Gastrin induces multidrug resistance via the degradation of p27^{Kip1} in the gastric carcinoma cell line SGC7901

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Abstract. Multidrug resistance (MDR) is one of the major reasons for the failure of chemotherapy-based gastric carcinoma (GC) treatments, hence, biologically based therapies are urgently needed. Gastrin (GAS), a key gastrointestinal (GI) hormone, was found to be involved in tumor formation, progression, and metastasis. In this study, quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical staining analysis revealed a high level of expression of GAS in drug-insensitive GC tissues (P<0.01) and similar results were revealed in GC cell lines SGC7901 and its multidrug-resistant variants SGC7901/VCR and SGC7901/ADR. We constructed a eukaryotic expression vector pCDNA3.1(+)/GAS for GAS overexpression and recombinant lentiviral vectors for specific siRNA (siGAS). Transfection of pCDNA3.1(+)/GAS increased (P<0.05) while transfection of siGAS (P<0.05) and co-treated with paclitaxel (TAX) and vincristine (VCR) combination (TAX-VCR) decreased (P<0.01) the cell viability of SGC7901, SGC7901/VCR and SGC7901/ADR. Apoptosis rates of SGC7901/VCR and SGC7901/ADR were reduced by pCDNA3.1(+)/GAS and increased by siGAS (P<0.05). The apoptosis rates of SGC7901/VCR, SGC7901/ADR and SGC7901 were all upregulated (P<0.01) when cells were co-treated with a combination of siGAS and TAX-VCR. Additionally, siGAS significantly downregulated the expression of Bcl-2 and multidrug-resistant associate protein (MRP1) and P-glycoprotein (Pgp) (P<0.05) in SGC7901/VCR and SGC7901/ADR cells. Moreover, GAS overexpression in SGC7901 cells significantly inhibited p27^{Kip1} expression but increased phosphorylation levels of p27^{Kip1} on Thr (187) and Ser (10) sites (P<0.05), as well as increasing nuclear accumulation of S-phase kinase-associated protein 2 (Skp2) and cytoplasmic accumulation of the Kip1 ubiquitination-promoting complex (KPC) (P<0.05). Silencing of Skp2 blocked the promoting effects of pCDNA3.1(+)/GAS on viability, the expression of MRP1 and Pgp and the inhibitory effects of pCDNA3.1(+)/GAS on apoptosis. In conclusion, we suggest that GAS contributes to the emergence of MDR of SGC7901 cells via the degradation of p27^{Kip1}.

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Introduction

Gastric carcinoma (GC) is one of the most common malignant tumors. Currently, combined surgical operation and chemotherapy play an important role in the comprehensive treatment of GC (1,2). However, multidrug resistance (MDR) and its adverse effectiveness on chemotherapy lead to a considerable number of cases where the effects of chemotherapy are not ideal (1,2). Therefore, studying the mechanism of MDR in GC is important in order to improve the efficacy of chemotherapy and prolong the survival time of patients.

It has been reported that there are several mechanisms whereby GCs become resistant to chemotherapeutic agents. One mechanism is that P-glycoprotein (P-gp, 170 kDa) and its coding gene multidrug resistance protein 1 (MDR1) play a role in drug-excreted pump function (3). Many studies have shown that MDR1/P-gp is closely related to the effectiveness of chemotherapy. For example, the survival rate of GC patients is short, the remission rate is low and the recurrence rate is high when MDR1 is positive (4). Cyclin-dependent kinase inhibitor 1B (p27^{Kip1}) is one of the anti-oncogenes that were first discovered by Polyak *et al* (5) in a variety of cancer cell MDR. p27^{Kip1} was significantly downregulated in paclitaxel (TAX), cisplatin and carboplatin-induced drug-resistant SKOV3 ovarian cancer cells (6). Overexpression of p27^{Kip1} caused cell cycle arrest at S phase, and promoted the pro-apoptotic reaction of cisplatin in human ovarian cancer SKOV3 cells (6).

A large number of studies have demonstrated that p27^{Kip1} plays an important role in the modulation of MDR through its degradation. In drug-resistant human myeloma cell lines, it was found that facilitation of S-phase kinase-associated protein 2 (Skp2) expression promoted cell cycle progression and p27^{Kip1} degradation (7). Cell adhesion-mediated drug resistance (CAM-DR) is a primary factor leading to relapse after chemotherapy (8). It was demonstrated that increasing the p27^{Kip1} level or disturbing p27^{Kip1} phosphorylation at the

Thr (187) site inhibited CAM-DR in human myeloma cell lines (9). Skp2 is an F-box family member that is the specific substrate recognition subunit of the skp1-cullin-F-box (SCF) ubiquitin ligase complex. Skp2 also promotes the degradation of p27^{Kip1} through the ubiquitin proteasome pathway (10). The degradation of p27^{Kip1} could also be increased by the Kip1 ubiquitination-promoting complex (KPC) (11). In a study of GC drug resistance, stable downregulation of the transcription factor E2F1 increased the sensitivity of cisplatin-resistant SGC7901/cisplatin cells, and inhibited the expression of drug resistance associated proteins, such as MDR1 and Skp2 (12). Other studies have also shown that E3 ubiquitin ligase Skp2 (nuclear) and KPC (cytoplasmic)-induced phosphorylation of p27^{Kip1} on Thr (187) and Ser (10) sites are prerequisites for p27^{Kip1} degradation (11,13).

GAS, an important gastrointestinal (GI) hormone, stimulates parietal cells to secret gastric acid (HCl) and participate in epithelial proliferation of the GI tract (14). There are five main types of GAS - GAS-14, -17, -34, -52 and -71. GAS-17 (GAS) accounts for >90% of total gastric secretion, deriving from gastroduodenal G-cells in most mammals (15,16). The secretion of GAS is elicited by GAS-releasing peptide (GRP), which regulates gastric acid secretion and enteric motor function (17). Importantly, it has been demonstrated that GAS plays a role in promoting cancer cell proliferation and anti-apoptosis. It has been suggested that GAS has the potential to modulate MDR in cancer cells (18). In CCK2 receptor (CCK2R) expressing human gastric adenocarcinoma cells, GAS reduced the expression of S-phase kinase associated protein 2 (p27^{Kip1}) through increasing the expression of miR-222 (19). Additionally, recent research has shown that in GC cells, GAS can significantly decrease the level of p27^{Kip1} and increase cyclin E protein expression through inducing the nuclear translocation of CacyBP/SIP (20).

Although GAS has been shown to regulate the growth of GC cells, the latest studies of GAS in our laboratory have found that GAS accelerated cell cycle progression and proliferation of GC and is associated with promoting the migration and invasion through the β-catenin/T-cell factor-4 (TCF-4) pathway (21). It is still necessary to investigate whether GAS functionally regulates the MDR of GC cells. The purpose of the present study was to evaluate the effects of GAS on MDR in GC. Hence, the effects and mechanisms of GAS on drug resistance of GC were investigated. The results revealed that the expression of GAS in GC tissues of drug-resistant patients and GC cell lines was significantly upregulated. Further studies indicated that suppression of GAS inhibited MDR through the degradation of p27^{Kip1} in multidrug-resistant variants of GC cell lines.

Materials and methods

Tissues. Surgical specimens of cancer tissues were collected from 17 patients with advanced GC. All patients (ten males and seven females with an average age of 58.3 years, ranging from 48 to 71 years) received neoadjuvant chemotherapy based on docetaxel (DTX) and cisplatin (CDDP) prior to surgical removal of the tumors (Table I). The response rates to chemotherapy for the patients were evaluated as follows: a complete response was considered as a good response, a partial response was considered as a moderate response, stable disease was considered as a poor response, and progressive disease was considered as no response. Twelve of the patients had no or poor response and the other five had moderate response to chemotherapy. The study was performed with the approval of the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was obtained from each patient before initiation of the study.

Cell culture. The human gastric adenocarcinoma cell line SGC7901 (obtained from Xijing Hospital, the Fourth Military Medical University, Xi'an, Shaanxi, China) and its multidrug-resistant variants SGC7901/VCR and SGC7901/ADR (established and maintained in our laboratory) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37˚C. To maintain the MDR phenotype, VCR (final concentration 1 μg/ml) and Adriamycin (ADR, final concentration 0.4 μg/ml) were added to the culture media of SGC7901/VCR and SGC7901/ADR cells, respectively.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA from GC tissues or cells was extracted using TRIzol (Invitrogen), and cDNA was synthesized using an M-MLV Reverse Transcriptase kit (Invitrogen). Then, the detection of specific products was performed using SYBR green (Applied Biosystems, Foster City, CA, USA) technology. Amplification involved a denaturation step (95˚C for 5 min, 1 cycle), and amplification and quantification were repeated for 40 cycles (95˚C for 5 sec and 60˚C for 1 min, respectively). The data of the relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and are presented as the fold change of transcripts for genes. The sample mean value was calculated and expressed as the cycle threshold (Ct). mRNA expression was calculated as the difference (ΔCt) between the Ct value of the target gene and the Ct value of the inner control. $2^{-\Delta\Delta Ct}$ means the fold change in the target mRNA expression. The mRNA expression expressed as the mean ± SD. Primer sequences were as follows: GAS, forward, 5'-GAGCTACCCT GGCTGGAGCAGCAG-3'; reverse, 5'-CTCATCCTCAGCA CTGCGGCGGCC-3'. Pgp, forward, 5'-GCAAGAGGAGCAG CTTAT-GAAG-3'; reverse, 5'-ACTCCCTACCTTCAAGT TGAGG-3'. β-actin, forward, 5'-AGGTCATCACCATTGGC AAT-3'; reverse, 5'-ACTCGTCATACTCCTGCTTG-3'.

Protein extraction. Total proteins from tissue and cell samples were extracted using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40 and 0.1% SDS). For nuclear protein and cytoplasmic protein extraction, protein was isolated using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime, Haimen, China) according to the manufacturer's instructions. Briefly, cells were harvested and washed with PBS, and then centrifuged at 8,000 g for 10 min. The cell sediments were collected and resuspended in 20 ml of PBS and mixed with 200 ml of buffer A containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After vortexing, cells were placed in an ice bath for 10 min. Thereafter, 10 ml of buffer B was added and the cells were again vortexed and placed in an ice bath for 1 min followed by centrifugation at 12,000 g for 5 min at 4˚C. The supernatants containing cytoplasmic protein were

Table I. Clinicopathological variables and the expression of GAS in GC patients.

Clinicopathological variable	Cases	GAS expression	t-value	P-value
Age (mean ± SD), 58.3±7.1 years (range) (48-71)			0.828	0.421
<59	9	1.488±0.495		
≥59	8	1.281±0.536		
Sex			1.050	0.310
Female	7	1.56±0.51		
Male	10	1.69±0.26		
Chemotherapy regimen			1.428	0.174
5-Fluorouracil	11	1.517±0.474		
Irinotecan	6	1.159±0.531		
Docetaxel (DTX)	17			
Cisplatin (CDDP)	17			
Response to chemotherapy			3.933	0.001
No or poor	12	1.62±0.46		
Moderate	5	0.68±0.72		
Metastasis			0.475	0.642
Yes	14	1.58±0.23		
No	3	1.20±0.70		

collected and used for further study. The remaining sediments were collected and resuspended in 50 ml of a nuclear protein extraction agent and subjected to an ice bath for 30 min with vortexing at intervals of 2 min. After centrifugation (12,000 g for 10 min at 4˚C), the supernatants containing nuclear protein were collected and stored at -70˚C for further analysis.

Western blotting (WB). The protein quantification was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Proteins (20 μg) were separated in a 10% SDS-PAGE gel and then electroblotted onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk and then incubated with rabbit anti-GAS (1:1,000, ab14182, Abcam, Cambridge, UK), Bcl-2 (1:700, ab59348, Abcam), MRP1 (1:600, ab32574, Abcam), Pgp (1:800, ab170904, Abcam), Skp2 (1:700, ab19877, Abcam), KPC (1:900, ab151317, Abcam), $p27^{Kip1}$ (1:600, Ab-10, Abcam), Thr (187) (1:600, sc-16324, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Ser (10) (1:400, ab36727, Abcam) and β-actin (1:1,000, ab8227, Abcam) at 4˚C overnight. Then, the membrane was incubated with HRP conjugated anti-rabbit IgG (1:10,000, ab97064, Abcam) for 1 h at room temperature. The signals were visualized using ECL reagents (Beyotime). The protein bands were visualized using an enhanced chemiluminescence detection system. Densitometry values were analyzed using ImageJ 1.43 software (National Institutes of Health, NIH, MD, USA), which were then normalized to β-actin.

Immunofluorescence (IF). We transfected SGC7901 cells with pCDNA3.1(+)/GAS or pCDNA3.1(+)/GAS -NC. At 48 h after the transfection, the cells were fixed in 3% paraformaldehyde in PBS at room temperature for 8 min, then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. After washing in PBS, the cells were incubated with Skp2 (1:400, Abcam) or KPC (1:600, Abcam) primary antibody at 4˚C overnight. After washing, the cells were incubated with FITC-labeled secondary antibody (Pierce, Rockford, IL, USA) at room temperature for 2 h avoiding light. The nuclear was stained with DAPI for 5 min and washed with PBS at three times. Adding anti-quenching reagent, and mounting was performed avoiding light. The cells were examined under a Nikon fluorescence microscope (Image Systems, Columbia, MD, USA).

Immunohistochemistry (IHC). GC tissues were fixed in neutral buffered formalin for 2 h at room temperature and for 18 h at 4˚C and then tissues were embedded in paraffin and sliced. The thickness of the tissue serial sections was 3.5 μm. The serial sections mounted on Superfrost Plus glass slides, and subjected to deparaffinization and rehydration. The endogenous peroxidase activity of was blocked by incubation in 0.3% H_2O_2 in methanol for 30 min. The sections were incubated overnight at 4˚C with rabbit anti-GAS antibodies (1:500, Abcam). The sections were exposed to biotin-labeled secondary antibodies for 1 h and then developed with DAB-H_2O_2. Immunohistochemical staining was performed as described previously (22,23).

Cell transfection assay. The construction of the GAS overexpression vector [pCDNA3.1(+)/GAS], and recombinant lentiviral vectors for siRNA specific for GAS (siGAS) and Skp2 (siSkp2) was performed by Sangon Biotechnology Co. Ltd. (Shanghai, China). All vectors were transfected into cells according to the manufacturer's instructions and following previous studies (24-29). Briefly, for the functional study of GAS, SGC7901/VCR and SGC7901/ADR cells were transfected with pCDNA3.1(+)/GAS or siGAS for 48 h; for mechanistic experiments, SGC7901/VCR and SGC7901/ADR or paclitaxel (TAX) and vincristine (VCR) combination (TAX-VCR) treated SGC7901 which were pre-transfected with pCDNA3.1(+)/GAS were further transfected with the siRNA of Skp2 (siSkp2) for 24 h. Cells transfected with null vectors served as negative controls (-NC). Following transfection, the medium was replaced with complete RPMI-1640 medium supplemented with serum and antibiotics.

MTT assay. To determine the drug sensitivity of the cells, SGC7901/VCR, SGC7901/ADR and SGC7901 cells were plated in a 96-well plate at a density of $5x10^3$ cells/well in RPMI-1640 containing 10% FBS and were transfected respectively with the above-mentioned vectors for 48 h. For the SGC7901/VCR and SGC7901/ADR groups, 20 μl of 5 mg/ml MTT was then added per well, and the culture was incubated for an additional 4 h. The supernatant was then discarded and 150 μl/well of dimethyl sulfoxide (DMSO) was added. The absorbance was measured at a wavelength of 490 nm (A490). For the SGC7901 group, TAX (1 μM, Sigma, St. Louis, MO, USA) (30) and VCR (10 μM, Sigma) (31) were added together in SGC7901 cells, but not SGC7901/VCR or SGC7901/ADR.

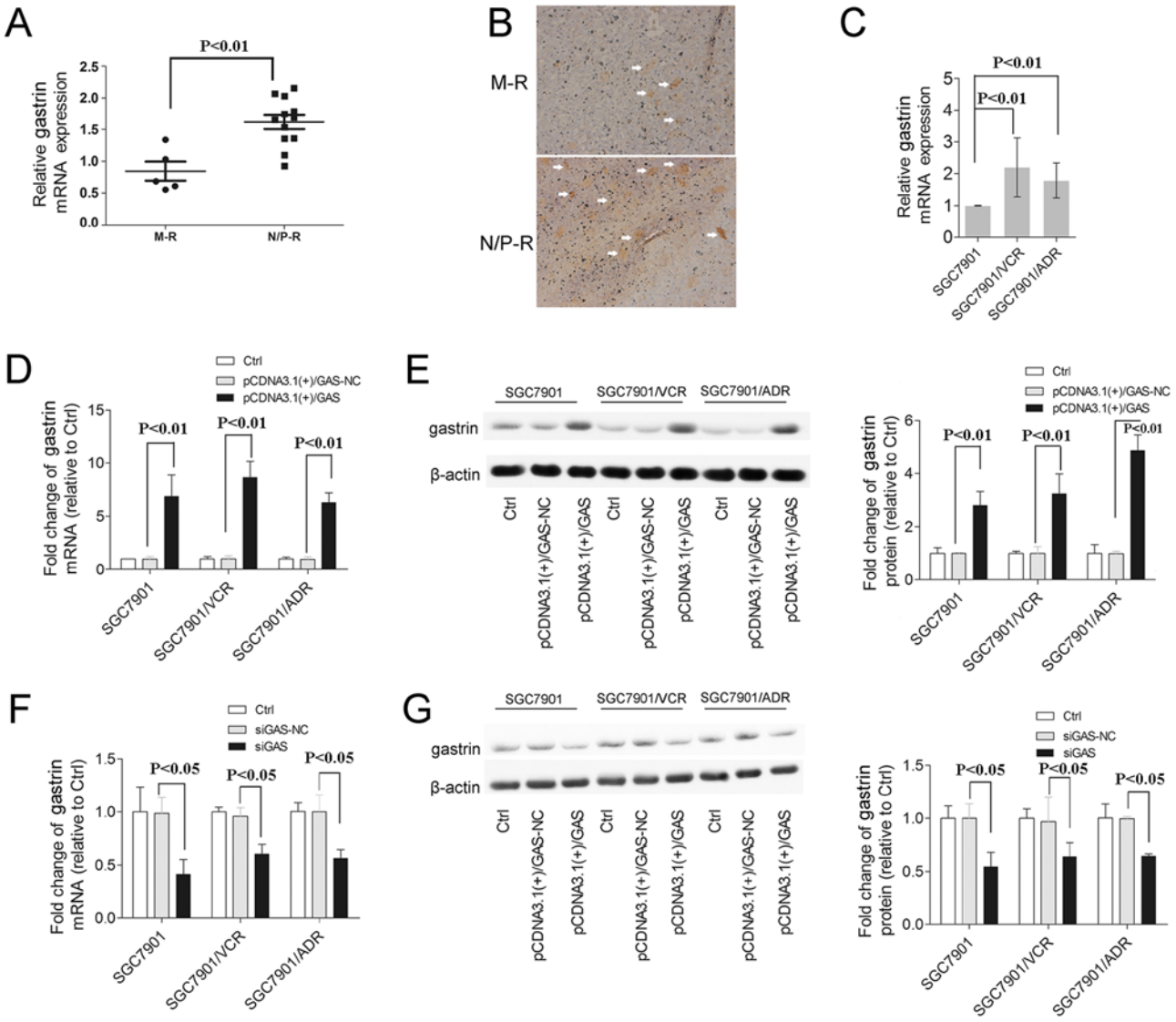

Figure 1. The expression of gastrin in tissues and the gancer cell line SGC7901. M-R, moderate responders; N/P-R, non-responders or poor responders. (A) The mRNA levels of gastrin in non-drug-resistant tissues and drug-resistant tissues in 17 patients with gastric cancer. (B) The expression of gastrin was detected by immunohistochemistry in 17 patients with gastric cancer (x400); arrows indicate the positive marks. (C) The mRNA levels of gastrin in SGC7901 and drug-resistant gastric cancer cell lines SGC7901/VCR and SGC7901/ADR. (D) The effects of gastrin overexpression plasmid [pCDNA3.1(+) GAS] on mRNA level were tested in three cell lines. (E) The effects of pCDNA3.1(+)GAS on protein level of gastrin were tested in SGC7901, SGC7901/VCR and SGC7901/ADR cell lines. (F) The effects of gastrin silence plasmid (siGAS) on mRNA level of gastrin were tested in SGC7901, SGC7901/VCR and SGC7901/ADR cell lines. (G) The effects of siGAS on protein level of gastrin were tested in SGC7901, SGC7901/VCR and SGC7901/ADR cell lines.

The culture medium and the drug were replaced at 24-h intervals to maintain the drug concentration. The following MTT assay steps are consistent with the former. Each assay was performed in triplicate.

Apoptosis assay. Each group of cells was collected and detected using an Annexin V Fluorescein Isothiocyanate kit (AV-FITC, BD Pharmingen, San Diego, CA, USA) on a BD FACSCalibur™ system (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. In brief, the groups of cells were consistent with the MTT assays and cells from the different conditions were suspended in 100 ml of binding buffer, at a density of 1x10^6 cells per ml. Then, cells were incubated with AV-FITC and propidium iodide (PI) for 15 min, and analyzed using Beckman CXP software (Beckman Coulter, Brea, CA, USA) on a FC-500 flow cytometer (Beckman Coulter).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using Student's t-test between two groups, and categorical data were examined using the χ^2 test. Data are presented as the mean ± SD. P<0.05 was considered statistically significant.

Results

GAS is overexpressed in drug-resistant GC cells. Recently, GAS was demonstrated to participate in the growth and metastasis of GC cells (21,32). Firstly, in order to determine the clinical relevance of GAS expression in chemoresistance of GC patients, we used qRT-PCR to evaluate the expression of GAS in human GC specimens. Interestingly, GAS was highly expressed in non-responders or poor responders (N/P-R). As shown in Fig. 1A,

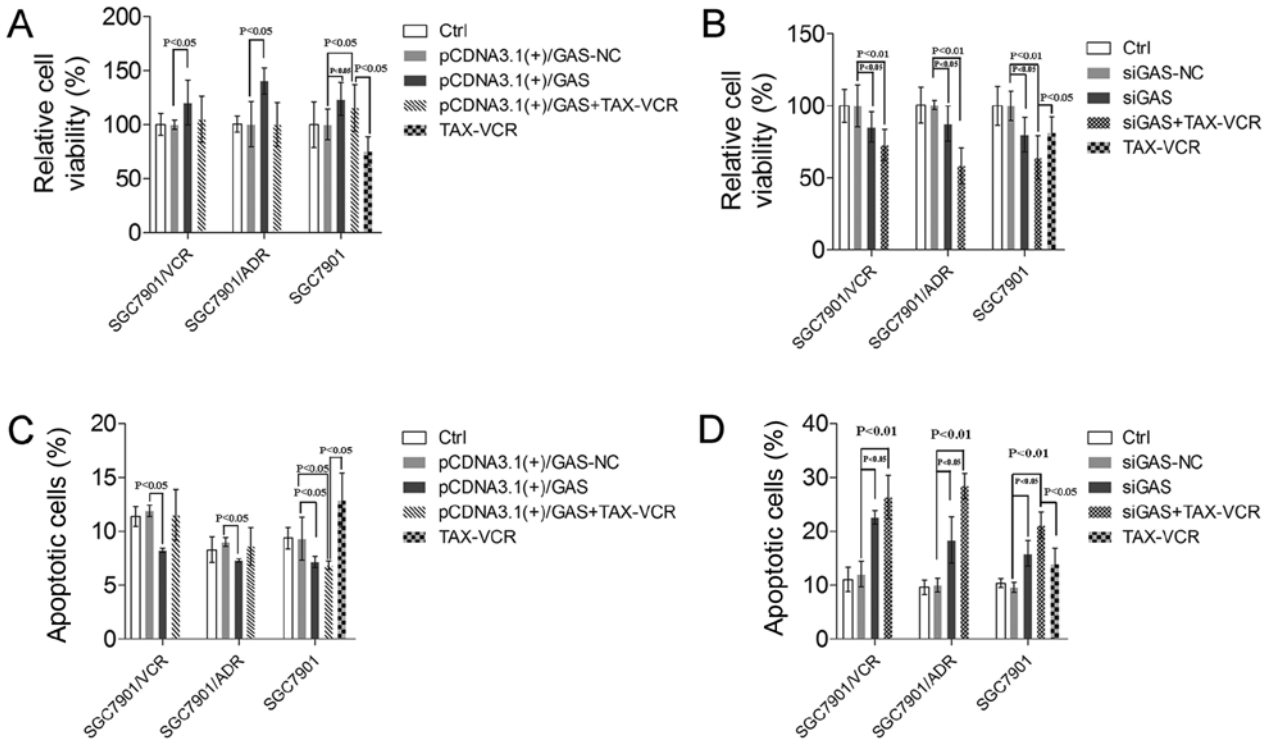

Figure 2. Effects of pCDNA3.1(+)GAS or siGAS on the gastric cancer cell viability and apoptosis rates in SGC7901, SGC7901/VCR and SGC7901/ADR cell lines. (A) Cell viability was detected by MTT assay. (B) Cell viability was detected by MTT assay. (C) Cell apoptosis rates were detected by flow cytometry. (D) Cell apoptosis rates were detected by flow cytometry.

the mRNA levels of GAS was ~2.0-fold higher in N/P-R than in moderate responders (M-R) (Fig. 1A, P<0.01).

Additionally, GAS-positive cells were stained brown. Obviously, GAS-positive cells in the N/P-R group are much more numerous than in the M-R group (Fig. 1B). Secondly, the mRNA level of GAS in drug-resistant cell lines was evaluated. Our results revealed a high expression of GAS in SGC7901/VCR and SGC7901/ADR cells compared with the SGC7901 group (Fig. 1C, P<0.01).

GAS inhibits drug sensitivity and increases viability of human gastric cancer cells. We constructed the GAS overexpression vector pCDNA3.1(+)/GAS and its silencing vector siGAS, and the effects of transfection were tested. After 48 h of transfection, we found that GAS was significantly upregulated in pCDNA3.1(+)/GAS vector-transfected SGC7901/VCR, SGC7901/ADR and SGC7901 cells (Fig. 1D and E) (P<0.01) and downregulated in siGAS transfected SGC7901/VCR, SGC7901/ADR and SGC7901 cells (P<0.05) compared with the NC groups (Fig. 1F and G).

To further investigate whether GAS contributes to MDR in GC, it was force expressed or knocked down in GC cell lines. MTT results showed that the transfection of pCDNA3.1(+)/GAS markedly increased the cell viability of the drug-resistant GC cell lines including SGC7901/VCR and SGC7901/ADR compared with pCDNA3.1(+)/GAS-NC-transfected cells (P<0.05) (Fig. 2A). The viability of pCDNA3.1(+)/GAS-transfected SGC7901 cell lines was still upregulated on treatment with a TAX-VCR combination compared with pCDNA3.1(+)/GAS-NC (Fig. 2A). Additionally, SGC7901/VCR and SGC7901/ADR cells were transfected with siGAS, revealing the opposite results. siGAS significantly decreased the viability of SGC7901/VCR and SGC7901/ADR cells (P<0.05) (Fig. 2B). We also found that the viability of siGAS-transfected SGC7901 cell lines was downregulated on treatment with the TAX-VCR combination compared with siGAS-NC (Fig. 2B).

siGAS increases the drug sensitivity of human gastric cancer cells through testing apoptosis. Previous studies have revealed that GAS play the role of anti-apoptosis in a variety of cancer cell types (18). Therefore, we tested the effects of GAS on the apoptosis of drug-resistant GC cell lines. The results showed that transfection of the pCDNA3.1(+)/GAS vector decreased the apoptosis rates in SGC7901/VCR, SGC7901/ADR and TAX-VCR co-treated SGC7901 cells compared with the pCDNA3.1(+)/GAS-NC group (Fig. 2C). However, siGAS groups revealed opposite results and siGAS promoted the sensitivity of SGC7901 to TAX-VCR (Fig. 2D). Further studies indicated that transfection of siGAS significantly down-regulated Bcl-2 MRP1 and Pgp expression in SGC7901/VCR, SGC7901/ADR and TAX-VCR co-treated SGC7901 cells (Fig. 3A-E, P<0.05). However, the opposite results were found in the GAS overexpression group (Fig. 3F-J, P<0.05). These results indicate that GAS confers decreased sensitivity to TAX or VCR and might be a key promoter of MDR in human GC cells.

GAS inhibits the expression of $p27^{Kip1}$ in GC cell lines. Previous studies have shown that GAS can inhibit the expression of $p27^{Kip1}$ and $p27^{Kip1}$ can participate in drug resistance in a variety of cancer cells (6,33). Overexpression of $p27^{Kip1}$ could

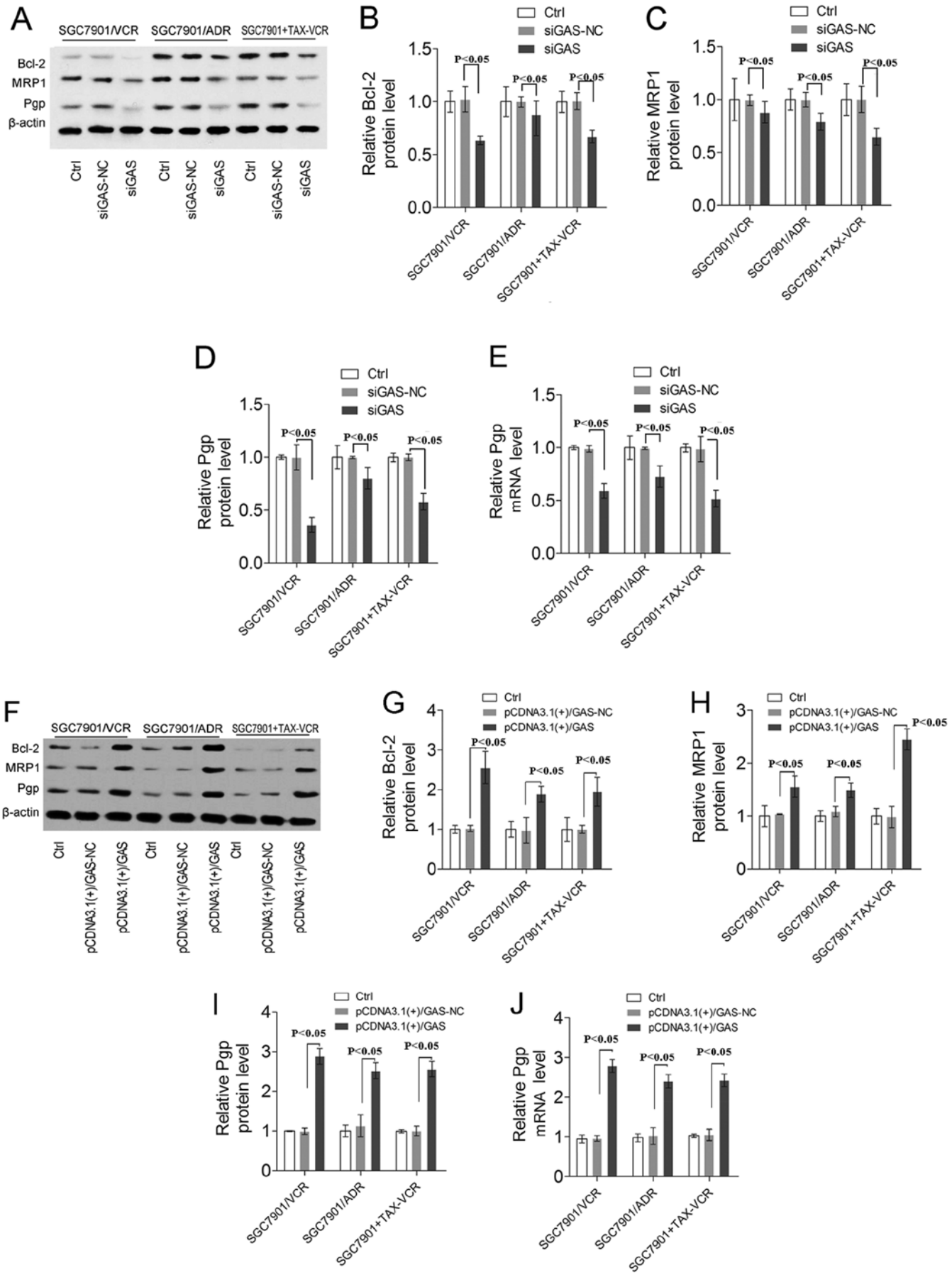

Figure 3. Effects of pCDNA3.1(+)GAS or siGAS on the multidrug resistance marker proteins in SGC7901/VCR and SGC7901/ADR cells and SGC7901 treated with TAX-VCR. (A and F) The expression of Bcl-2, MRP1 and Pgp were detected in the SGC7901 cells by western blotting. [(B-D) and (G-I)] Relative protein expression levels were quantified using ImageJ 1.43 software and normalized to β-actin. (E and J) Relative mRNA expression levels of Pgp and normalized to β-actin.

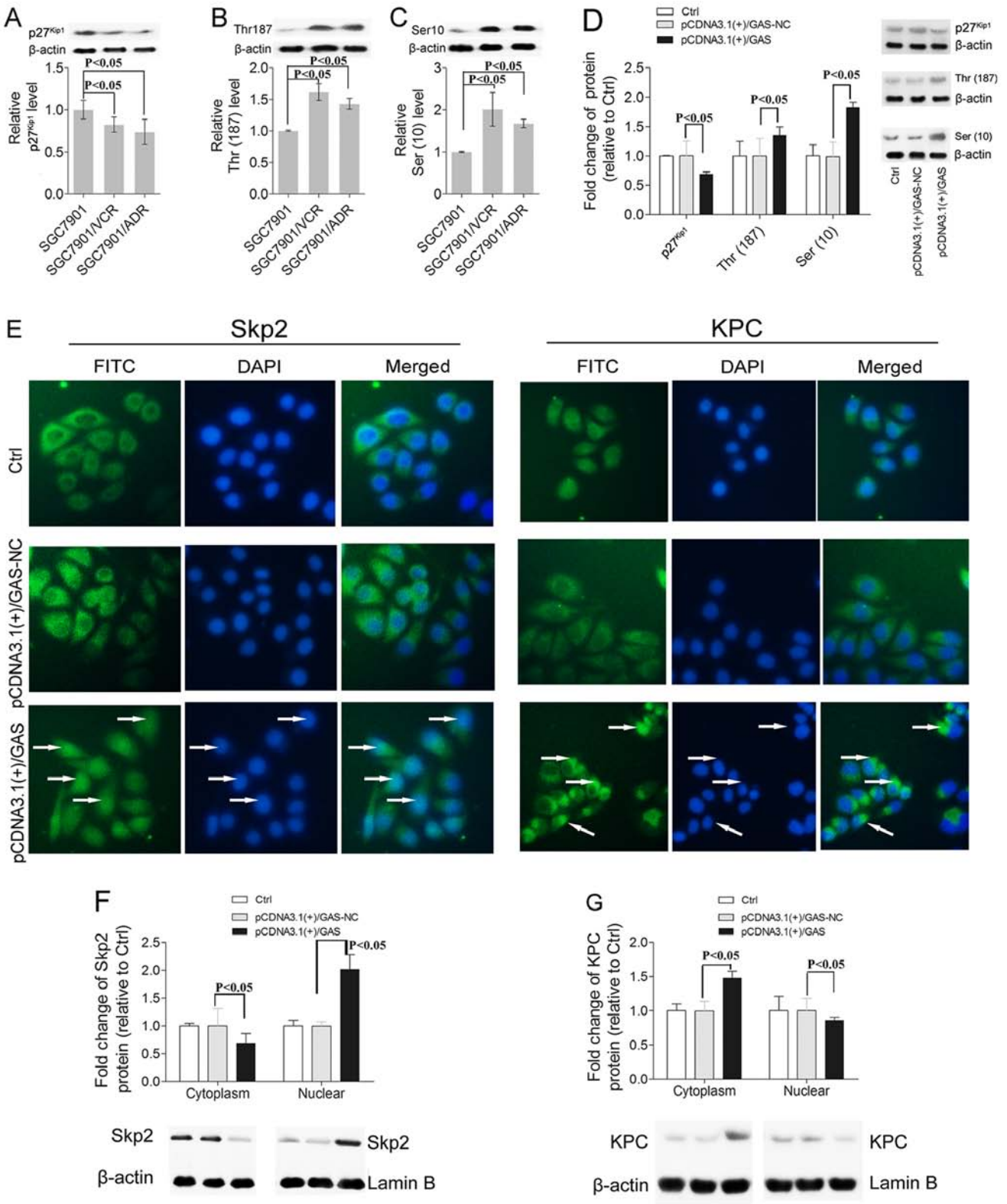

Figure 4. Gastrin promotes the degradation of $p27^{Kip1}$ during the drug resistance of gastric cancer cells. (A-C) The protein level changes of $p27^{Kip1}$ and the phosphorylation of $p27^{Kip1}$ at Thr (187) and Ser (10) sites in SGC7901, SGC7901/VCR and SGC7901/ADR cells were detected by western blotting. (D) The protein level changes of $p27^{Kip1}$ and the phosphorylation of $p27^{Kip1}$ at Thr (187) and Ser (10) sites in pCDNA3.1(+)/GAS-transfected SGC7901 were detected by western blotting. (E) Skp2 and KPC expression were analyzed by immunofluorescence staining (x400); DAPI showed the nulear by blue staining, FITC showed Skp2 or KPC by green staining, magnification; arrows indicate positive marks. (F and G) The protein level changes of Skp2 and KPC in pCDNA3.1(+)/GAS-transfected SGC7901 were detected by western blotting.

lead to cell cycle arrest in S phase, and promote the pro-apoptosis response of cisplatin (6). In our study, we found that the phosphorylation levels of $p27^{Kip1}$ on Thr (187) and Ser (10) sites were higher in SGC7901/VCR and SGC7901/ADR cells than in normal SGC7901 cells, but the levels of $p27^{Kip1}$ were lower (Fig. 4A-C, $P<0.05$). In pCDNA3.1(+)/GAS transfected

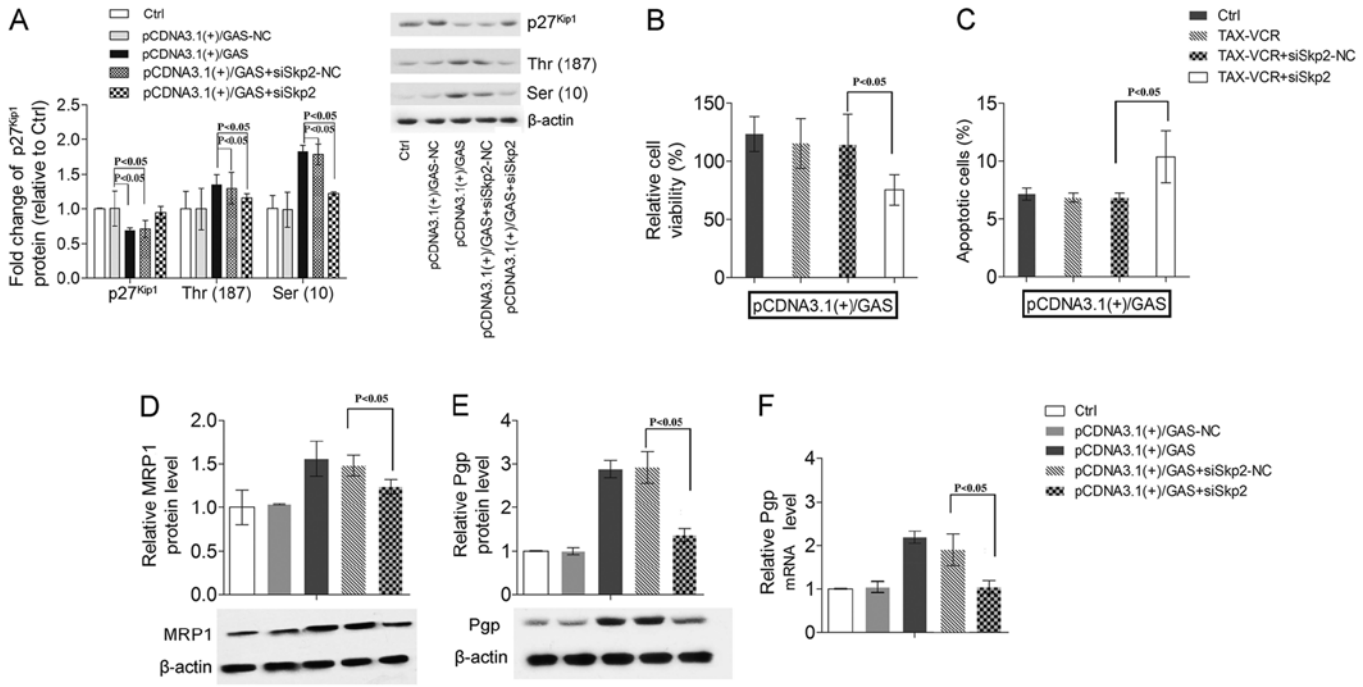

Figure 5. Effects of pCDNA3.1(+)GAS and Skp2 silence (siSkp2) combination on cell viability and apoptosis of gastric cancer cell lines and the level changes of MRP1 and Pgp. (A) The protein level changes of p27^{Kip1} and the phosphorylation of p27^{Kip1} at Thr (187) and Ser (10) sites in pCDNA3.1(+)GAS and siSkp2 co-treated SGC7901 were detected by western blotting. (B) The viability of pCDNA3.1(+)GAS, siSkp2 and TAX-VCR co-treated SGC7901 cells were detected by MTT assay. (C) The apoptosis rates of pCDNA3.1(+)GAS, siSkp2 and TAX-VCR co-treated SGC7901 cells were detected by flow cytometry. (D and E) The expression of MRP1 and Pgp were detected in the pCDNA3.1(+)GAS and siSkp2 co-treated SGC7901 by western blotting. (F) The expression of Pgp was detected in the pCDNA3.1(+)GAS and siSkp2 co-treated SGC7901 by qRT-PCR.

SGC7901 cells, we found similar results: pCDNA3.1(+)/GAS significantly inhibited p27^{Kip1} expression, but increased the phosphorylation levels of p27^{Kip1} on Thr (187) and Ser (10) sites (Fig. 4D, P<0.05). Additionally, using immunofluorescence localization methods and WB, we found that the nuclear accumulation of Skp2 and the cytoplasmic accumulation of KPC were increased by GAS overexpression (Fig. 4E-G, P<0.05). These results indicate that GAS may regulate and induce the degradation of p27^{Kip1} during the emergence of drug resistance in human gastric cancer cells.

p27^{Kip1} induces drug sensitivity of pCDNA3.1(+)/GAS-transfected SGC7901. Studies have shown that depletion of Skp2 could inhibit the degradation of p27^{Kip1} (34). Therefore we constructed recombinant lentiviral siRNA vectors specific for Skp2 (siSkp2). SGC7901 cells that were transfected with pCDNA3.1(+)/GAS were further transfected with siSkp2. We tested the phosphorylation levels of p27^{Kip1} at Thr (187) and Ser (10) sites, and found that they were all downregulated and p27^{Kip1} levels were upregulated compared with the siSkp2-NC group (Fig. 5A, P<0.05). SGC7901 cells transfected with pCDNA3.1(+)/GAS and treated with TAX-VCR were further transfected with siSkp2, and we found the cell viability was significantly inhibited and the cell apoptosis was markedly promoted compared with the siSkp2-NC group (Fig. 5B and C, P<0.05). Additionally, siSkp2 attenuated the high level of MRP1 and Pgp in SGC7901 cells induced by GAS-overexpression (Fig. 5D-F, P<0.05).

These results indicated that inhibition of p27^{Kip1} degradation could attenuate the effects of GAS overexpression. All of these results suggested that GAS induces the MDR of SGC7901 via the degradation of p27^{Kip1}.

Discussion

In this study, we demonstrated a stimulatory role of GAS in MDR in human GC cells. Firstly, we provided evidence that GAS is upregulated in the SGC7901/VCR and SGC7901/ADR cell lines and in non- or poorly-responding gastric tumor patients. Secondly, further analysis demonstrated that the overexpression of GAS partly abolished the drug sensitivity of SGC7901 cells while the silencing of GAS increased the drug sensitivity of SGC7901, SGC7901/VCR and SGC7901/ADR cells. A mechanistic assay confirmed that the degradation of p27^{Kip1} was the downstream mechanism of GAS in regulating MDR in GC cells. These findings suggest a potentially important role for GAS in regulating MDR.

MDR is a great impediment to the success of chemotherapy for GC. It is believed that GAS plays an important role in the process of cell migration and might be involved in the regulation of cancer development and drug resistance in digestive tract and other cancers (18). Firstly, it was reported that GAS mediates proliferative responses in a variety of cancer cell model systems (21) and in particular has been implicated in accelerating the development of gastrointestinal cancers (35). Secondly, Bcl-2 is known as a key regulator of the progression of apoptosis and MDR in multiple types of cancer (36). In a study of large intestine carcinoma, high expression of GAS in large intestine cancer tissue samples was accompanied by high expression of Bcl-2, which indicates that the regulation and

control of GAS in cell apoptosis of large intestine carcinoma may be directly related to the abnormal expression of bcl-2 (37) Furthermore, in a clinical study of resistance to chemotherapy in small cell lung cancer (SCLC), pro-GAS releasing peptide (pro-GRP) was identified as a surrogate marker of Bcl-2 amplification and changes correlated with changes in tumor volume (38). Thirdly, the secretion of GAS was controlled by proton pump inhibitors (PPI), which could protect colorectal cancer (CRC) cells from developing chemo- or radiotherapeutics resistance (39). Rintoul and Sethi (40) suggested that SCLC cells produce a variety of growth factors, cytokines and inflammatory mediators, including insulin-like growth factor-1, GRP and interleukin-8 via autocrine and paracrine effects, and these proteins are able to protect SCLC cells from chemotherapy-induced apoptosis. In the present study, we found direct evidence of GAS downregulating the drug sensitivity of GC cells. The MDR of SGC7901 was upregulated by GAS overexpression and the MDR of SGC7901/VCR and SGC7901/VCR was downregulated by GAS silencing. These results demonstrated that GAS could play a positive role in the formation and development of human GC.

SGC7901/VCR and SGC7901/VCR are the MDR variants of SGC7901, and we found remarkable upregulation of GAS along with MRP1, Pgp and Bcl-2 overexpression, while the downregulation of $p27^{Kip1}$ was also demonstrated in these two cell lines compared with SGC7901. Interestingly, we overexpressed GAS using pCDNA3.1(+)/GAS in SGC7901, and found the overexpression of GAS upregulated MRP1, Pgp and Bcl-2, but accelerated the degradation of $p27^{Kip1}$ with a coincident upregulation of the phosphorylation levels of $p27^{Kip1}$ on Thr (187) and Ser (10) sites. Recently, $p27^{Kip1}$ has been shown to be involved in MDR in a variety of cancer cell lines. It was reported that $p27^{Kip1}$ was significantly downregulated in TAX, cisplatin and carboplatin-induced drug resistance in ovarian cancer cells, and when the expression of $p27^{Kip1}$ was restored, the sensitivity of cisplatin cells was increased (6). Overexpression of $p27^{Kip1}$ causes cell cycle arrest at S phase, and promotes the pro-apoptotic reaction of cisplatin (6). More importantly, GAS could inhibit the expression of $p27^{Kip1}$ by increasing the expression of miR-222 (19). In GC cells, GAS induced the nuclear translocation of CacyBP/SIP. CacyBP-SIP nuclear translocation significantly decreased the level of the cell cycle inhibitor $p27^{Kip1}$, increasing cyclin E protein expression (20), so CacyBP/SIP nuclear translocation might be one of the potential mechanisms of the loss of $p27^{Kip1}$. More importantly, we found that nuclear accumulation of Skp2 was also upregulated by gastrin. However, in the study of Niu *et al*, CacyBP/SIP nuclear translocation did not affect the level of Skp2 (20). Thus we believe that there is an intimate connection between $p27^{Kip1}$ and GAS in regulating drug resistance of GC, and nuclear accumulation of Skp2 might be the key mechanism that can correlate between increased gastrin and degradation of $p27^{Kip1}$.

A large number of studies have demonstrated that the degradation of $p27^{Kip1}$ plays an important role in the modulation of MDR. First, in drug-resistant human myeloma cell lines, it was found that facilitation of Skp2 expression promoted cell cycle progression and suppressed $p27^{Kip1}$ expression (7). Second, cell adhesion-mediated drug resistance (CAM-DR) in many types of cancer is a primary factor leading to relapse after chemotherapy (8). It was demonstrated that increasing $p27^{Kip1}$ level or disturbing $p27^{Kip1}$ phosphorylation at Thr (187) inhibited CAM-DR in human myeloma cell lines (9) Third, Skp2 can promote the degradation of $p27^{Kip1}$ through the ubiquitin proteasome pathway (10), while the degradation of $p27^{Kip1}$ can also be increased by KPC (11). In a study of drug resistance in GC, stable downregulation of the transcription factor E2F1 increased the sensitivity of SGC7901/cisplatin (a cisplatin-resistant GC cell line), and inhibited the expression of drug resistance associated proteins such as MDR1 and Skp2 (12). Other studies also show that E3 ubiquitin ligase Skp2 (nuclear) and KPC (cytoplasmic)-induced phosphorylation of $p27^{Kip1}$ on Thr (187) and Ser (10) sites is a prerequisite for $p27^{Kip1}$ degradation (11,13).

In the present study, we found a significant accumulation of Skp2 in the nucleus and accumulation of KPC in the cytoplasm of GC cells. Our results are consistent with previous studies. It has been shown that the accumulation of $p27^{Kip1}$ was increased in Skp2 defect-mice. Skp2 can interact with $p27^{Kip1}$ ubiquitinating it (11,41-43). E3 ubiquitin ligase Skp2 (in the nucleus) and KPC (in the cytoplasm)-induced phosphorylation of $p27^{Kip1}$ on Thr (187) and Ser (10) sites is a prerequisite for degradation (11,13). In agreement with these studies, $p27^{Kip1}$ restored the MDR that was inhibited by silencing of GAS. The silencing of both Skp2 and KPC showed similar results. All of these studies indicate that the degradation of $p27^{Kip1}$ is a key component of the pathway downstream of GAS in modulating multidrug resistance of GC cells.

In conclusion, our findings indicated that GAS promotes the introduction of MDR in GC cells by $p27^{Kip1}$ degradation through a mechanism promoting phosphorylation levels of $p27^{Kip1}$ on Thr (187) and Ser (10) sites and inducing the accumulation of E3 ubiquitin ligase Skp2 (nuclear) and KPC (cytoplasmic). Given its triggered effect on MDR, GAS holds promise to be a novel therapeutic target for chemotherapy in GC patients.

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