

Original Article

p38 mitogen-activated protein kinase regulates chemoresistance in human gastric cancer via epithelial mesenchymal transition

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Abstract: Gastric cancer is the most common cancer throughout the world. Acquired chemoresistance and epithelial mesenchymal transition (EMT) emerge as critical steps in the progression of gastric cancer. p38 mitogen-activated protein kinase (MAPK) signaling is involved in the anti-apoptotic and EMT process. Our current work aimed to evaluate the effects of p38 MAPK in adriamycin (ADM)-resistant human gastric cancer cells. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Real-time polymerase chain reaction (RT-PCR) and Western blot analysis were performed to detect the mRNA and protein expression levels. Compared with the parental cells, the p38 MAPK activation was remarkably increased in SGC7901/ADM and BGC823/ADM cells. Downregulation of p38 expression by p38 siRNA sensitized chemoresistant tumor cells to ADM administration. In addition, the EMT related markers E-cadherin or vimentin was up-regulated or down-regulated upon the inhibition of p38 MAPK in gastric cancer cells. Further studies identified the inhibition of p38 partially reversed the EMT changes found in this cell system, as illustrated by decreased gene expression of the EMT marker zinc finger E-box-binding homeobox 2 (ZEB2) protein and mRNA levels, a known EMT promoter, concomitant with increased E-cadherin protein. Taken together, our results demonstrated that downregulation of p38, which might be associated with the inactivation of the p38 MAPK signaling pathway, sensitizes gastric cancer cells to ADM via EMT, suggesting that p38 MAPK is a promising target for the design of targeted therapies for overcoming drug-resistant gastric cancer cells.

Keywords: Gastric cancer, p38 MAPK, drug resistance, epithelial mesenchymal transition

Introduction

Gastric cancer (GC) remains as one of the leading causes of cancer-related deaths worldwide, constituting an important public health problem [1]. It is estimated that about one million new GC cases are diagnosed, and about 700,000 people die of this disease, thus representing up to 10% of the cancer-related deaths every year in the world [2]. Gastric cancer is prone to metastasis in the early stages of the disease [3]. In addition to surgical resection, systemic chemotherapy has become one of the important adjuvant therapies of GC [4].

Adriamycin (ADM), also named doxorubicin, is a well-known chemotherapeutic treatment used in solid tumors of the prostate, breast, thyroid, adrenal cortex, neck, lung and ovary [5]. ADM is widely used to treat GC in combination regi-

mens with cisplatin and 5-fluorouracil [6]. However, chemoresistance to ADM significantly limits its efficacy [7]. Resistance to ADM is often associated with the activation of p38 MAPK [8].

A lot of studies have indicated that epithelial mesenchymal transition (EMT), a process vital for morphogenesis during embryonic development, is found in the most aggressive metastatic cancer [9]. A hallmark of EMT is loss of epithelial cell markers, including the cell adhesion protein, E-cadherin, and ZEB2 [10]. EMT is a crucial event leading to invasion and metastasis in solid tumors, including GC [11]. Accumulating evidence shows that EMT contributes chemoresistance, however, little is known about whether chemotherapy can induce EMT in GC [12]. Emerging evidence has shown that p38 MAPK play an important role in chemoresistance by modulating EMT [13]. However, lit-

Table 1. The sequences of primer

Primer names	Sequences (5'→3')	Application
p38/forward	GCGTAGACTTGACAACATC	Real-time PCR
p38/reverse	GGTGAAGGGTAAGCAGAG	Real-time PCR
ZEB2/forward	TGACAGACTCCGACTCCT	Real-time PCR
ZEB2/reverse	TTCTTTCTCGTGCTCCTT-3	Real-time PCR
E-cadherin/forward	GGGTCTTGCTATGTTGCC	Real-time PCR
E-cadherin/reverse	GTTCCGCTCTGTCTTTGG	Real-time PCR
Vimentin/forward	CCAGGCAAAGCAGGAGTC	Real-time PCR
Vimentin/reverse	GGGTATCAACCAGAGGGAGT	Real-time PCR
Actin/forward	GTCCACCGCAAATGCTTCTA	Real-time PCR
Actin/reverse	TGCTGTACCTTCACCGTTC	Real-time PCR

weeks prior to the experiments. All the cells were cultured a humidified atmosphere containing 5% CO₂ and 95% air at 37°C.

Antibodies and reagents

ADM was purchased from ALEXIS Biochemicals (Lausen, Switzerland). The primary antibodies against human p38 and phospho-p38 (Cell Signaling Technology, Beverly, MA, USA), ZEB2, E-cadherin and vimentin (Santa Cruz Biotechnology, CA, USA) were used in Western blot analysis.

tle is known about its role in the induction of EMT by chemoresistance in GC.

In the current study, we found that phosphorylated (phospho)-p38 expression was upregulated in ADM-resistant GC cells. Downregulation of p38 expression by p38 siRNA inhibited EMT and promoted the sensitivity of ADM-resistant GC cells. The study aimed to evaluate the relevance of p38 MAPK in drug resistance of GC cells and further explore the underlying molecular mechanism.

Materials and methods

Cell culture

The human gastric carcinoma cell lines SGC-7901 and BGC823 were obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The ADM-resistant SGC7901/ADM cell line and BGC823/ADM cell line were obtained from the SGC7901 and BGC823 cells, based on a previously described method [14]. In brief, the SGC-7901/ADM and BGC823/ADM cell cells were induced *in vitro* by continuous exposure of SGC7901 and BGC823 parent cells to adriamycin (ADM) at concentrations of 0.25 µg/L to 25 µg/L. Cell lines capable of sustained growth in medium containing 25 µg/L ADM were considered to be resistant after 3 months. The SGC-7901 and BGC823 cells were cultured in RPMI 1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 10 U/mL penicillin, and 10 µg/mL streptomycin in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. The SGC7901/ADM and BGC823/ADM cells were cultured in the same medium with 1 µg/ml DM, and were grown without ADM for 2

3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

All gastric cancer cell lines (SGC7901, BGC823, SGC7901/ADM and BGC823/ADM) were seeded into 96-well plates (6.0 × 10³ cells/well) and allowed to attach overnight. After cellular adhesion, freshly prepared ADM at the appropriate concentration (0, 0.03, 0.3, 3 µM) was added. The viability of the cells was evaluated using an MTT assay according to the manufacturer's specifications (Roche Applied Science, Indianapolis, IN, USA). Briefly, MTT was added at a concentration of 500 mg/L, and the cells were incubated for 4 hours (h) at 37°C. The absorbance reading of each well was determined using a computer-controlled microtiter plate reader at a wavelength of 570 nm. The cell growth inhibitory rates were defined as the relative absorbance of treated vs. untreated cells.

Real-time polymerase chain reaction

The mRNA expression levels were analyzed using a real-time polymerase chain reaction assay. Total RNA (1 µg) was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed with the First-Strand cDNA Synthesis Kit (TOYOBO, Osaka, Japan) according to the manufacturer's instruction. We performed real-time PCR with the Applied Biosystems SYBR Green master mix kit using a Rotor-Gene 3000 thermal cycling instrument (Corbett Life Science, Sydney, Australia). The PCR conditions consisted of 30 cycles, with 5 s denaturation at 95°C, 30 s annealing at 60°C, and 60 s extension at 72°C. And cDNA was used as the template for the real-time PCR reaction with the following specific primers (Table 1). The human actin gene was amplified as an endogenous control. For determination of

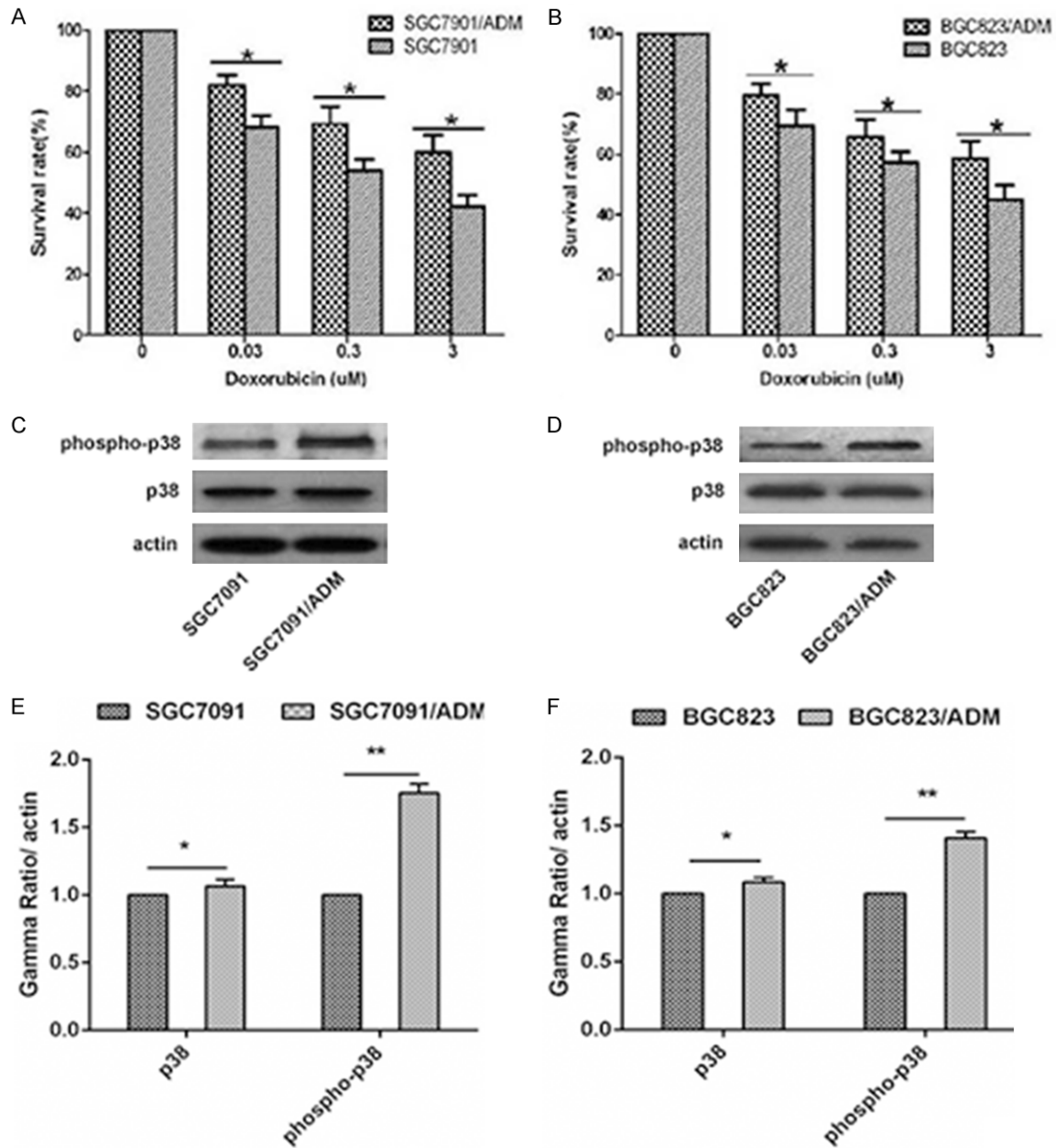


Figure 1. Up-regulation of phosphorylated p38 (p-p38) in chemically resistant GC cells. A and B. Effects of various concentrations (0.03 μ M, 0.3 μ M and 3 μ M) of ADM on cells (SGC7901/ADM cells, SGC7901 cells, BGC823/ADM cells, and BGC823 cells) for 24 h, $*P < 0.05$. C-F. Western blot analysis of the protein level of p-p38 and p38 in SGC7901/ADM cells, SGC7901 cells, BGC823/ADM cells, and BGC823 cells, $*P < 0.05$, $**P < 0.01$. Actin was used as an internal control. All experiments were performed in triplicate.

relative quantification, the expression levels of the genes were calculated and expression based on the method of $2^{-\Delta\Delta Ct}$.

Western blot assay

Protein expression levels were analyzed by Western blot. Briefly, the cells were washed with PBS (phosphate buffered saline, pH 7.4) and

lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 50 μ g/ml leupeptin, 30 μ g/ml aprotinin, and 1 mM PMSF). Protein was loaded at a concentration of 40 μ g per lane, separated on a 12.5% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel, and then transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a wet transfer system (Bio-Rad, Hercules,

CA, USA). Next, the membrane was blocked with 10% nonfat dry milk in TBST (Tris buffered saline with Tween-20, pH 8.0) and then incubated with primary antibodies: p38 (Abcam; no.: ab170099; dilution: 1:1000), phospho-p38 (Abcam; no.: ab4822; dilution: 1:1000), E-cadherin (Abcam; no.: ab40772; dilution: 1:15000), vimentin (Abcam; no.: ab45939; dilution: 1:1000), ZEB2 (Abcam; no.: ab138222; dilution: 1:800) and actin (Abcam; no.: ab5694; dilution: 1:800) overnight at 4°C. The appropriate horseradish peroxidase (HRP) conjugated secondary antibodies (Abcam; no.: ab205718; dilution: 1:3000) were used at 1:3000 for all antibodies. Positive antibody reactions were detected with the enhanced chemoluminescence system and Hyperfilm X-ray film.

ZEB2 luciferase reporter vector

We designed a dual luciferase reporter vector by ZEB2 promoter region cloned into pGL3-Basic plasmid. Firstly, ZEB2 promoter region was PCR-amplified from human genomic DNA with primers containing flanking XbaI recognition sequences. The PCR primers used to amplify the ZEB2 were 5'-GCTCTAGAACTACTGC-ATTTAAGC-3' (forward) and 5'-GCTCTAGACTT-AGTTTGGCTACATTT-3' (reverse). The PCR products were ligated at the XbaI restriction site downstream of the firefly luciferase coding region of the pGL3 vector (Promega).

Promoter activity assays

GC cells were plated onto 12-well dishes and transfected with the pGL3 luciferase reporter plasmid (0.5 mg/well), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For normalization of luciferase activity, the pRL-TK control vector containing the herpes simplex virus thymidine kinase promoter encoding Renilla luciferase was used, resulting in its constitutive expression in a variety of cell types (Promega). The pRL-TK vector was used (0.05 mg/well) together with pGL3 plasmids for co-transfection. For all experiments, cells were cultured for 48 h after transfection and lysed with the Passive Lysis Buffer (Promega). Lysates were analyzed using the Dual-Luciferase Reporter Assay System kit (Promega). Luminescence was measured on an AutoLumat LB 953 Luminometer (Berthold Technologies, Bad Wildbad, Schwarzwald, Germany). All experiments were repeated at least three times.

Statistical analysis

All statistical analyses were performed with the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). All experimental data were shown as the Mean \pm SD. The statistical significance of differences between two groups was analyzed by means of *t*-test. $P < 0.05$ was considered statistical significance.

Results

The phosphorylation of p38 MAPK is up-regulated in chemoresistant gastric cancer cells

Firstly, in order to compare and verify the differences of resistance between ADM treated cells and its parental cells, different concentrations of ADM were used to treated cells. Sensitivity of the cells to ADM, is reflected by the survival rate directly. The ADM sensitivity of SGC7901 and BGC823 cells showed that SGC7901/ADM cells were less sensitive to ADM treatment compared with their parental cells (ADM: 0.03 μ M, $P < 0.05$; ADM: 0.3 μ M, $P < 0.05$; ADM: 3 μ M, $P < 0.05$) (**Figure 1A**). Similarly, we also found that BGC823/ADM cells became more resistant to ADM compared with BGC823 cells (ADM: 0.03 μ M, $P < 0.05$; ADM: 0.3 μ M, $P < 0.05$; ADM: 3 μ M, $P < 0.05$) (**Figure 1B**). To evaluate the biological relevance of p38 MAPK in drug resistance, we measured the expression of phosphorylation of p38 in ADM sensitive/resistant GC cells. Data from Western blot assay showed that phosphorylation of p38 was significantly up-regulated in SGC7901/ADM and BGC823/ADM cells compared with their parental cells ($P < 0.01$; $P < 0.01$) (**Figure 1C-F**). These results suggested that p38 MAPK might play a critical role in the drug resistance of GC cells.

p38 MAPK is involved in the chemoresistance of GC cells

Next, in order to further confirm the important role of p38 in cell resistance, p38 siRNA was transfected into SGC7901/ADM and BGC23/ADM cells to confirm whether p38 MAPK was involved in the ADM sensitivity in GC cells. As shown in **Figure 2**, with transfection of p38 siRNA, real-time PCR and Western blot assay showed that SGC7901/ADM cells transfected with p38 siRNA exhibited decreased p38 levels

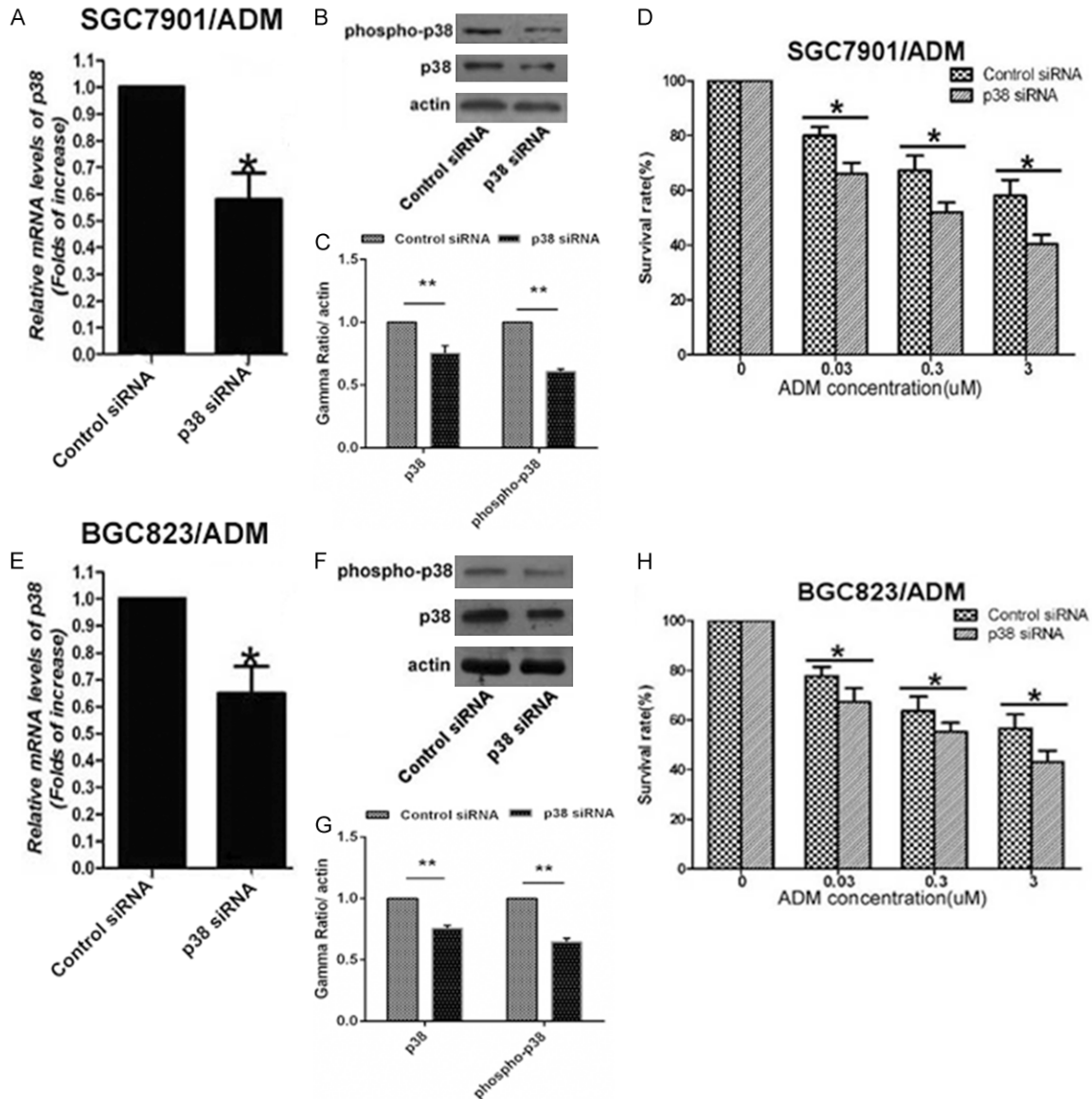


Figure 2. p38 MAPK was involved in chemoresistance of GC cells. A-C and E-G. SGC7901/ADM and BGC823/ADM cells were transfected with p38 siRNA or control siRNA, expression of p38 mRNA and protein (p38 and p-p38) detected by real-time PCR and Western blot, respectively. Actin expression was used as an internal control for mRNA and protein, respectively, $**P < 0.01$. D and H. Cells viability (SGC7901/ADM and BGC823/ADM) were evaluated using MTT assay after transfection with p38 siRNA and control siRNA, $*P < 0.05$. Values are presented as the Mean \pm SD. from 3 independent experiments.

compared to those transfected with negative controls. At the same time, the mRNA expression of the phospho-p38 markedly downregulated with the decreased of p38 levels, which was the key factor in p38 MAPK pathway activation (Figure 2A-C and 2E-G). As a result, downregulation of p38 remarkably enhanced the ADM sensitivity in SGC7901/ADM cells transfected with p38 siRNA (Figure 2D). In addition, transfection of p38 siRNA into BGC823/ADM cells also decreased the expression of

p38, and consequently, suppressed cells proliferation (Figure 2H). Taken together, these data obviously suggested that down-regulation of p38 contributed to the chemotherapeutics sensitivity of GC cells to ADM.

Down-regulation of p38 inhibited ZEB2 expression in GC cells

Data from real-time PCR showed that downregulation of p38 inhibited the mRNA expression

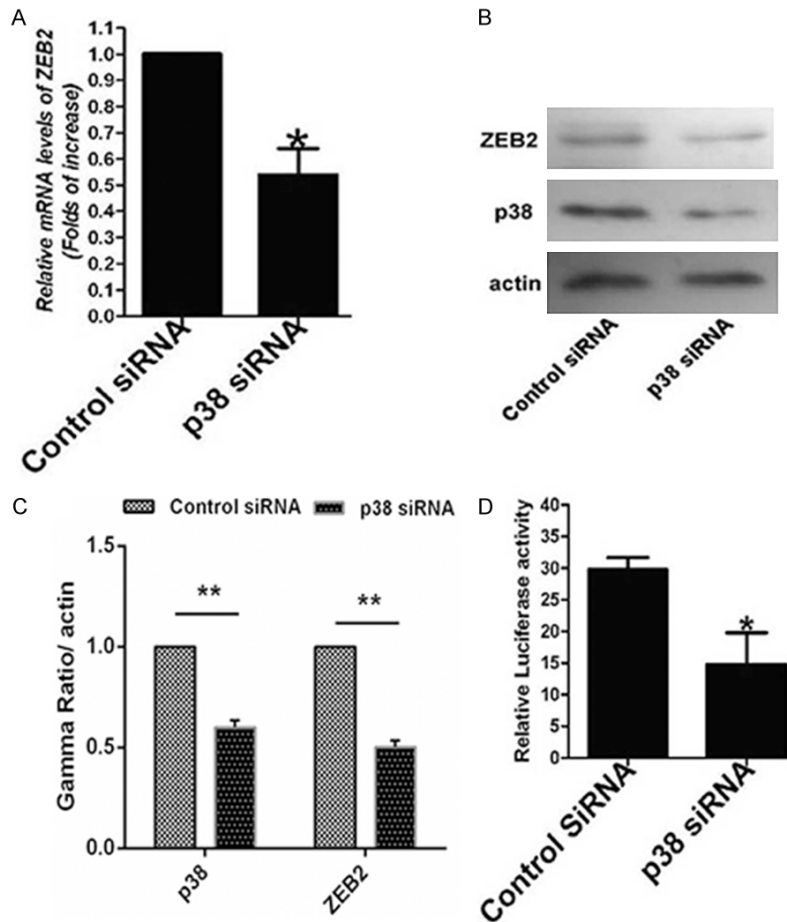


Figure 3. ZEB2 was a downstream target of p38 MAPK in GC cells. A-C. SGC7901/ADM cells were transfected with p38 siRNA or control siRNA, expression of ZEB2 mRNA and protein detected by real-time PCR and Western blot, respectively. Actin expression was used as an internal control for mRNA and protein, respectively, * $P < 0.05$. D. ZEB2 transactivation was mediated by p38 MAPK. SGC7901/ADM cells were transfected for 9 h with the ZEB2-luciferase reporter construct. SGC7901/ADM cells were transfected together with control siRNA or p38 siRNA. Luciferase reporter assay was performed to measure the relative luciferase activity, * $P < 0.05$. Values are the Mean \pm SD. from three different experiments.

of ZEB2 in SGC7901/ADM cells (**Figure 3A**). Consistently, the protein expression of ZEB2 was also reduced in p38 siRNA-transfected tumor cells (**Figure 3B, 3C**). To further confirm that ZEB2 was a downstream target of p38 MAPK, ZEB2 promoter region was cloned into pGL3-Basic plasmid and the transcriptional regulation of p38 was investigated through a dual-luciferase reporter assay. Co-transfected p38 siRNA and a negative control with pGL3-Basic plasmid into SGC7901/ADM cells, respectively. As shown in **Figure 3D**, a marked reduction in luciferase activity was observed in SGC7901/ADM cells after transfection with p38 siRNA. These results demonstrated that ZEB2 was inhibited by p38 MAPK in GC cells.

Effects of p38 MAPK on GC cell epithelial-mesenchymal transition (EMT) program

The involvement of EMT has been reported in many vital cellular processes in malignant tumors, and thus we determined the association between p38 MAPK and EMT. Results showed that downregulation of p38 in SGC7901/ADM cells led to up-regulation of E-cadherin and down-regulation of vimentin at the mRNA levels (**Figure 4A, 4B**). Moreover, Western blot analysis revealed that the epithelial marker E-cadherin was significantly up-regulated, and the mesenchymal marker vimentin was down-regulated in p38 siRNA transfected GC cells (**Figure 4C, 4D**). Together, these data indicated that p38 siRNA might reverse EMT in GC, at least in part, via the direct inhibition of ZEB2. And these results also suggested that EMT was involved in the downregulation of p38 expression and enhanced ADM sensitivity in GC cells.

Discussion

ADM-based chemotherapy serves as one of the standard chemotherapy protocols for GC treatment, but resistance to ADM usually results in failure in clinical practice [15]. In recent years, increasing evidence suggest that p38 MAPK and EMT are important regulators of chemoresistance in a wide range of cancers [16]. Therefore, the revelation of the biological roles of p38 MAPK may contribute to find novel therapeutic targets in treatment. In the current work, we demonstrated that inhibition of p38 MAPK sensitized GC cells to ADM via reversing EMT program.

Previous studies have suggested that p38 MAPK is up-regulated in several cancers such as

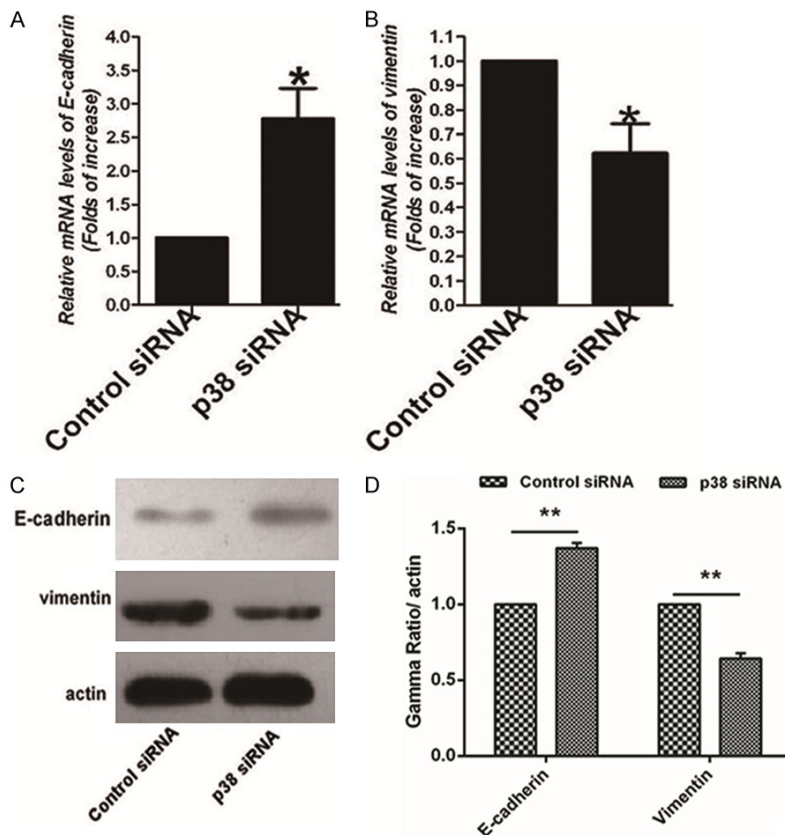


Figure 4. p38 MAPK regulated EMT in GC cells. A and C, D. SGC7901/ADM cells were transfected with p38 siRNA or control siRNA, expression of E-cadherin mRNA and protein detected by real-time PCR and Western blot, respectively. Actin expression was used as an internal control for mRNA and protein, respectively, $*P < 0.05$, $**P < 0.01$. B-D. SGC7901/ADM cells were transfected with p38 siRNA or control siRNA, expression of vimentin mRNA and protein detected by real-time PCR and Western blot, respectively. Actin expression was used as an internal control for mRNA and protein, respectively, $*P < 0.05$, $**P < 0.01$. Values are the Mean \pm SD. from three different experiments.

leukemia, esophageal squamous cell carcinoma and anaplastic thyroid cancer [17]. Documented biological roles of p38 MAPK include induction of proliferation, inhibition of apoptosis, and unsensitization to chemotherapeutics in cancer cells [18]. In addition, research has shown that the expression of p38 MAPK phosphorylation was up-regulated in ADM-resistant GC cells (Figure 1). In this study, we found that inhibition of p38 expression by p38 siRNA could decrease the expression of the phosphorylation of p38 MAPK, an effect that was reversed upon ADM-resistant restoration in GC cells (Figure 2).

EMT refers to the complicated progress in which tumor cell loses epithelial properties and becomes motile mesenchymal cells with capacity for metastasis. Previous studies have shown

that EMT is involved in stem cell behaviors, such as wound healing and development, and so on [19]. Emerging evidence suggest that EMT also plays diverse roles in cancer cell growth and three major steps of malignant cancer progression: invasion, dissemination and metastasis [20]. In addition, it is widely accepted that tumors undergoing EMT may resist conventional chemotherapy [21]. An investigation of squamous cell carcinoma has revealed that p38 MAPK serves as a multi-functional molecular regulator and plays critical roles in EMT program [22]. In this current work, we found that inhibition of p38 MAPK by p38 siRNA promoted the epithelial marker E-cadherin and suppressed the mesenchymal marker vimentin at mRNA and protein level (Figure 4), suggesting that silence the p38 could serve as an EMT-suppressive regulator in GC cells.

ZEB2, a member of the zinc finger E-box-binding homeobox family, plays a vital role as a regulator to suppress the expression of adhesion molecules and to assist the escape of tumor cells from cell death during EMT [23, 24]. ZEB2 possesses DNA binding capacity and recognizes the E-box elements in the promoter region of its target genes, which include E-cadherin [23]. Binding of ZEB2 to its cognate E-box often leads to the suppression of gene transcription. ZEB2 is frequently expressed in many types of tumor cells in which E-cadherin expression is reduced [25]. In this study, ZEB2 expression was promoted in ADM-resistant GC cells, but inhibition of p38 expression by p38 siRNA reversed the increased ZEB2 expression, which suggests that ZEB2 expression in vitro is dependent on the p38 MAPK signaling pathway (Figure 3). As we all known, the expression of ZEB2 suppressed the expression of E-cadherin

protein [23]. Although the underlying mechanism of ZEB2 inhibition remains to be elucidated, the finding that p38 MAPK is able to target ZEB2, which is a key EMT-regulatory gene, highlights a fundamental step in the p38 MAPK signaling pathway in high ADM-induced EMT. Moreover, inhibition of ZEB2 by p38 siRNA could suppress the migration, invasion and chemoresistance of GC cells.

Taken together, our works demonstrated that silencing of p38 expression via RNAi increased the sensitivity of GC cells to ADM through regulation of EMT regulator ZEB2. These findings provide new insight into the mechanism responsible for the chemoresistance in human GC and imply that inhibition of p38 MAPK may serve as a potential therapeutic candidate in drug-resistant GC patients.

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Disclosure of conflict of interest

None.

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