan G, Liu M, Yang G, Wang C, Fan P. CDKN2B is critical for verapamil-mediated reversal of doxorubicin resistance in hepatocellular carcinoma. Oncotarget 2017; 8: 110052-110063.

15) Yeh JM, Tramontano AC, Hur C, Schrag D. Comparative effectiveness of adjuvant chemoradiotherapy after gastrectomy among older patients with gastric adenocarcinoma: a SEER-Medicare study. Gastric Cancer 2017; 20: 811-824.

16) Li XQ, Wang L, Lei Y, Hu T, Zhang FL, Cho CH, To KK. Reversal of P-gp and BCRP-mediated MDR by tariquidar derivatives. Eur J Med Chem 2015; 101: 560-572.

17) Monden N, Abe S, Hishikawa Y, Yoshimura H, Kinugasa S, Dhar DK, Tachibana M, Nagasue N. The role of P-glycoprotein in human gastric cancer xenografts in response to chemotherapy. Int J Surg Investig 1999; 1: 3-10.

18) Kumar A, Soprano DR, Parekh HK. Cross-resistance to the synthetic retinoid CD437 in a paclitaxel-resistant human ovarian carcinoma cell line is independent of the overexpression of retinoic acid receptor-gamma. Cancer Res 2001; 61: 7552-7555.

19) Kang HC, Kim IJ, Park JH, Shin Y, Ku JL, Jung MS, Yoo BC, Kim HK, Park JG. Identification of genes with differential expression in acquired drug-resistant gastric cancer cells using high-density oligonucleotide microarrays. Clin Cancer Res 2004; 10: 272-284.

20) Chiu LY, Ko JL, Lee YJ, Yang TY, Tee YT, Sheu GT. L-type calcium channel blockers reverse docetaxel and vincristine-induced multidrug resistance independent of ABCB1 expression in human lung cancer cell lines. Toxicol Lett 2010; 192: 408-418.

21) Yang G, Fan G, Zhang T, Ma K, Huang J, Liu M, Teng X, Xu K, Fan P, Cheng D. Upregulation of Ubiquitin Carboxyl-Terminal Hydrolase L1 (UCHL1) mediates the reversal effect of verapamil on chemo-resistance to adriamycin of hepatocellular carcinoma. Med Sci Monit 2018; 24: 2072-2082.

22) Liu Y, Lu Z, Fan P, Duan Q, Li Y, Tong S, Hu B, Lv R, Hu L, Zhuang J. Clinical efficacy of chemotherapy combined with verapamil in metastatic colorectal patients. Cell Biochem Biophys 2011; 61: 393-398.

23) Jia W, Zhu Z, Zhang T, Fan G, Fan P, Liu Y, Duan Q. Treatment of malignant ascites with a combination of chemotherapy drugs and intraperitoneal perfusion of verapamil. Cancer Chemother Pharmacol 2013; 71: 1585-1590.

24) Huang J, Zhang T, Ma K, Fan P, Liu Y, Weng C, Fan G, Duan Q, Zhu X. Clinical evaluation of targeted arterial perfusion of verapamil and chemotherapeutic drugs in interventional therapy of advanced lung cancer. Cancer Chemother Pharmacol 2013; 72: 889-896.

25) Huang J, Duan Q, Fan P, Ji C, Lv Y, Lin X, Qian L, Yu X. Clinical evaluation of targeted arterial infusion of verapamil in the interventional chemotherapy of primary hepatocellular carcinoma. Cell Biochem Biophys 2011; 59: 127-132.

26) Wen C, Duan Q, Zhang T, Liu Y, Wu Y, Ma K, Fan P, Jia W. Studies on assessment methods of malignant ascites residue and changes of verapamil concentration in intraperitoneal perfusion chemotherapy. Cancer Chemother Pharmacol 2014; 74: 473-478.

27) Fan GF, Pan JJ, Fan PS, Zhang TY, Liu YB, Huang J, Weng CT, Liu M, Duan QH, Wu Y, Tang LL, Yang GH, Dai HB, Zhu ZQ. The clinical observation of verapamil in combination with interventional chemotherapy in advanced gastric cancer. Eur Rev Med Pharmacol Sci 2018; 22: 5508-5518.

28) Lin CF, Chen CL, Lin YS. Ceramide in apoptotic signaling and anticancer therapy. Curr Med Chem 2006; 13: 1609-1616.

29) Bleicher RJ, Cabot MC. Glucosylceramide synthase and apoptosis. Biochim Biophys Acta 2002; 1585: 172-178.

30) Reynolds CP, Maurer BJ, Kolesnick RN. Ceramide synthesis and metabolism as a target for cancer therapy. Cancer Lett 2004; 206: 169-180.