

Autophagy inhibition enhanced 5-FU-induced cell death in human gastric carcinoma BGC-823 cells

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Abstract. The exact molecular mechanism of 5-fluorouracil (5-FU) in human gastric cancer cells remains to be elucidated. Cultured BGC-823 human gastric carcinoma and AGS cell lines were treated with 5-FU. Autophagosome formation was investigated through multiple approaches, including the quantification of green fluorescent protein-microtubule-associated protein 1A/1B-light chain 3 (LC3) puncta, LC3 conversion and electron microscopy observations. Additionally, autophagy was inhibited using 3-methyladenine (3-MA) and beclin-1 ablation, to determine its role in 5-FU-mediated cell death. In addition, the present study assessed alterations in sirtuin expression following 5-FU treatment with reverse transcription-quantitative polymerase chain reaction. 5-FU treatment induced apoptosis and inhibited proliferation in BGC-823 and AGS gastric cancer cells. It is of note that the 5-FU treatment only promoted autophagy in BGC-823 cells. Additionally, inhibition of autophagy by either 3-MA or beclin-1 ablation increased 5-FU-induced cell death in BGC-823 cells. The present study quantified changes in sirtuin (SIRT1, SIRT3, SIRT5, and SIRT6) expression following 5-FU treatment and using a specific inhibitor, sirtinol, the present study investigated their involvement in 5-FU-mediated autophagy. Autophagy inhibition through manipulation of sirtuin proteins may increase the therapeutic efficacy of the 5-FU chemotherapeutic drug against gastric carcinoma.

Introduction

Gastric cancer is one of the major causes of cancer-associated mortality worldwide, especially in Asia-Pacific regions (1). The incidence rate is high in Asian populations, including Korea, China and Japan. Treatment usually involves complete resection by surgery. However, only 20-30% of patients with

advanced gastric cancer have their survival rate improved by surgery alone, and these suffer a high risk of recurrence and metachronous metastases after surgery (2). It has therefore been determined that adjuvant chemotherapy may have a subordinate role in clinical trials for advanced or metastatic stomach cancers (3). Therapeutic application of several chemotherapeutic agents, including 5-fluorouracil (5-FU), doxorubicin, mitomycin, carmustine and cisplatin led to a low response rate, ranging from 15-30% in patients with advanced gastric cancer, when used as single treatments. Combination chemotherapy, such as the widely used regimen of 5-FU, doxorubicin, and mitomycin, increased the response rate to 30-40% for a period of 5 to 6 months, and patient overall survival time increased by 7 to 8 months (4).

From these different chemotherapeutic agents, the present study specifically focused on the molecular mechanism of 5-FU treatment in gastric cancer (5). 5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of a hydrogen, and is a major chemotherapeutic agent used in therapies against malignancies, including stomach, colon and breast carcinomas. It has been previously determined that cytotoxicity may be induced through a mechanism involving the incorporation of fluoro-nucleotides into RNA and DNA and by inhibiting the nucleotide synthetic enzyme thymidylate synthase (6). The cytotoxic effect of 5-FU may activate programmed cell death pathways and induce apoptosis in cancer cell lines, such as colorectal cancer cells (7). Autophagy induction by 5-FU has been frequently observed in various cancer cells, including colon, pancreatic and hepatocellular carcinoma cells (8-10). Autophagy is an evolutionarily conserved process involving the degradation of long-lived proteins, organelles and bulk cytoplasm which occurs during cell development, stress or starvation (11). However, its role in cancer is controversial as previous studies (12,13) have reported its role in tumor growth, whereas others report its tumor suppressive function. It has also been previously reported that autophagy delays apoptosis in response to chemotherapy and its inhibition increases drug-induced apoptosis in human cancer cell lines. However, anti-tumor agents in some conditions, such as at high doses, appear to augment autophagic cell death (14).

The sirtuins (SIRT), a family of class III histone deacetylase enzymes, are highly conserved mammalian homologues of the yeast Sir2 gene. They have been identified to have an important role in cancer biology, by acting as tumor promoters and tumor suppressors in different cancer types (15). The

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sirtuin super family of proteins has seven members (SIRT1-7) and SIRT1 has been identified to modulate autophagy in gastric cancer (16). Previous studies have also implicated other family members, including SIRT2, SIRT3, SIRT5 and SIRT6 in autophagy regulation (17-20).

The present study aimed to determine the molecular mechanism of 5-FU treatment in gastric cancer and to evaluate if a combinatory therapeutic approach, including the use of autophagy inhibitors may enhance the therapeutic efficacy of 5-FU against gastric cancer.

Materials and methods

Chemical reagents and antibodies. 5-FU was purchased from Xudong Haipu Pharmaceuticals (Shanghai, China), 3-methyladenine (3MA), sirtinol, rapamycin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). The antibodies purchased were: Microtubule-associated protein 1 light chain 3 (LC3; L7543; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), which recognizes human LC3-I and LC3-II by immunoblotting (18 kDa and 16 kDa respectively), p70S6 kinase (9202) and Phospho-p70 S6 kinase (Thr389; 9205; Cell Signaling Technology, Inc., Danvers, MA, USA), Beclin 1 (sc-10086) and β -actin (sc-81178; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The GFP-LC3 plasmid was provided by Dr Mengqiang Li (Peking University School of Medicine, Peking, China).

Culturing of gastric cell lines. BGC-823 and AGS cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium, supplemented with 10% (v/v) fetal bovine serum, 100 kIU/l penicillin and 100 mg/l streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere incubator with 5% CO₂. Cells were sub-cultured every two days with a split ratio of 1:2.

To investigate the role of sirtuins in autophagy, sirtinol was co-treated with 5-FU. The BGC-823 cells were either treated individually or together with 5-FU, 3-MA, or sirtinol in the following six groups: i) untreated (Ctr); ii) 5 mM 3MA (3MA); iii) 40 μ g/ml 5-FU (5-FU); iv) 40 μ g/ml 5-FU+5 mM 3MA (5-FU+3MA); v) 40 μ g/ml of 5-FU+10 μ M sirtinol (5-FU+sirtinol); and vi) 40 μ g/ml of 5-FU+10 μ M sirtinol+5 mM 3MA (5-FU+sirtinol+3MA).

MTT assay. Cells were seeded into 96-well flat bottom microtiter plates, at a density of 10⁴-10⁵ cells/well in 100 μ l culture medium. The seeded cells were incubated for 24-48 h with 5-FU at concentrations of 5, 10, 25, 50, 100, and 200 μ g/ml. Next, 100 μ l MTT reagent (5 mg/ml, Sigma-Aldrich; Merck KGaA) was added to each well, and the cells were further incubated for 4 h at 37°C in a CO₂ incubator. Finally, the crystals were dissolved in 100 μ l DMSO, by pipetting up and down, and the absorbance was quantified at 570 nm in a microtiter plate reader (BD Biosciences, Franklin Lakes, NJ, USA).

Confocal microscopy. In order to quantify the green fluorescent protein (GFP)-microtubule-associated

protein 1A/1B-light chain 3 (LC3) puncta formation in cells treated with 5-FU and untreated cells were used as controls, the cells at ~60% confluence were transfected with GFP-LC3 cDNA using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were exposed to treatment for 48, 24 h after the transfection. Subsequently, LC3 puncta formation was assessed under confocal microscopy (Olympus Corporation, Tokyo, Japan) and the distribution was quantitatively evaluated using ImageJ version 1.48 (National Institutes of Health, Bethesda, MD, USA). The percentage of GFP-LC3 puncta distribution was calculated from 5 non-overlapping fields selected at random (magnification, x600) and the statistical significance was evaluated from three repeated independent experiments.

Transmission electron microscopy. The cells were harvested by trypsin digestion (Sigma-Aldrich; Merck KGaA) and washed twice with PBS before fixing with ice-cold glutaraldehyde (3% in 0.1 M cacodylate buffer, pH 7.4; Sigma-Aldrich; Merck KGaA) for 30 min. After washing again with PBS, the cells were post-fixed in OsO₄ at room temperature for 1 h and embedded in Epon resin overnight at room temperature (Sigma-Aldrich; Merck KGaA). The 0.1-mm sections were stained at room temperature for 10 min with uranyl acetate/lead citrate (Fluka Chemie AG, Buchs, Switzerland) solution and then observed under a JEM1230 electron microscope (JEOL, Ltd., Tokyo, Japan).

Flow cytometry. After trypsinization, the cells were washed with PBS twice, and then suspended (5x10⁵ cells/500 μ l) in the binding buffer, prior to incubation with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) solutions, according to the manufacturer's protocol of Annexin V-FITC apoptosis detection kit (Biovision Inc., Milpitas, CA, USA). The cells were analyzed using a flow cytometer FACSaria (BD Biosciences, Franklin Lakes, NJ, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA (1 μ g) was extracted by the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse-transcribed into cDNA using a reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, the cDNA was amplified using the following parameters: Pre-denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.) with SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers used for the qPCR are presented in Table I. The data was normalized against house-keeping gene GAPDH, and analysis was performed using the 2^{- $\Delta\Delta$ Cq} method (21), where $-\Delta\Delta Cq = \Delta Cq$ (treated sample) - ΔCq (untreated), and $\Delta Cq = Cq$ (target gene) - Cq (housekeeping gene). Changes in gene expression, reported as fold-change of relative mRNA expression are expressed as the target/reference ratio of the sample divided by the target/reference ratio of the control and the experiments were repeated three times.

RNA interference. The cells were seeded at 40% confluence in each well (1.5x10⁴/well) of 96-well plates overnight. The next day, the cells were transfected with Beclin-1 siRNA or

Table I. List of target primer pairs used for gene specific amplification in quantitative polymerase chain reaction.

Gene	Forward (5'-3')	Reverse (5'-3')
SIRT1	ACTTGTACGACGAAGACGAC	GATCTGTGCCAATCATAAGA
SIRT2	GCAGGAGGCTCAGGACTCA	TGACTCTGCGACAGCGTTC
SIRT3	CCTCCAGCAGTACGATCTCC	GGTTCCATGAGCTTCAACCA
SIRT4	GGGTTATTTGTGCCAGCAAG	AAGTTTCTCGCCCAGTACCG
SIRT5	GTTCAAGTATGGCAGATTTTCG	CTCCAATAACCTCCAGCTCC
SIRT6	CCAAGTTCGACACCACCTTT	CGGACGTACTGCGTCTTACA
SIRT7	ATCAGCACGGCAGCGTCTAT	AGGTGGAGCCCGTCACAG
GAPDH	ACGGATTTGGTCGTATTGGG	TGATTTTGGAGGGATCTCGC

SIRT, sirtuin.

control siRNA duplex (Santa Cruz Biotechnology, Inc.) using Lipofectamine 2000 reagent. The knockdown of the specific gene was confirmed using western blot analysis 48 h after transfection.

Western blot analysis. 5-FU-treated and control cells were lysed by incubation in a lysis buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, with freshly added proteinase inhibitor cocktail) on ice for 30 min. The pelleted cell lysates were centrifuged at 20,817 x g for 15 min at 4°C and the supernatant was collected. The protein concentration was quantified using BCA assay reagent (Pierce; Thermo Fisher Scientific, Inc.) and subsequently proteins were separated on 10% SDS-PAGE gel. This was followed by their transfer to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk, the membranes were incubated with the primary antibodies diluted at 1:1,000 overnight at 4°C. The following day, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rat (7077; Cell Signaling Technology, Inc.) at 1:5,000 dilution for 2 h at room temperature. The protein bands were visualized on X-ray film using an enhanced chemiluminescence system (Pierce; Thermo Fisher Scientific, Inc.). To determine the linear range of the chemiluminescent signals, X-ray films were quantitatively analyzed via densitometry analysis by ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean \pm standard error of the mean. Significant differences between groups were analyzed using Student-Newman-Keuls analysis. Non-normal distribution data were presented as mean without an error bar and analyzed using the Mann-Whitney test to determine the significance of the difference between two groups. $P < 0.05$ was considered to indicate statistically significant difference.

Results

5-FU inhibits gastric cancer cell proliferation and induced apoptosis. The MTT assay indicated that 5-FU treatment

inhibited BGC-823 and AGS cell viability in a dose- and time-dependent manner. The 5-FU concentrations from 5 to 200 μ g/ml generated varied cellular cytotoxicity profiles after treatment duration of 24, 48 and 72 h. An IC_{50} of 40 μ g/ml after 48 h 5-FU treatment in BGC-823 cells and 60 μ g/ml in AGS cells was observed (Fig. 1A). Therefore, these two 5-FU concentrations (40 μ g/ml in BGC-823 and 60 μ g/ml in AGS cells) were used for subsequent experiments.

The microscopic observation of cells after 5-FU treatment revealed cells to be round, lifting off the culture surface, and having multiple membrane blebs (Fig. 1B), indicating the probable onset of apoptosis and necrosis. To verify these observations, the cells were analyzed using flow cytometry to detect cell death, using an Annexin V-FITC/PI assay. As presented in Fig. 1C, the number of Annexin V positive cells increased significantly in AGS and BGC-823 cells, respectively, after 5-FU treatment when compared with the untreated group. This data indicated that 5-FU treatment promoted apoptosis in gastric cancer cells.

5-FU induces autophagy only in BGC-823 cells. A previous study determined that 5-FU induces autophagosome formation in some cancer cells (22); therefore, the present study tested this in both gastric cancer cell lines. LC3, a mammalian homologue of the yeast Atg8 protein located in the autophagosome membrane, acts as a specific marker of autophagy. Therefore, in order to determine the formation of autophagosomes, both BGC-823 and AGS cells were transfected with a GFP-LC3 plasmid. After 48 h of 5-FU treatment, GFP-LC3 puncta were accumulated in BGC-823 cells compared with untreated cells (Fig. 2A). Subsequently, the levels of LC3-II (16 kDa), a mature and spliced form of LC3 that represents autophagic activity (23), were also assessed by western blotting. It is of note that increased expression of LC3-II was observed only in BGC-823 cells, treated with 5-FU for 48 h. This was consistent with GFP-LC3 immunofluorescence data. Rapamycin treatment with 10 nM concentration at room temperature for 48 h (a notable agonist of autophagy) was used as control, upregulated LC3-II levels in both cell lines were observed as expected, and 3MA (a well-known inhibitor of autophagy) treatment downregulated LC3-II levels in BGC-823 cells, and no change in AGS cells was observed (Fig. 2B). The present

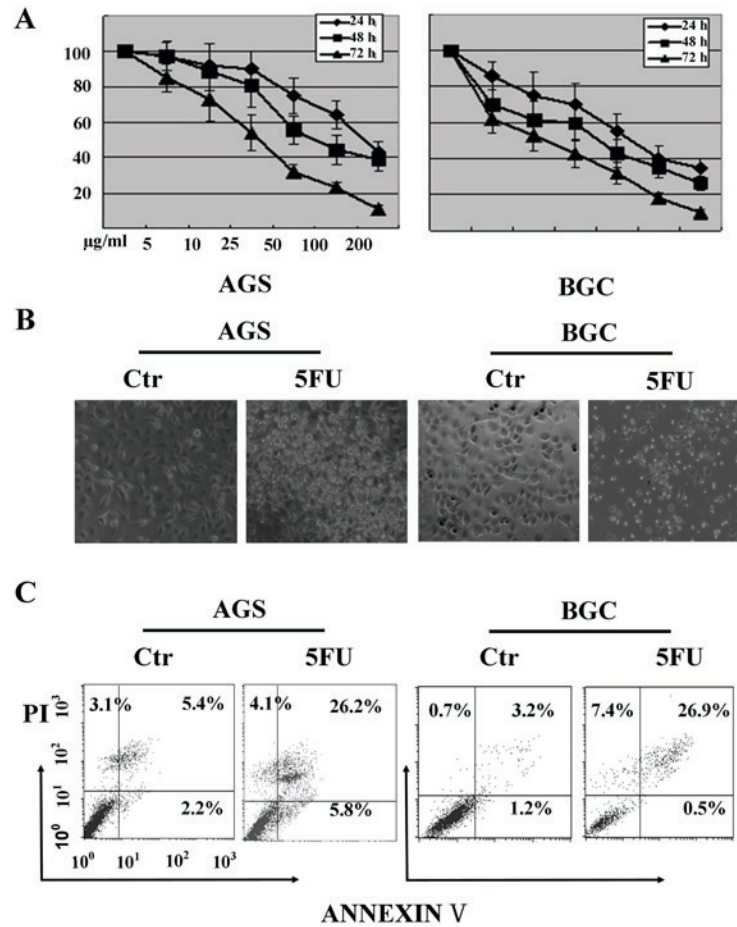


Figure 1. 5-FU inhibited cell proliferation and induced apoptosis in gastric cancer cells. (A) Effects of 5-FU on BGC-823 and AGS cell viability, after 24, 48 and 72 h treatment, using different 5-FU concentrations (0-200 $\mu\text{g/ml}$) as assessed by MTT assay. All experiments were repeated independently three times. (B) Estimation of apoptotic percentage through microscopy (x100) in gastric cancer cells after treatment with 5-FU or without (Ctrl) for 48 h. (C) Annexin V/PI staining analysis through flow cytometry, in gastric cells with or without 5-FU treatment for 48 h. Ctrl, control; PI, propidium iodide; 5-FU, 5-fluorouracil.

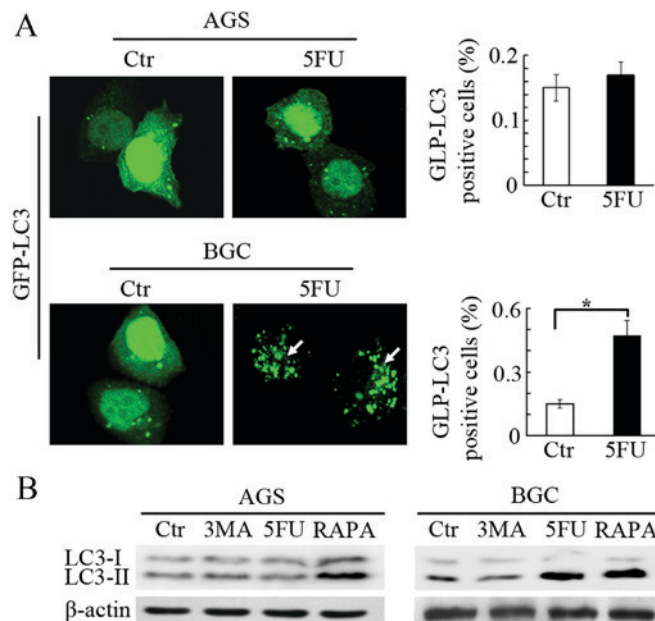


Figure 2. 5-FU induced autophagy only in BGC-823 cells. (A) Cells were treated with 5-FU for 48 h and GFP-LC3 puncta were identified using fluorescent microscopy. Representative images have been shown and GFP-LC3 puncta/granules are marked by white arrows (x600). Upper panel, AGS cells, lower panel, BGC cells. * $P < 0.05$. (B) Western blotting analysis of LC3 expression in cells treated with 5-FU or control. β -actin expression was used as protein loading control. RAPA treatment was used as a positive control, whereas 3MA treatment was negative control. Ctrl, control; 5-FU, 5-fluorouracil; RAPA, rapamycin; 3MA, 3-methyladenine; GFP.

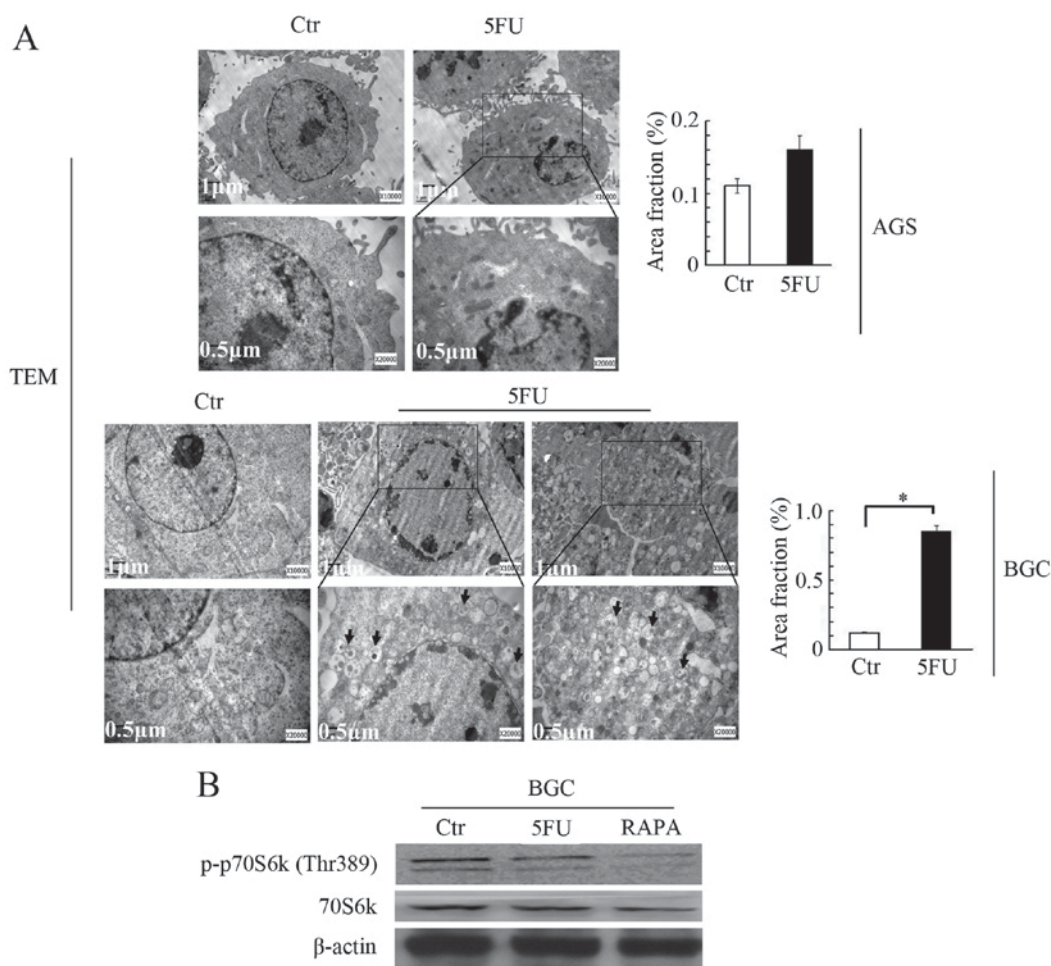


Figure 3. 5-FU induced autophagy in BGC-823 cells via an mTOR-dependent pathway. (A) Upper panel shows representative electron microscope images of cells treated with 5-FU and control cells. Typical autophagosomes or autolysosomes are indicated by black arrows. Lower panel depicts the autophagosome area ratio to cytoplasm, measured using ImageJ version 1.48 and analyzed by a Mann-Whitney test, in cells either treated with 5-FU or controls. * $P < 0.05$. The area ratio data had non-normal distribution and thus presented as the mean of at least 20 cell measurements in each group. These experiments were repeated independently three times. (B) Immunoblotting analysis of the expression of p-p70S6k (Thr389) in 5-FU-treated BGC-823 cells, compared with Ctrl cells. RAPA treatment was used for positive control comparison. β -actin was used as the equal loading control. Ctrl, control; 5-FU, 5-fluorouracil; RAPA, rapamycin.

study also verified the effect of 5-FU on autophagosome formation induction using transmission electron microscopy (TEM). The present study observed multiple autophagosomes (a double membrane structure) and autolysosomes (a single membrane structure) in the cytoplasm of BGC-823 cells treated with 5-FU when compared to untreated cells (Fig. 3A). Additionally, the present study identified a significant difference in the ratio of autophagosome area to cytoplasm between 5-FU treated cells and control cells (Fig. 3A, lower panel). However, autophagosomes and autolysosomes were rarely observed under TEM in AGS cells following 5-FU treatment. Overall, this data indicated that 5-FU promoted autophagosome formation in BGC-823 cells only and had no effect on AGS cells.

5-FU reduces the phosphorylation of p70S6 kinase in BGC-823 cells. Considering that 5-FU promoted the autophagic process only in BGC-823 cells, and not in AGS cells, the present study aimed to determine the molecular mechanism behind its process. Among the various signaling pathways regulating autophagy, the mTOR pathway inhibition is the one that has been widely reported to promote autophagy (24). Therefore,

this pathway was monitored by analyzing the phosphorylation of downstream kinase 70S6 kinase at the Threonine 389 site. As presented in Fig. 3B, 5-FU treatment reduced the phosphorylation at Threonine 389 in BGC-823 cells compared with untreated cells. Rapamycin treatment was used as a positive control. These results indicated that 5-FU-induced autophagy may be dependent on mTOR inhibition.

3-MA augments 5-FU-induced cell death in BGC-823 cells. Autophagy tends to execute a dual function (promotion of cell death or cell survival) in a context-dependent manner (25). The present study aimed to assess the influence of the autophagy inhibitor 3-MA (5 mM) on 5-FU effects in BGC-823 cells (Fig. 4). GFP-LC3-transfected BGC-823 cells were treated with 5-FU in the presence or absence of 3-MA, to test autophagosome formation. As presented in Fig. 4A, the number of GFP-LC3 puncta was significantly lower in cells treated with a combination of 5-FU and 3-MA compared with those subjected to 5-FU treatment alone. Microscopic assessment of cells treated with 5-FU and 3-MA presented variable cell viability, where the majority of the cells appeared round, lifted off from the culture surface, with multiple membrane

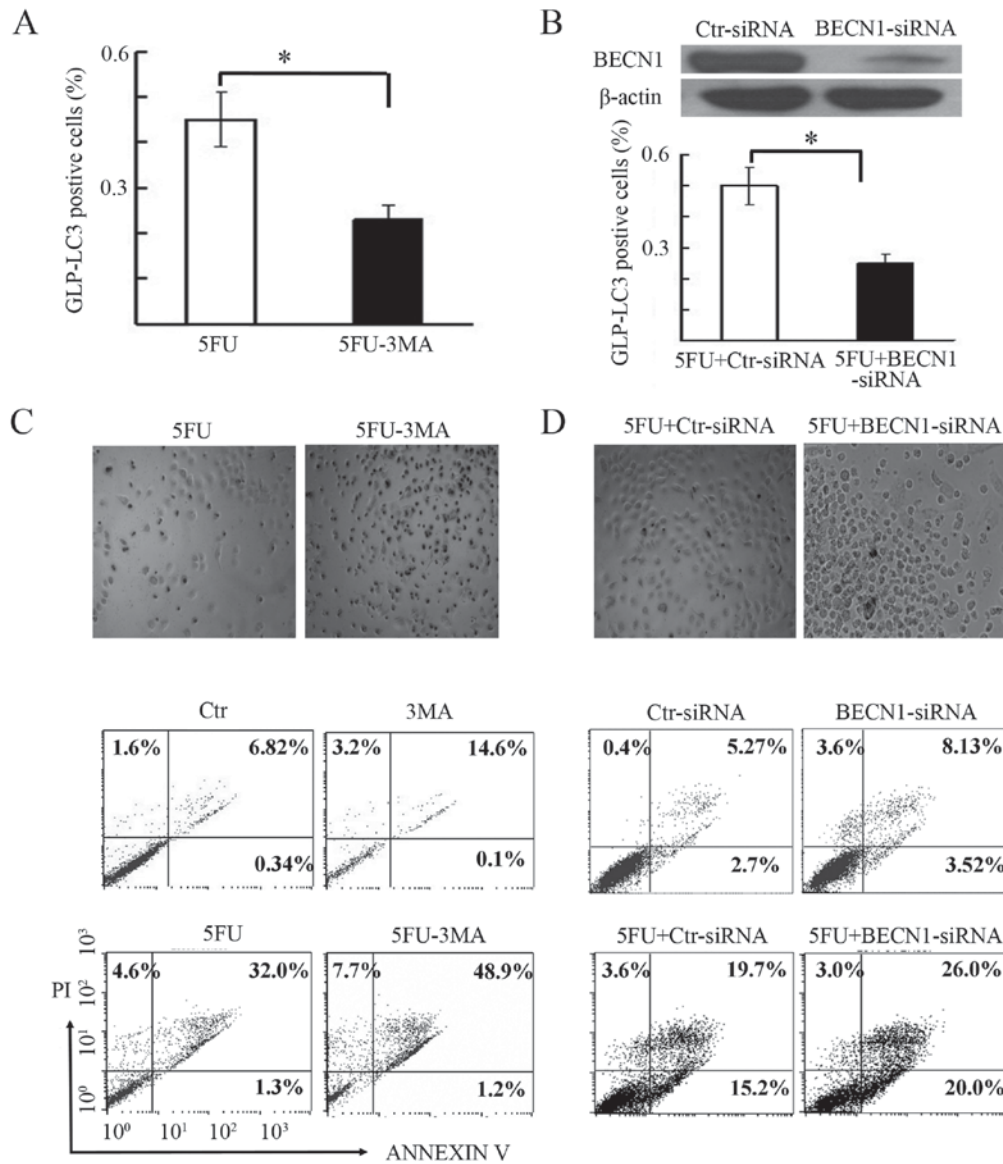


Figure 4. Autophagy inhibition enhanced 5-FU-induced cell death. (A) Assessment of GFP-LC3 positive puncta (%) in BGC-823 cells treated with 5-FU and 5-FU+3MA. * $P < 0.05$. The GFP-LC3 positive cells (%) are presented as the mean \pm standard error of the mean from three independent experiments. (B) Beclin-1 ablation by its specific siRNA in BGC-823 cells, in comparison to control siRNA (Ctr-siRNA). β -actin expression was used as the protein loading control. The lower panel shows the effect of Beclin-1 ablation on GFP-LC3 positive cells, after 5-FU treatment. * $P < 0.05$. (C) Microscopy (x100) and flow cytometry-based detection of apoptosis after autophagy inhibition by 3MA. The upper panel shows a representative image of apoptotic cells obtained through microscopy, whereas the lower panel shows the percentage of Annexin V and/or PI positive cells, after treatment with 5-FU alone and with 3MA co-treatment (5-FU+3MA). (D) Microscopy (x100) and flow cytometry based detection of apoptosis after autophagy inhibition by Beclin-1 ablation. Upper panel shows a representative image of apoptotic cells obtained through microscopy, whereas the lower panel shows the percentage of Annexin V and/or PI positive cells, after treatment with 5-FU and Beclin-1 siRNA (5-FU+BECN1-siRNA) or control siRNA (5-FU+Ctrl-siRNA). Ctr, control; 5-FU, 5-fluorouracil; BECN1-siRNA, Beclin-1 small interfering RNA; 3MA, 3-methyladenine; PI, propidium iodide, LC3, microtubule-associated protein 1A/1B-light chain 3; GFP, green fluorescent protein.

blebs (Fig. 4C, upper panel), indicating the possible onset of apoptosis and necrosis. In order to verify these observations, cell death was investigated using flow cytometry. As presented in Fig. 4C (lower panel), the number of Annexin V and PI positive cells in the 5-FU+3MA group was 19.9% higher compared with the 5-FU group alone. This indicated that autophagy inhibition by 3-MA treatment increased 5-FU-induced cell death.

Inhibition of autophagy by Beclin-1 siRNA enhances 5-FU-induced cell death. To independently confirm the induction of cell death by 5-FU following autophagy inhibition, the present study blocked Beclin-1 expression, an important

component of the autophagy pathway (26-27). The BGC-823 cells transfected with Beclin-1 siRNA or scrambled siRNA for 24 h were later treated with 5-FU for another 48 h. As presented in Fig. 4B (upper panel), the Beclin-1 expression was ablated by 90% with its specific siRNA duplex compared to scrambled siRNA. The effect of Beclin-1 siRNA on autophagy inhibition was confirmed by monitoring GFP-LC3 puncta (Fig. 4B, lower panel). Further examination of siRNA-transfected cells by microscopy and flow cytometry confirmed these findings. The cells exhibited rounded morphology, were lifted off the culture surface and had multiple membrane blebs, after treatment with 5-FU in combination with Beclin-1

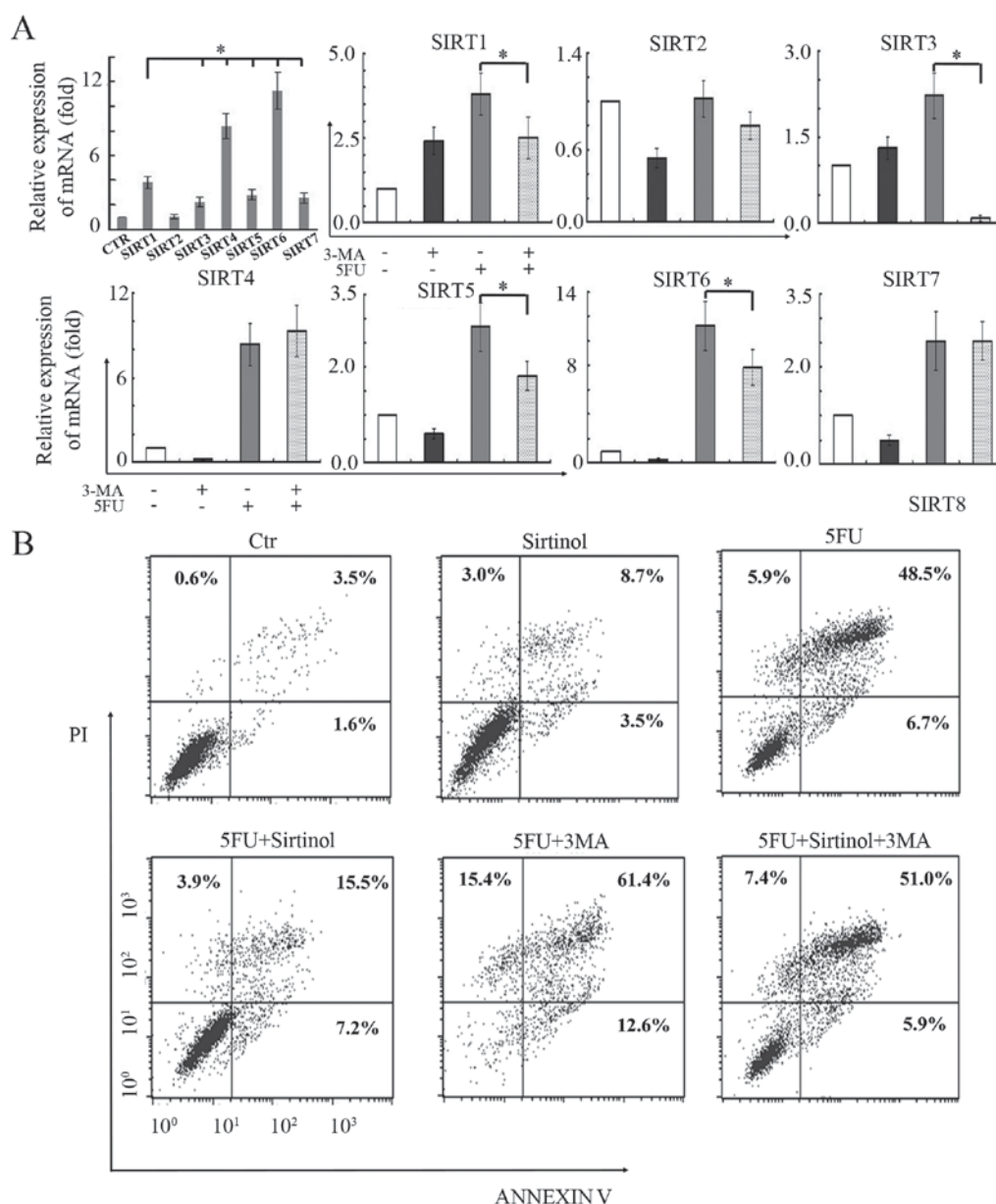


Figure 5. Sirtuin family may be involved in 5-FU-induced autophagy in BGC-823 cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression of SIRT1-7 in BGC-823 cells after exposure to 5-FU when compared with Ctr treatment (top left panel). * $P < 0.05$. Three independent experiments were performed. (B) Flow cytometry analysis of BGC-823 cells following different experiments. After these treatments, cells were analyzed for Annexin V/PI staining through flow cytometry to assess apoptosis. * $P < 0.05$. SIRT1-7, sirtuin 1-7; Ctr, control; 5-FU, 5-fluorouracil; 3MA, 3-methyladenine; PI, propidium iodide.

ablation (Fig. 4D, upper panel). Flow cytometry analysis also revealed a higher percentage (49%) of Annexin V and PI positive cells after 5-FU treatment and Beclin-1 ablation than control ablation (38.5%; Fig. 4D, lower panel). This confirmed the observation that 5-FU-induced gastric cancer cell death was enhanced by autophagy inhibition.

Sirtuins are involved in 5-FU-induced autophagy-mediated cell death in BGC-823 cells. The involvement of the sirtuin family of proteins was determined in 5-FU-induced autophagy induction in gastric carcinoma cells. The RT-qPCR data revealed that the mRNA levels of six sirtuins, including SIRT1 and SIRT3-7, were upregulated after 5-FU treatment in BGC-823 cells (Fig. 5A, top left). It is of note that the levels of SIRT4 and SIRT6 mRNA had the highest expression compared

with the remaining sirtuins. Subsequently, the present study assessed the sirtuin levels in BGC-823 cells treated with 5-FU in the presence of 3-MA (5 mM). RT-qPCR analysis indicated that increases in SIRT1, SIRT3, SIRT5 and SIRT6 mRNA expression induced by 5-FU was attenuated by 3-MA (Fig. 5A). These findings indicate that four sirtuins, SIRT1, SIRT3, SIRT5, and SIRT6 may be involved in 5-FU-induced autophagy in BGC-823 cells. Additionally, the present study tested the effect of sirtuin inhibition, using sirtinol (a specific inhibitor of SIRT), on 5-FU-induced cell death. The BGC-823 cells were co-treated with 5-FU, 3-MA and sirtinol. Following these treatments, the cells were analyzed by flow cytometry for Annexin V-FITC/PI staining to detect cell death. The findings of the present study demonstrated that sirtinol treatment abrogated both 5-FU-induced and 5-FU+3MA-induced

cell death (Fig. 5B). The percentage of cell death decreased from 61.1% in the 5-FU treatment group to 26.6% in the 5-FU+sirtinol treatment group, whereas it decreased from 89.4% in the 5-FU+3MA treatment group to 64.3% in the 5-FU+sirtinol+3MA treatment group. Therefore, the protective role of SIRT proteins in 5-FU-induced cell death is evident.

Discussion

Despite reports of 5-FU inducing apoptosis and inhibiting cell proliferation in gastric cancer cells (28), its exact molecular mechanism remains to be elucidated. Consistent with these previously published studies, the present study also observed that 5-FU promoted gastric cancer cell apoptosis in BGC-823 and AGS cell lines and inhibited their proliferation. Microscopic observation of cells following 5-FU treatment revealed rounded morphology, cells starting to lift off the culture surface and exhibited multiple membrane blebs. This is indicative of very early apoptosis and necrotic behavior and then confirmed by the Annexin V-FITC/PI assay. In terms of the molecular mechanisms of many chemotherapeutic drugs, it has been previously suggested that they induce autophagy in cancer cell lines and animal models (25). 5-FU induced autophagy, was not observed under conventional microscopy, GFP-LC3 fluorescence signals (indicative of puncta formation), LC3 immunoblotting, and TEM were used instead (29). Similarly, the present study determined that 5-FU induced autophagosome formation in the BGC-823 cell line, which may be dependent on mTOR inhibition. Conversely, autophagosome formation through 5-FU treatment was not observed in the AGS cell line. This discrepancy may be due to the cellular origins of these two different cell types. AGS cells were derived from well-differentiated gastric adenocarcinoma, whereas BGC-823 cells originated from poorly-differentiated gastric adenocarcinoma. The induction of autophagic processes following 5-FU chemotherapy in poorly-differentiated gastric adenocarcinoma cells may be a defensive response under stress conditions, whereas in well-differentiated gastric adenocarcinoma cells, the lack of autophagosome formation, usually as a guardian mechanism, may promote tumor cell apoptosis under 5-FU treatment. The contribution of autophagy to cell death or survival is controversial; however, in the current experiments, autophagy inhibition was determined to have enhanced 5-FU-mediated apoptosis. This suggests that upregulation of autophagy by 5-FU does not support cell death. Therefore, alteration in the phenotypic changes in BGC-823 cells treated with 5-FU may be directly attributed to modulation of apoptosis, as confirmed by the Annexin V-FITC/PI assay.

It is well-known that autophagy has a dual role in cancer cells after chemotherapy. In some cases, it promotes survival, whereas in other instances it facilitates cell death (12). It is of note that in the current study a specific autophagy inhibitor, 3-MA, enhanced 5-FU-induced apoptosis in BGC-823 cells. Additionally, it was also confirmed that the augmentation of cell death by 5-FU, after inhibiting autophagy through Beclin-1 ablation in BGC-823 cells. These observations indicated that autophagy induction in BGC-823 cells delayed cell death; however, inhibition of this pathway enhanced the 5-FU mediated cell death. Similar observations have been made in our previous studies, where matrine-treated or etoposide-treated

hepatoma cells exhibited a delayed cell death response due to autophagosome formation; however, autophagy suppression augmented the cell death (30-32). Autophagy is stimulated by stress conditions like nutrition deprivation and starvation and is involved in the removal of long-lived proteins and damaged organelles, in addition to providing amino acids for maintaining the metabolism, essential for survival in poor conditions (11). Autophagy generally provides cancer cells with a rescue mechanism (33,34). It has been previously reported that in human skin squamous carcinoma cells, autophagy inhibition with 3-MA enhanced 5-FU mediated apoptosis (35). In another previous study, in the absence of caspase activation, GAPDH-enhanced autophagy protected cells from caspase-independent cell death (36). In apoptosis-defective cells, autophagy has been identified to promote survival after metabolic stress and autophagy inhibition induced necrotic cell death (37). It is also possible that autophagy sequesters and degrades proteins or organelles, such as mitochondria damaged by 5-FU treatment and releases amino acids, which in turn maintains gastric cancer cell viability.

Furthermore, when investigating the mechanistic role of 5-FU in gastric cancer cells the present study determined that SIRT1 and SIRT3-7 were upregulated, but not SIRT2, in 5-FU-treated BGC-823 cells. It is of note that 3MA co-treatment with 5-FU reduced the expression of four sirtuins, SIRT1, SIRT3, SIRT5 and SIRT6, suggesting that sirtuins were important in the 5-FU-induced autophagy in gastric cancer cells. SIRT3 and SIRT5 proteins were primarily localized in the mitochondria, whereas SIRT1 and SIRT6 were in the nucleus. Previous studies have established the role of these four sirtuins in autophagy induction and formation (38,39). SIRT1 has predominantly been implicated in autophagosome formation (16), with SIRT3, SIRT5, and SIRT6 more recently revealed to be associated with autophagy (18,19,40). The SIRT1 mechanism of modulating autophagy occurs via deacetylation of autophagy-related gene 5 (Atg5), Beclin-1, Atg7, Atg8, and other autophagy mediators, which affects autophagosome induction, maturation, and tumor growth (16,41,42). Sirtuins appear to promote cellular proliferation and may also be involved in 5-FU-induced autophagy, epithelial-mesenchymal transition, cell invasion and chemoresistance in gastric cancer cells (16). It is of note that the present study identified SIRT inhibition by sirtinol reduced 5-FU-induced apoptotic cell death, suggesting that SIRT1 inhibition may protect against cell death and function as a cell survival mechanism under chemotherapy. However, this observation is preliminary and further studies are warranted, for example using SIRT siRNA, to elucidate the specific role of SIRT proteins in 5-FU-induced autophagy and cancer cell death.

In summary, the present study demonstrated that 5-FU promoted apoptosis and autophagy in gastric cancer cells. However, autophagy inhibition enhanced 5-FU-induced apoptosis. Furthermore, the present study noted the probable involvement of four sirtuin proteins, SIRT1, SIRT3, SIRT5, and SIRT6, in 5-FU mediated autophagy induction. In conclusion, the current study revealed that manipulation of autophagy for therapeutic purposes may be useful in order to overcome chemoresistance and the use of autophagy inhibitors along with 5-FU may enhance its therapeutic efficacy in gastric carcinoma.

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