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Regenerating Family Member 4 (Reg4) Enhances 5-Fluorouracil Resistance of Gastric Cancer Through Activating MAPK/Erk/Bim Signaling Pathway

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Background: Reg4, a member of the Reg multigene family, is highly upregulated in many gastrointestinal cancers including gastric cancer (GC). The enhanced expression of Reg4 is associated with the resistance of GC to 5-fluorouracil (5-FU), while the underlying mechanism is not clear. The aim of the present study was to explore the resistant mechanism underlying 5-FU resistance.


Material/Methods: Reg4 expression was assessed by Western blot analysis for SGC-7901, BGC-823, AGS, MKN28, and MKN45. Synthetic short single strand RNA oligonucleotides and Flag-Reg4 plasmid were used to investigate the biological function of Reg4 *in vitro*. The cell viability assay was performed by MTT. Flow cytometry was carried out to measure the apoptosis caused by 5-FU. Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) was used to examine the expression of 5-FU metabolism related enzymes. The effect of Reg4 on intracellular signaling was evaluated by Western blot.

Results: Western blot analysis of 5 GC cells showed that Reg4 was low or null in SGC-7901 and BGC-823, while high in AGS, MKN28, and MKN45. Over-expression of flag-Reg4 in SGC-7901 led to an increase in cell viability and lower apoptosis with 5-FU treatment. In contrast, siRNA knockdown of Reg4 enhanced 5-FU induced apoptosis. However, over-expression or knockdown of Reg4 had no significant influence on the expression of 5-FU metabolic enzymes. Further investigation revealed that Reg4 could activate Erk1/2-Bim-caspase3 cascade.

Conclusions: Reg4 inhibited apoptosis through regulating MAPK/Erk/Bim signaling pathway and thereby enhanced the resistance of GC to 5-FU.

MeSH Keywords: **Apoptosis • Fluorouracil • Stomach Neoplasms**

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Background

Gastric cancer (GC) is the third leading cause of cancer-related mortality in China [1]. While early stage gastric cancer has a favorable prognosis [2]; most GC patients are diagnosed at advanced stages when effective therapeutics are not readily available [3]. As a result, the 5-year survival rate of late stage GC is less than 20%–25% and the median survival time is only 6–15 months [4,5].

The 5-fluorouracil (5-FU) based chemotherapy is a standard treatment for patients with advanced GC [6]. Although combining 5-FU with cisplatin, docetaxel, or oxaliplatin can improve treatment response [7], resistance to 5-FU in GC is still an intractable issue in the clinic. It was reported that upregulation of the enzymes associated with 5-FU metabolism such as thymidylate synthase (TS), orotate phosphoribosyl transferase (OPRT) and dihydropyrimidine dehydrogenase (DPD) were involved in the 5-FU-resistance [8]. Besides, alterations of apoptosis pathway [9] or proteins in response to DNA damage [10,11] can lead to resistance to 5-FU.

Our previous study [12] indicated that the mRNA levels of regenerating family member 4 (Reg4) were strongly related to the intrinsic resistance of GC cells to 5-FU. As a member of the REG gene family, Reg4 was originally identified by high-throughput sequencing analysis of an inflammatory bowel disease library [13], and its overexpression was proven to be closely correlated to the carcinogenesis in some types of cancer [14–19]. In the present study, the role of Reg4 in promoting 5-FU-resistance in GC cells was confirmed and its mechanism was investigated.

Material and Methods

Cell lines and culture

Human gastric carcinoma cell lines SGC7901, AGS, MKN28, MKN45, and BGC823 were bought from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. AGS cells were maintained in F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco); other cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS. All cells were cultured in a 5% CO₂ and 37°C incubator.

Western blot

Cells were harvested and lysed in NP40 buffer with protease inhibitor cocktails added. The concentration of total protein was measured using the Bradford calorimetric assay (Bio-Rad Laboratories, CA, USA). Thirty micrograms of protein were separated on 12% SDS-PAGE gel and transferred to a polyvinylidene

difluoride membrane (Millipore, Bedford, MA, USA). Then the membrane was blocked with 5% bovine serum albumin (Amresco, USA) at room temperature for two hours and then incubated with antibodies against Reg4 (1: 1,000, R&D Systems Inc., USA), P44/42 (1: 1,000, Cell Signaling Technology, USA), phosphorylated P44/42 (1: 1,000, Cell Signaling Technology, USA), Bim (1: 1,000, Cell Signaling Technology, USA), Bcl-2 (1: 1,000, Epitomics, USA), Bax (1: 1,000, Epitomics, USA), active-caspase3 (1: 1,000, Antibody Revolution Inc., USA) at 4°C overnight. In the next day, the membrane was covered with HRP-conjugated secondary antibody (1: 3,000, R&D Systems Inc., USA) at room temperature for two hours and detected using enhanced chemiluminescence reagent (ECL, Millipore). Tubulin (1: 1,000, Antibody Revolution Inc., USA) was applied as a loading control.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA were extracted from cells with the TRIzol reagent (Invitrogen). Five hundred nanograms of RNA were reverse transcribed to cDNA using PrimerScript RT Reagent (Takara Biotechnology, Dalian, China). The Quantitative PCR was carried out using SYBR Premix Ex Taq II kit (Takara Biotechnology) on ABI 7500 Real-time PCR System (Life Technology, Foster City, CA, USA). All the primers were obtained from Invitrogen and the sequences of primers were

5'-GAGTGTCAGTCTTACGGAAACG-3' (Reg4 forward),
5'-TCGTGCAGGCCAATCCATATC-3' (Reg4 reverse),
5'-GAAGGTGAAGTCCGGAGTC-3' (GAPDH forward),
5'-GAAGATGGTGATGGGATTTC-3' (GAPDH reverse),
5'-GCCTCGGTGTGCCTTCA-3' (TS forward),
5'-CCCCTGATGTGCGCAAT-3' (TS reverse),
5'-TAGTGTGTTGGAAACTG TTAGAGTT-3' (OPRT forward),
5'-TGGCATCAGTGACCTCAAGCCC TCCT-3' (OPRT reverse),
5'-AGGACGCAAGGAGGGTTTG-3' (DPD forward),
5'-GTCCGCCGAGTCCTTACTGA-3' (DPD reverse).

Cycling conditions were 95°C for 10 minute, 40 repeats of 95 °C for 15 seconds followed by 60°C for one minute. The expression levels of mRNAs were calculated using the 2^{-ΔΔCt} method which was described before [20].

Reg4 knockdown and overexpression

Specific siRNAs targeting human Reg4 (siReg4-1 and siReg4-2) and non-targeting control siRNA (siCtrl) were designed and provided by Shanghai GenePharma Co., Ltd, China. The siReg4-1 sequences were 5'-AAGUGGCUAUCAGAGAAGCTT-3' and 5'-GCUUCUCUGAUAGCCACUUTT-3'. The siReg4-2 sequences were 5'-GACAGAAGGAAGAAACUCATT-3' and 5'-UGAGUUUCUUCUUCUGUUCTT-3'. The siCtrl sequences were 5'-UUCUCCGACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAG AATT-3'. For overexpression of Reg4 gene, its cDNA was

amplified using polymerase chain reaction (PCR) and subcloned into the p3 × FLAG-CMV-10 vector (Sigma, St. Louis, MO, USA). The p3 × FLAG vector was used as control. All transfections were performed using Lipofectamine3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection for 24 hours, the alterations of Reg4 expression were evaluated by Western blot and RT-qPCR.

Treatment of 5-FU and cell viability assay

The 5-FU was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of 5-FU were prepared in sterile DMSO and stored at 4°C prior to use.

Twenty-four hours post transfection, cells were seeded at a density of 3,000 cells per well in 96-well plates. The next day, different concentrations of 5-FU were added into the plates and after 48 hours, cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma) assay.

Two proper concentrations of 5-FU for each cell line were chosen from the results of cell viability assay. Transfected cells were treated with 5-FU in these two concentrations and then harvested for flow cytometry measurement, Western bolt and RT-qPCR analyses.

Flow cytometry analysis of apoptosis cells

Those 5-FU treated transfected cells were harvested by Accutase-Enzyme Cell Detachment Medium (eBiosciences, San Diego, CA, USA) and stained with the PE Annexin V Apoptosis Detection kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Measurements were performed on a FACS Calibur flow cytometer and results were analyzed by CellQuest software (BD Biosciences, San Diego, CA, USA).

Statistical analysis

SPSS 21.0 (IBM) was used for data analysis. Data from a minimum of three independent experiments were expressed as means ± standard deviations (SD). Comparisons between groups were carried out using the one-way ANOVA with Bonferroni post hoc test or Student's *t*-test, and a *p* value of <0.05 was considered statistically significant.

Results

Reg4 knockdown and overexpression

First, we evaluated the expression of Reg4 in five GC cell lines. As shown in Figure 1A, the protein expression of Reg4 was

high in AGS, MKN28, and MKN45, but low in BGC-823, and null in SGC7901. Hence AGS and SGC7901 cells were selected to do knockdown and overexpression of Reg4, respectively. As illustrated in Figure 1B–1E, both on protein and mRNA levels, AGS-siReg4-1 and AGS-siReg4-2 expressed less Reg4 than AGS-siCtrl, and SGC-Flag-Reg4 cells expressed more Reg4 than SGC-Flag cells.

Reg4 mediated 5-FU resistance of GC cells

The cell viability assay showed that after 5-FU treatment, AGS-siCtrl cells had better viability than AGS-siReg4-1 and AGS-siReg4-2 cells (Figure 2A) and similarly, SGC-Flag-Reg4 cells had higher cell viability than SGC-Flag cells (Figure 2B). The concentrations of 5-FU for next assays were set as 3 µg/mL and 30 µg/mL for AGS, 0.5 µg/mL and 5 µg/mL for SGC7901.

Expression of the enzymes associated with 5-FU metabolism

Results from RT-qPCR indicated that there was no obvious difference in expression of enzymes associated with 5-FU metabolism (TS, OPRT, and DPD) between control cells and AGS knockdown or overexpression cells (Figure 3).

Reg4 inhibited apoptosis of GC cells

As displayed in Figure 4, after treated with 5-FU, in both of the two concentrations, AGS-siReg4-1 and AGS siReg4-2 cells had significantly higher percentages of apoptosis cells than AGS-siCtrl cells, and SGC-Flag-Reg4 cells had lower apoptosis cells content than SGC-Flag-Ctrl cells.

Reg4 regulated the MAPK/Erk/Bim signaling pathway

The Western blot showed that compared with AGS-siCtrl cells, AGS-siReg4-1 and AGS-siReg4-2 cells expressed less phosphorylated Erk1/2 (p-Erk1/2), more apoptosis proteins including Bim, Bax, as well as active-caspase3, and comparable total-Erk1/2 (T-Erk1/2) and Bcl-2 (Figure 5). Consistent trend was detected in SGC-Flag-Reg4 and SGC-Flag cells (Figure 5).

Discussion

The present study showed that Reg4 increased the 5-FU-tolerability of GC cells *in vitro*, which was consistent with some previous findings that GC patients with high-expression Reg4 had poor response to 5-FU treatment in comparison to those with low-expression of Reg4 [21].

As has been reported in pancreatic cancer studies, that Reg4 has anti-apoptotic properties when exposed to chemotherapy [22],

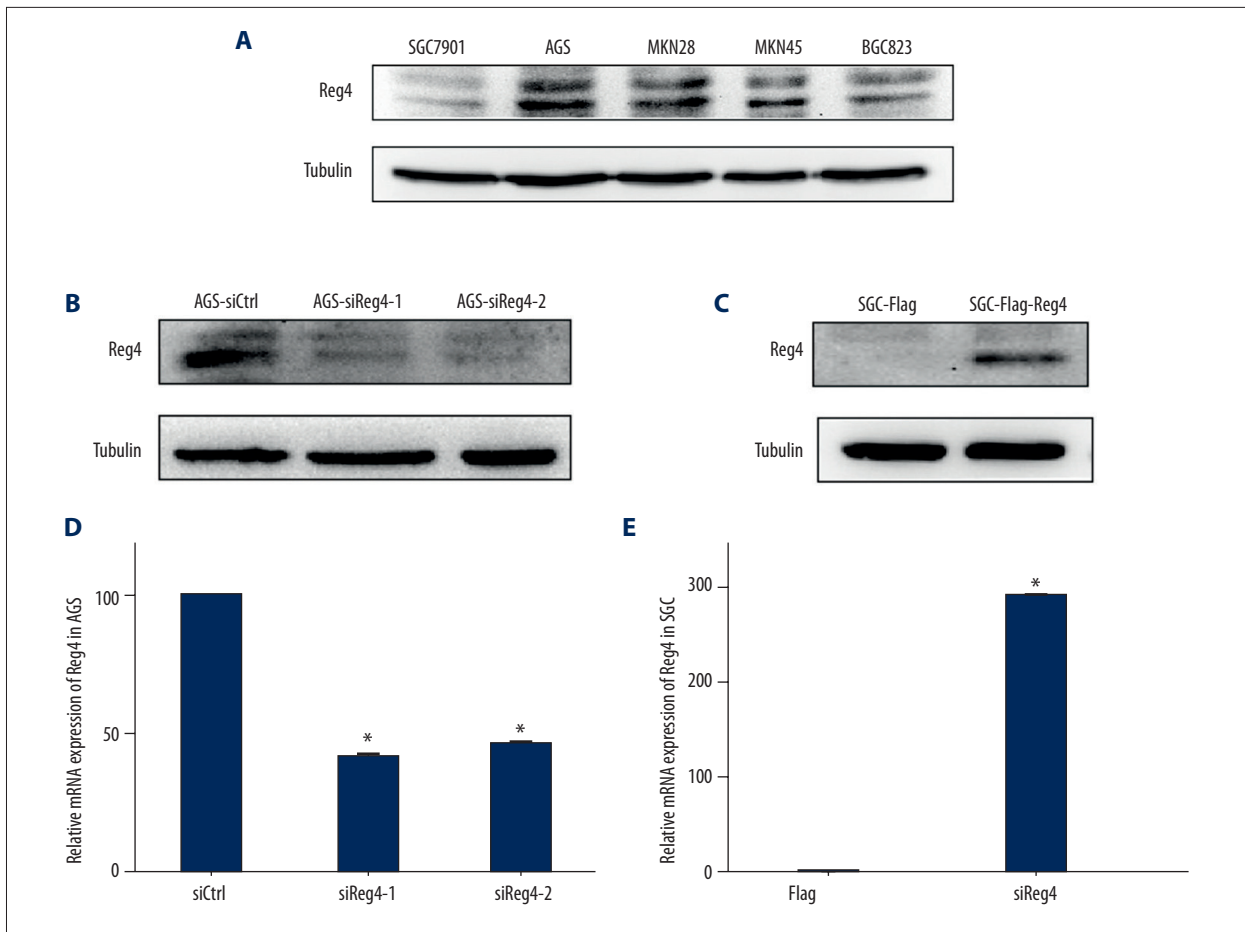


Figure 1. Expression of Reg4 in gastric cancer cell lines. **(A)** Lysates prepared from SGC7901, AGS, MKN28, MKN45, and BGC823 cells were subjected to Western blot analysis with anti-Reg4 antibody. Tubulin served as a loading control. **(B)** Lysates prepared from AGS cells transfected with siRNA targeting Reg4 were subjected to Western blot analysis with anti-Reg4 antibody. Tubulin served as a loading control. **(C)** Lysates prepared from SGC cells transfected with Flag carrying Reg4 were subjected to Western blot analysis with anti-Reg4 antibody. Tubulin served as a loading control. **(D)** RT-qPCR analysis of Reg4 was performed using total RNA from AGS cells transfected with siRNA against Reg4. GAPDH served as an internal control. Values represent the mean \pm SEM, (n=3). **(E)** RT-qPCR analysis of Reg4 was performed using total RNA from SGC cells transfected with Flag Reg4. GAPDH served as an internal control. Values represent the mean \pm SEM, (n=3), * $p < 0.05$.

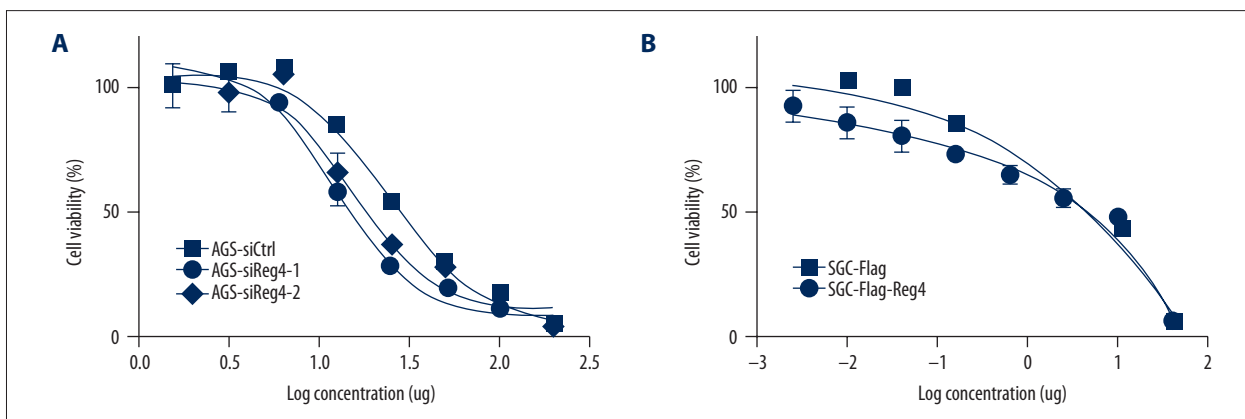


Figure 2. Forced Reg4 expression enhanced 5-FU resistance in GC cells. Cell viability was assessed by MTT assay at 48 hours after 5-FU treatment. Bars and error bars represent mean and SD, respectively, from three different experiments. Values represent the mean \pm SEM, (n=3).

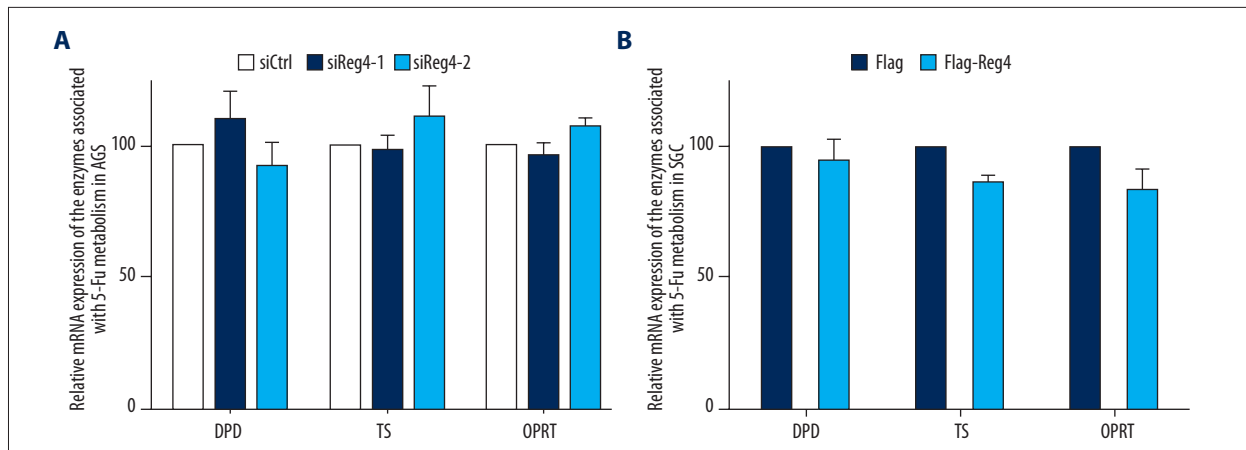


Figure 3. Expression of the enzymes associated with 5-fluorouracil metabolism. **(A)** RT-qPCR analysis of the mRNA expression of DPD, TS, and OPRT in AGS-siCtrl, AGS-siReg4-1, and AGS-siReg4-2. Values represent the mean \pm SD, (n=3), $p>0.05$. **(B)** RT-qPCR analysis of the mRNA expression of DPD, TS, and OPRT in SGC-Flag and SGC-Flag-Reg4 cells. Values represent the mean \pm SD, (n=3), $p>0.05$.

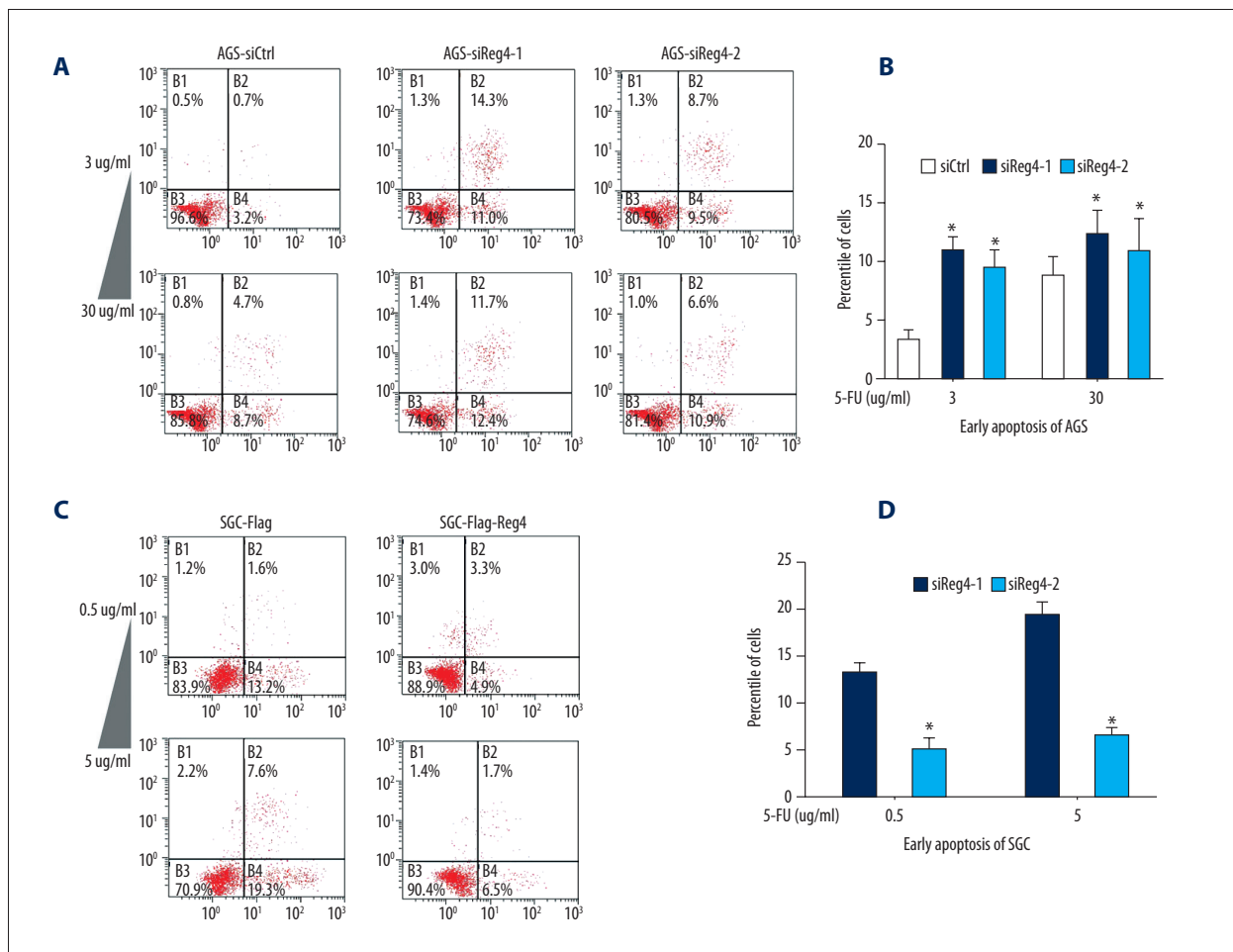


Figure 4. High expression of Reg4 decreases apoptosis induced by 5-FU. **(A, B)** Flow cytometry assays were performed using AGS cells that had been transfected with Reg4 or control siRNA and treated with 3 ug/mL and 30 ug/mL 5-FU for 24 hours. Values represent the mean \pm SD, (n=3), * $p<0.05$. **(C, D)** Flow cytometry assays were performed using SGC-Flag and SGC-Flag-Reg4 treated with 0.5 ug/mL and 5 ug/mL 5-FU for 24 hours. Values represent the mean \pm SD, (n=3), * $p<0.05$.

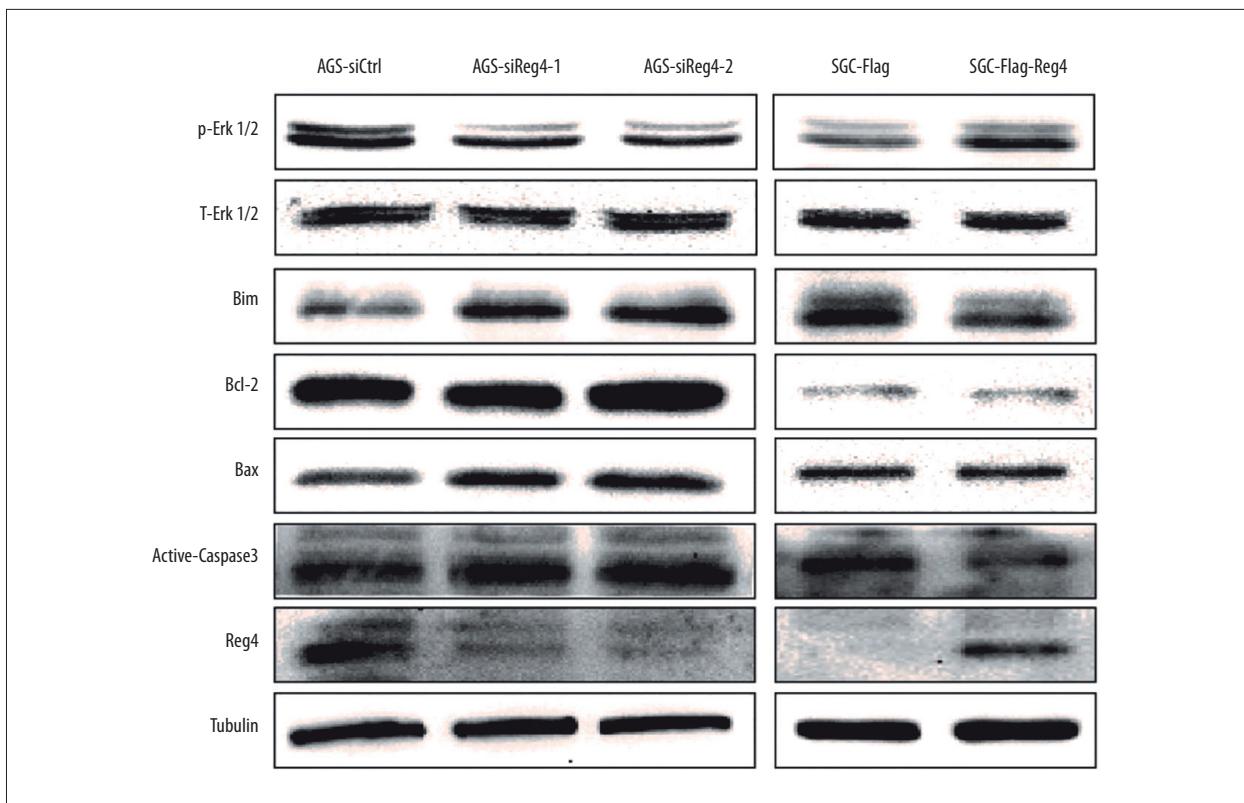


Figure 5. High expression of Reg4 enhances p-Erk1/2 and some apoptotic proteins' expression. Lysates prepared from AGS-siCtrl, AGS-siReg4-1, AGS-siReg4-2, SGC-Flag, and SGC-Flag-Reg4 cells were subjected to Western blot analysis with p-Erk1/2, T-Erk1/2, Bim, Bcl-2, Bax, and active-caspase3 antibody. Tubulin served as a loading control.

our results from flow cytometry assay indicated Reg4 decreased 5-FU inducing apoptosis in GC cells. Moreover, our study found that the MAPK/Erk pathway was activated when Reg4 exerted anti-apoptotic effects. It is well acknowledged that the MAPK/Erk pathway plays a crucial role in multiple cellular processes such as cell proliferation, differentiation, adhesion, and migration [23]. Furthermore, it has been reported that Reg4 could modulate receptor tyrosine kinases (RTKs) in prostate cancer and colon cancer [24]. With the addition of acknowledgement that the MAPK pathway is one of the signaling transduction downstream of many RTKs [25], we may suppose that Reg4 may activate the MAPK pathway through influencing RTKs. The mechanism needs to be further explored.

As for apoptotic cascade, MAPK/Erk signaling could repress pro-apoptotic proteins such as Bad and Bim to achieve cell survival [26,27]. In this study, Bim was found to be reduced when Reg4 was overexpressed. Bim, a BH3-domain-only molecule belonging to the Bcl-2 family, is responsible for mitochondrial stress-induced apoptosis [28,29]. As suggested before, Bim can directly bind to and activate Bax; its binding to pro-survival Bcl-2 homologs can indirectly promote the activation of Bax [29]. Thereupon the primed Bax enables the caspase pathway which ultimately leads to cell death [28]. Our

results from Western blot analysis (Figure 5) were consistent with the aforementioned understanding, and therefore, we speculated that Reg4 enhanced 5-FU-resistance through affecting the MAPK/Erk/Bim pathway.

However, the present study did not find any alteration of enzymes involved in 5-FU metabolism (DPD, TS, and OPRT) which are known to be important in the formation of 5-FU-resistance [21]. In addition, several studies concentrating on colorectal cancer found that Reg4 could inhibit the expression of Bcl-2 [30,31] while in this study there was no difference in Bcl-2 expression among cells with diverse levels of Reg4. We considered that these disparities were partly caused by the different cell lines used in experiments.

Conclusions

The present study demonstrated that Reg4 inhibited apoptosis through MAPK/Erk/Bim pathway and thereby enhanced the resistance of GC cells to 5-FU. These observations provide clues for further understanding of 5-FU-resistance in GC and other cancers.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of interest

No potential conflicts of interest were disclosed.