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MiR-195 reverses 5-FU resistance through targeting HMGA1 in gastric cancer cells

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Abstract. – OBJECTIVE: To investigate the role of micro ribonucleic acid (miR)-195 in acquired resistance to 5-fluorouracil (5-FU) in gastric cancer and its potential mechanism.

MATERIALS AND METHODS: The drug resistance of AGS/5-FU and SGC-7901/5-FU cells compared with their parental cells was verified *via* methyl thiazolyl tetrazolium (MTT) assay, and the expression levels of miR-195 and high-mobility group protein A1 (HMGA1) in AGS/5-FU and SGC-7901/5-FU cells were detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and Western blotting. MiR-195 mimic and miR-195 inhibitor were transfected into AGS/5-FU and AGS cells, respectively, the changes in HMGA1 expression were detected *via* qRT-PCR and Western blotting, and the sensitivity of cells to 5-FU after transfection was detected *via* MTT assay. After the wild-type and mutant-type luciferase reporter plasmids of HMGA1 were co-transfected with miR-195 mimic or miR-195 NC into cells, the luciferase activity was analyzed using the dual-luciferase reporter system. Finally, the rescue experiment was performed to confirm whether the changes in HMGA1 expression promote the formation of drug resistance in gastric cancer.

RESULTS: Both AGS/5-FU and SGC-7901/5-FU cells were significantly resistant to 5-FU compared with their parental cells, and miR-195 was down-regulated in AGS/5-FU and SGC-7901/5-FU cells, while HMGA1 was up-regulated in AGS and SGC-7901 cells. The transfection with miR-195 mimic could suppress the expression level of HMGA1 in AGS/5-FU cells, while the transfection with miR-195 inhibitor could up-regulate the expression level of HMGA1 in AGS cells. Moreover, miR-195 could bind to HMGA1 3'-untranslated region (3'UTR) in a targeted way, thereby inhibiting its expression. It was confirmed via a rescue experiment that the changes in HMGA1 expression promoted the formation of drug resistance in gastric cancer.

CONCLUSIONS: The down-regulation of miR-195 induces the resistance to 5-FU in gastric cancer through promoting the expression of HMGA1.

Key Words

MiR-195, HMGA1, Gastric cancer, 5-FU, Drug resistance.

Introduction

Gastric cancer is a common malignant tumor with a high morbidity rate in East Asian countries, and its occurrence is the result of the combined action of multiple oncogenes and tumor suppressor genes. Gastric cancer is one of the digestive tract tumors with poor prognosis. The main reason for the treatment failure of gastric cancer is the production of chemotherapy resistance. Fluorouracil (FU) is the most commonly used chemotherapeutic drug for gastric cancer, which exerts an anti-cancer effect through interfering in the DNA synthesis of tumor cells. Studies have demonstrated that tumor cells can inhibit the key enzymes required for the activation of 5-FU, enhance the activity of 5-FU metabolic enzymes, degrade the thymidylate synthase (TS) reduced folate substrate and alter the TS activity, thus exerting an anti-5-FU effect. With the deepening of understanding of micro ribonucleic acids (miRNAs), the direction of recent studies on chemotherapy resistance of gastric cancer has been changed to miRNAs.

MiRNAs are a group of small non-coding single-stranded RNAs with about 21-25 nucleotides in length, which are highly conserved in structure and function in different species, with space-time specificity in the expression. They, through complete or partial binding to the 3'-untranslated region (3'UTR) of the target gene mRNA, mediate the cleavage or inhibit the translation of target gene mRNA, and regulate the gene expression mainly at the post-transcriptional level[1]. One miRNA can regulate hundreds of target genes and widely participate in various biological process-

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es, such as embryonic development, cell proliferation, differentiation, apoptosis, cell cycle, and angiogenesis, through forming a complex regulatory network with target genes[2]. In recent years, it has been found in tumor research that there are many miRNAs involved in the occurrence, development, metastasis, and drug resistance of tumors, which are highly or lowly expressed in tumor cells and play similar roles to oncogenes or cancer suppressor genes[3], affecting a variety of biological behaviors of tumors.

Studies have proved that miR-195 is abnormally expressed in a variety of tumors and closely associated with tumorigenesis. Besides, it has also been demonstrated that miR-195 is highly or lowly expressed in different tumors, down-regulated in tongue squamous cell carcinoma, bladder tumor, gastric cancer, and hepatocellular carcinoma but up-regulated in breast cancer, adrenocortical carcinoma, and chronic lymphocytic leukemia. Therefore, it can be seen that the expression of miR-195 varies from tumor to tumor, and its role is also different in the occurrence and development of tumors[4,5]. In the present work, the effect of miR-195 on the chemotherapy sensitivity of gastric cancer cells to 5-FU was explored, and the mechanism of resistance to 5-FU in gastric cancer cells was investigated.

Materials and Methods

Cell Lines, Culture, and Reagents

Human gastric cancer SGC-7901 and AGS cells were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China), and cultured in the Dulbecco's Modified Eagle Medium (DMEM, Thermo Scientific Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific Hyclone, Waltham, MA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin under 5% CO_2 at 37°C. 5-FU-resistant gastric cancer cell lines, SGC-7901/5-FU and AGS/5-FU, were constructed from their parental cells SGC-7901 and AGS through persistent gradient exposure to 5-FU for about 6 months, and the drug concentration was increased from 0.05 μM until the cells acquired the resistance to 1 μM 5-FU (Sigma-Aldrich, St. Louis, MO, USA). The SGC-7901/5-FU and AGS/5-FU cell lines were cultured in the same conditions as above.

Methyl Thiazolyl Tetrazolium (MTT) Assay

Gastric cancer cells were digested with Tyrisin, collected and inoculated into a 96-well plate (6×10^3 cells/well). On the next day, 5-FU in a gradient concentration was added, the wells without 5-FU added were used as the control group, and 3 replicates were set for each concentration. The cells were cultured for 48 h after drug administration, the absorbance was measured, and the cell survival rate was calculated.

RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total mRNA was extracted from cells with TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into the complementary deoxyribose nucleic acid (cDNA) under the following conditions: 25°C for 10 min, 50°C for 30 min, and 85°C for 5 min, followed by detection using the fluorescence qRT-PCR kit. The primer sequences are as follows: miR-195: forward: 5'-GATAGCAGCACAGAAATATTGGC-3', and reverse: 5'-TGCGGGTGCTCGCTTCGGCAGC-3'. High-mobility group protein A1 (HMGA1): forward: 5'-GGCCCAAATCGACCATAAAGG-3', and reverse: 5'-GGACAAATCATGGCTACCCCT-3'. GAPDH (internal reference): forward: 5'-CAATGACCCCTTCATTGACC-3', and reverse: 5'-TGGAAGATGGTGATGGGATT-3'. The fluorescence qRT-PCR conditions are as follows: 95°C for 5 min, 95°C for 15 s, and 60°C for 1 min for a total of 40 cycles. The temperature of the dissolution curve was set at 60-95°C, and 3 replicates were set for each sample.

Western Blotting

The cells were inoculated into a 6-well plate and collected after corresponding treatment. The protein was extracted from the cells with RIPA lysis buffer and quantified using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) in each group. An equal amount of protein was taken in each group, isolated *via* 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) using a semi-dry method and sealed with 5% skim milk at room temperature for 2 h. After the milk was discarded, the VEGFA primary antibody (1:1000) was added for incubation at 4°C overnight. On the next day, the unbound primary antibody was washed away with the phosphate-buffered saline, and the secondary antibody

was added for incubation at room temperature for 1 h. Finally, enhanced chemiluminescence (ECL) was dropwise added for exposure and image development, and the expression of the corresponding protein was detected in each group.

Transfection

At 1 d before transfection, the cells in the logarithmic growth phase were digested with Tyrisin, counted and inoculated into the plate (4×10^5 cells/well) containing 2 mL media with antibiotic-free serum, making the cell density 90.95% at transfection. Then, miR-195 mimic (50 nM), miR-195 mimic control (200 nM), miR-195 inhibitor (100 nM), and miR-195 control inhibitor control (100 nM) to be transfected were diluted in the serum-free medium. The Lipofectamine 2000 transfection reagent was diluted with the serum-free medium, followed by transfection according to the instructions. After 48 h, the cells were collected for subsequent analysis. AGS/5-FU and SGC-7901/5-FU cells were transfected with either siHMGA1 or scrambled non-targeting siRNA, while AGS and SGC-7901 cells were transfected with either HMGA1 overexpression vector or empty vector. After transfection, the expression of miR-195 was determined by qRT-PCR, and the expressions of HMGA1 mRNA and protein were determined by qRT-PCR and Western blotting, respectively.

Luciferase Activity Assay

The wild-type (WT) and mutant-type (MT) dual-luciferase reporter vectors of HMGA1 3'UTR were constructed through subcloning the human HMGA1 mRNA 3'UTR and mutant-type 3'UTR sequences into the pGL3 dual-luciferase reporter vectors. AGS cells (5×10^4) were inoculated into a 24-well plate, incubated for 24 h and then transfected with the WT or MT 3'UTR vectors and miR-195 mimic. After 48 h, the cells were assayed for luciferase activity using the Dual-Luciferase Reporter System following the manufacturer's protocol. The firefly luciferase activity was normalized to Renilla luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Data were represented as mean ± SD (Standard Deviation). The *t*-test was used for analyzing measurement data. $p<0.05$ indicated the significant difference.

Results

AGS /5-FU and SGC-7901/5-FU Cells Were Significantly Resistant to 5-FU

5-FU-resistant cells were constructed using 5-FU induction in gradient concentration. The parental cells were cultured in the complete medium containing 5-FU at an initial concentration of 1 nM. The 5-FU concentration was increased after passage every time until it reached 100 nM, under which the cells were cultured for 3 months. The sensitivity of the parental cells AGS and SGC-7901, AGS/5-FU and SGC-7901/5-FU cells to 5-FU was detected *via* MTT assay. The results revealed that the half maximal inhibitory concentration (IC50) of 5-FU was 12.15 μM and 53.72 μM for AGS and AGS/5-FU cells, respectively, and 7.36 μM and 39.24 μM for SGC-7901 and SGC-7901/5-FU cells, respectively (Figure 1A, 1B). It can be seen that the resistance of AGS/5-FU and SGC-7901/5-FU cells to 5-FU was significantly increased compared with their parental cells, and the differences were statistically significant ($p<0.05$). Furthermore, the expression of miR-195 in the 4 kinds of cells was detected *via* RT-PCR. It was found that the expression of miR-195 in AGS/5-FU and SGC-7901/5-FU cells was significantly lower than that in their parental cells, and there were statistically significant differences ($p<0.05$) (Figure 1C). What is more, the expression of HMGA1 in the 4 kinds of cells was detected *via* Western blotting. We found that the expression of HMGA1 in AGS/5-FU and SGC-7901/5-FU cells was significantly higher than that in their parental cells, and there were statistically significant differences ($p<0.05$) (Figure 1D).

MiR-195 Induced Sensitivity of Gastric Cancer Cell Lines to 5-FU

The above results indicate that the expression level of miR-195 is significantly down-regulated in resistant cells. To investigate whether the down-regulation of miR-195 leads to the drug resistance, the cells were transfected with miR-195 mimic and miR-195 inhibitor to observe the changes in the sensitivity of resistant cells and parental cells to 5-FU after miR-195 was regulated. First, the results of qRT-PCR confirmed that the transfection with miR-195 mimic significantly up-regulated the expression level of miR-195 in AGS/5-FU cells ($p<0.05$), while the transfection with miR-195 inhibitor down-regulated the expression level of miR-195 in AGS cells (Figure 2A, 2B). Then, the cell viability in each group was detected *via* MTT assay after treatment with 5-FU in corresponding concentration.

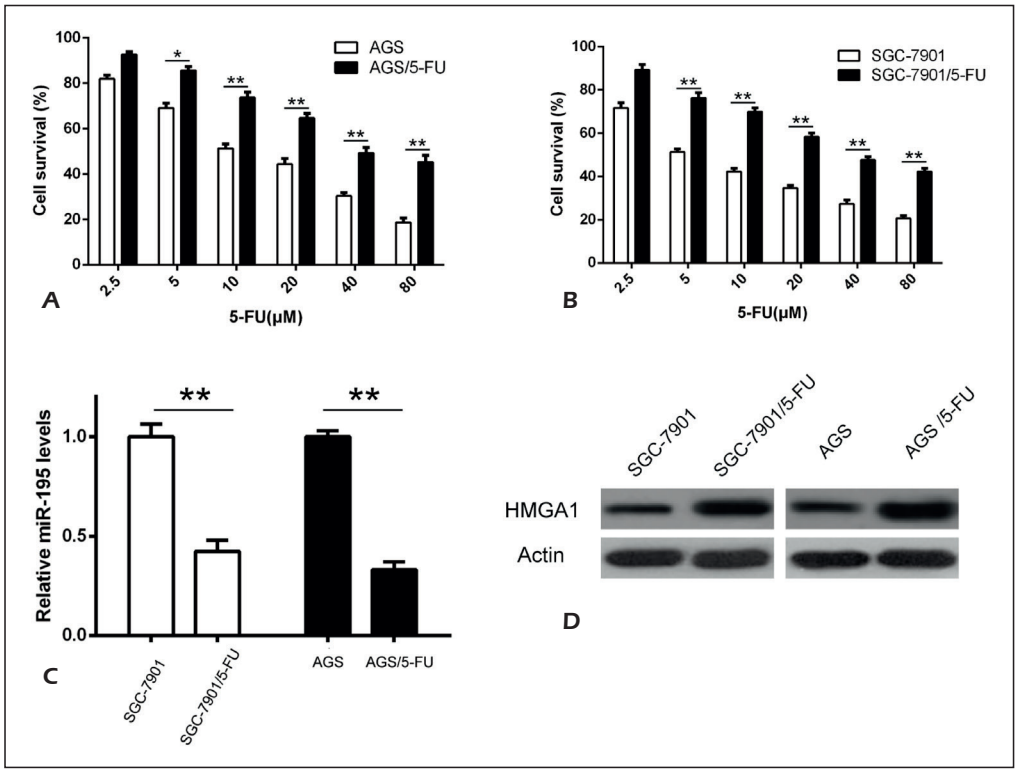

Figure 1. AGS /5-FU and SGC-7901/5-FU cells were significantly resistant to 5-FU. **A**-**B**, Cell vitality of acquired 5-FU resistant gastric cancer cell lines (AGS/5-FU, SGC-7901/5-FU) and their parental cell lines (AGS, SGC-7901) was determined by CCK8 assay after treated with indicated concentrations of 5-FU for 48 h. **C**, The expression levels of miR-195 was detected in the above cells by qRT-PCR. **D**, The expression levels of HMGA1 were detected in the above cells by Western blotting. Data are presented as means ± SD (n = 3). *p<0.05, **p<0.01 *vs.* control group.

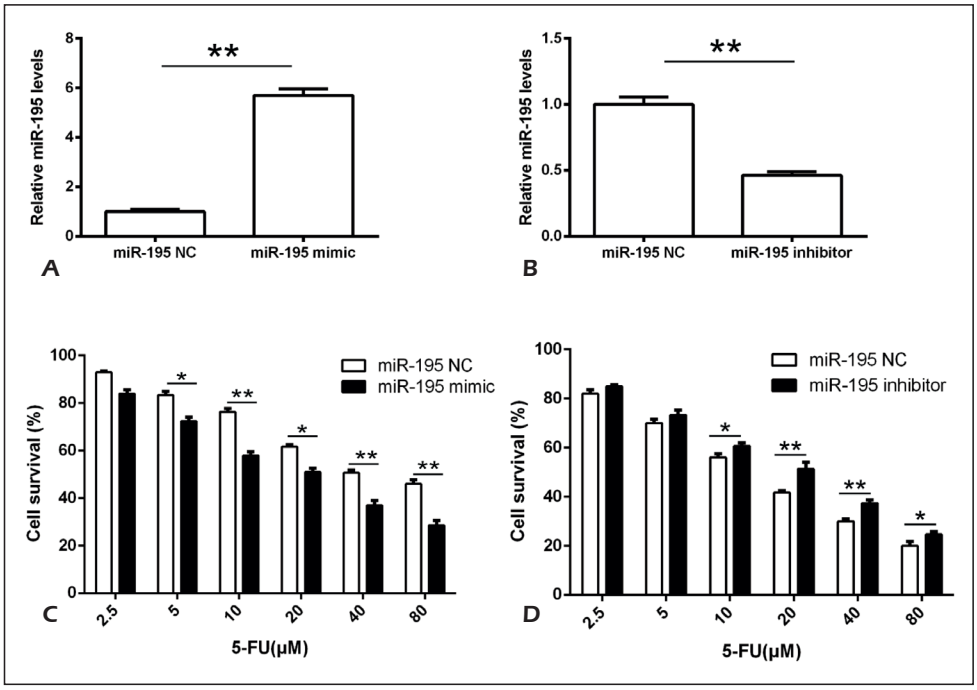

Figure 2. MiR-195 induced sensitivity of gastric cancer cell lines to 5-FU. **A**, Expression of miR-195 in AGS/5-FU cells transfected with miR-195 mimic or miR-195 negative control of mimic (NC). **B**, Expression of miR-195 in AGS cells were transfected with miR-195 inhibitor or miR-195 NC. **C**-**D**, The cell viability of AGS/5-FU and AGS cells were tested by CCK8 assay after transfected with the above reagents. Data are presented as means ± SD (n = 3). *p<0.5, **p<0.01 *vs.* control group.

As shown in Figure 2C and D, it was found that the overexpression of miR-195 could markedly increase the sensitivity of AGS/5-FU cells to 5-FU (IC50=49.55 μM and 19.68 μM in both cells), while the down-regulation of miR-195 could remarkably promote the resistance of AGS cells to 5-FU (IC50=13.97 μM and 19.73 μM in both cells). To sum up, altering the expression level of miR-195 can regulate the sensitivity of gastric cancer AGS cells to 5-FU.

MiR-195 Targeted HMGA1 and Regulated Its Expression

Researches have demonstrated that miR-195 can regulate the HMGA1 expression level in a targeted way, but its role in drug resistance has not been studied yet. To verify the target of miR-195 in resistance to 5-FU in gastric cancer, the dual-luciferase reporter gene assay was performed to detect whether miR-195 binds to the corresponding 3'UTR of HMGA1 mRNA to negatively regulate its expression. The WT or MT 3'UTR-containing miR-195 binding sites (Figure 3A) were cloned into the downstream of the luciferase reporter gene. The AGS cells were subsequently co-transfected with miR-195 mimic or miR-195 NC and the cloned luciferase reporter. The dual-luciferase assay showed that miR-195 mimic significantly decreased luciferase activity in AGS cells with WT reporter vectors. However, no evident decline in luciferase activity due to miR-195 mimic was observed in AGS cells with MT reporter vectors (Figure 3B). The above experimental results demonstrate that miR-195 targets HMGA1 and can suppress its expression. Then, the regulatory effect of miR-195 on HMGA1 was further verified in AGS/5-FU and AGS cells. The results showed that the HMGA1 protein and mRNA expressions could be inhibited after AGS/5-FU cells were transfected with miR-195 mimic, while it could be increased after AGS cells were transfected with miR-195 inhibitor (Figure 3C, 3D). To sum up, miR-195 can inhibit the HMGA1 expression in a targeted way.

MiR-195 Targeted HMGA1 to Regulate Sensitivity of Gastric Cancer to 5-FU

To further determine the role of HMGA1 in 5-FU resistance of AGS/5-FU cells with down-regulation of miR-195, rescue experiment was further performed. AGS/5-FU cells were co-transfected with miR-195 mimic and HMGA1 vectors, or miR-195 mimic and empty vector control, while AGS cells were co-transfected with miR-195 inhibitor and si-HMGA1 or miR-195 inhibitor and si-NC. Then, the expression level of

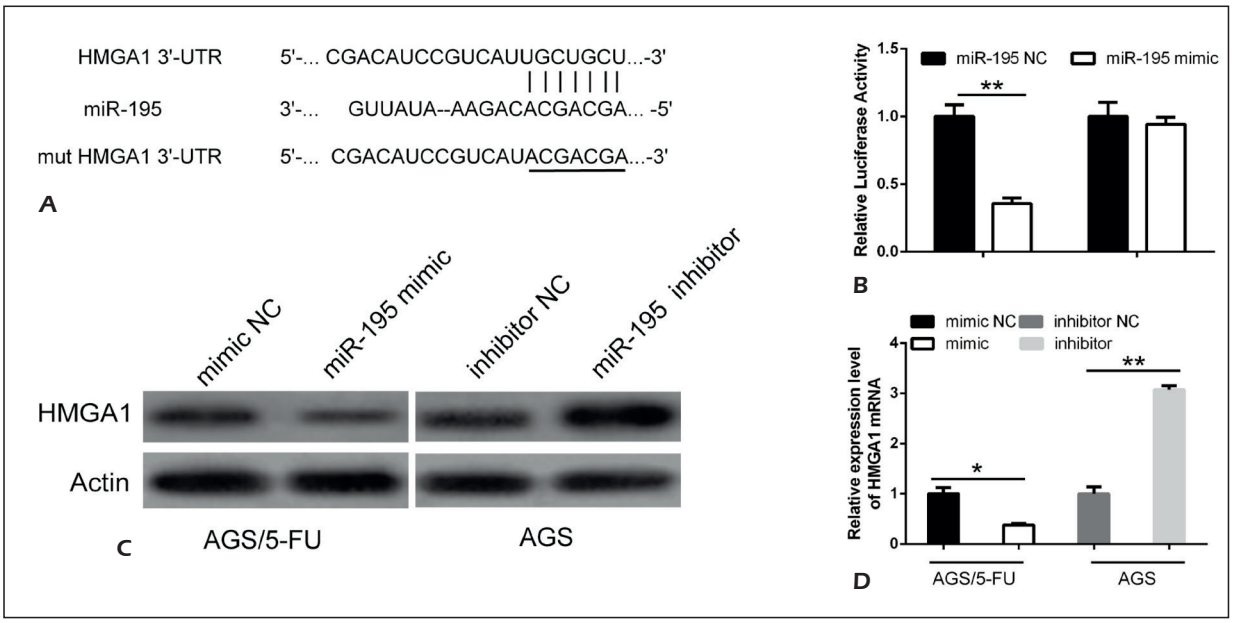

Figure 3. MiR-195 targeted HMGA1 and regulated its expression. **A**, We constructed wild genotype/mutant genotype (mut) of the miR-195-binding site in HMGA1 3'-UTR. **B**, Luciferase activity of mimic group was inhibited significantly co-transfected with wild 3'-UTR construct compared to mutant construct groups. **C-D**, Overexpression of miR-195 decreased the HMGA1 protein and mRNA expression levels in AGS/5-FU and inhibition of miR-195 increased the levels in AGS. Data are presented as means ± SD (n = 3). *p<0.05, **p<0.01 *vs.* control group.

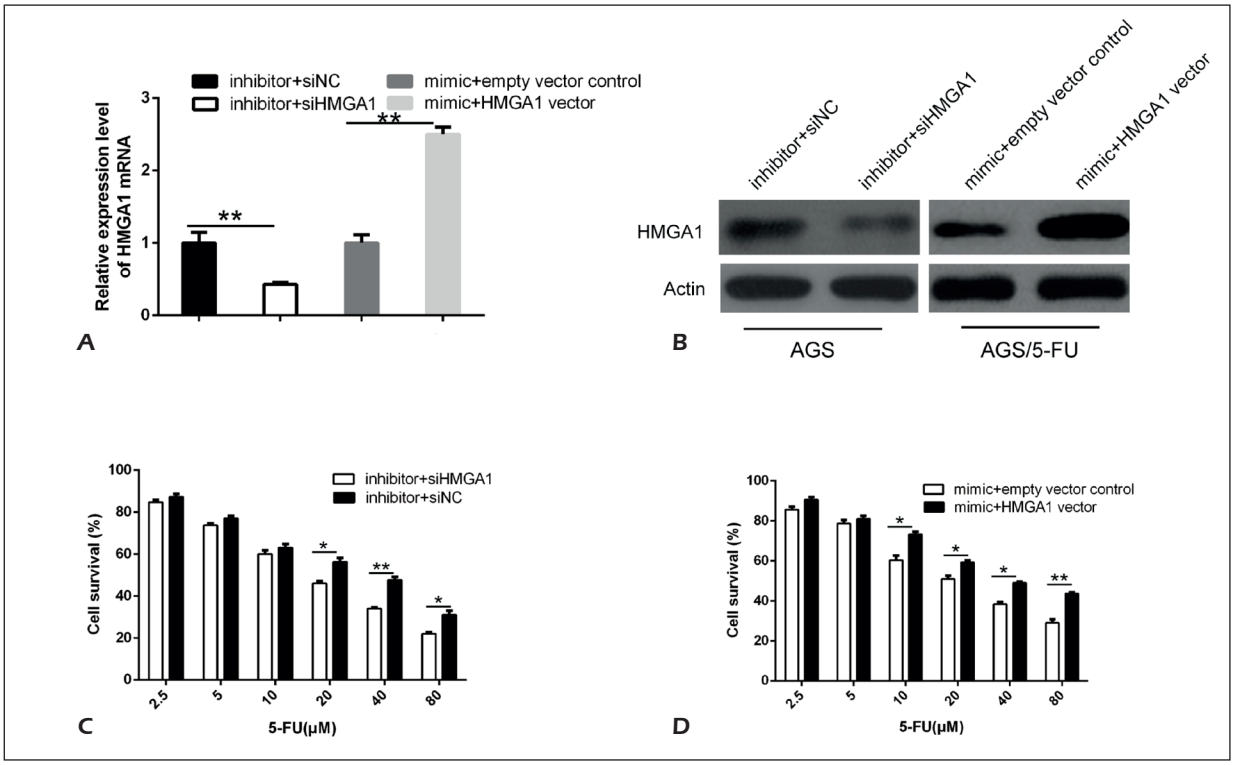

Figure 4. MiR-195 targeted HMGA1 to regulate the sensitivity of gastric cancer to 5-FU. AGS cells were co-transfected with miR-195 inhibitor along with siHMGA1or siNC. AGS/5-FU cells were co-transfected with miR-195 mimic along with HMGA1 vector or empty vector control. **A**-**B**, qRT-PCR and Western blotting was performed to detect the mRNA and protein expression of HMGA1 in gastric cancer cells. **C**, After siHMGA1 treatment, inhibition of miR-195 induced 5-FU-resistance was downregulated in AGS cells. **D**, Overexpression of miR-195 induced 5-FU-sensitivity was reversed by overexpressed HMGA1 in AGS/5-FU cells. Data are presented as means ± SD (n = 3). *p<0.05, **p<0.01 *vs.* control group.

HMGA1 after co-transfection was detected *via* qRT-PCR and Western blotting. As shown in Figure 4A and 4B, the mRNA and protein expression levels of HMGA1 in AGS/5-FU cells transfected with miR-195 inhibitor and si-HMGA1 were significantly lower than those in cells transfected with miR-195 inhibitor and si-NC, while they were significantly higher in AGS cells transfected with miR-195 mimic and HMGA1 vectors than those in cells transfected with miR-195 mimic and empty vector control. Then, MTT assay was performed after successful transfection. The results (Figure 4C and 4D) showed that the sensitivity to 5-FU remarkably declined in AGS/5-FU cells transfected with miR-195 mimic and HMGA1 vectors compared with that in cells transfected with miR-195 mimic and empty vector control (IC50=43.39 μM and 21.81 μM, p<0.05). Moreover, the sensitivity to 5-FU also remarkably declined in AGS cells transfected with miR-195 inhibitor and si-NC compared with that in cells transfected with miR-195 inhibitor and si-HMGA1 (IC50=28.36 μM and 17.16 μM, p<0.05). The above results suggest that the down-regulation of HMGA1 can reduce the drug resistance induced by the miR-195 inhibitor, while the up-regulation of HMGA1 in AGS/5-FU cells can restore the sensitivity of cells overexpressing miR-195 to 5-FU. In other words, HMGA1 plays an important role in resistance to 5-FU induced by the down-regulation of miR-195. The above investigations also prove that HMGA1 is the target of miR-195 in AGS/5-FU cells.

Discussion

The resistance of gastric cancer to 5-FU has been paid much attention to, and the mechanism of drug resistance is complex and involves multiple genes and processes, which is the result of combined action of multiple factors[6]. Studies[7,8] have confirmed that the abnormal expression of resistance-related genes will lower the concentration of anti-tumor drugs, affecting the efficacy of

drugs. In recent years, reports have shown that miRNAs are also directly or indirectly involved in regulating the drug resistance and sensitivity of tumors through a variety of pathways[9], making it possible to affect the sensitivity of tumors to drugs through miRNAs[10,11]. In gastric cancer, miRNAs, as oncogenes or cancer suppressor genes, are involved in such important cellular signaling pathways as MAPK, JAK/STAT, and P13K/AKT, thereby affecting the occurrence and development of gastric cancer. Moreover, researches have revealed that miR-21-5, miR-145, etc. are involved in the regulatory process of chemotherapy sensitivity of gastric cancer and its mechanism[12]. Therefore, the miRNA can serve as not only an important biomarker for early screening and diagnosis of gastric cancer, but also a key therapeutic target for gastric cancer.

MiR-195 has been further studied in drug resistance of tumor. Qu et al[13] found that miR-195 is significantly down-regulated in resistant colon cancer HT29/DOX and LOVO/DOX cells. According to the *in vitro* cell viability assay, the knockout of miR-195 in HT29 and LOVO cells significantly inhibits the cytotoxicity caused by doxorubicin, producing the drug resistance of parental colon cancer cells to doxorubicin and reducing the apoptotic activity, while the overexpression of miR-195 enhances the sensitivity of resistant cells to doxorubicin and increases the apoptotic activity. The mechanism research revealed that miR-195 can target the 3'UTR of Bcl-2 mRNA, thereby inhibiting the Bcl-2 expression. Yang et al[14] studied and showed that miR-195 is lowly expressed in 5-FU-resistant liver cancer BEL-7024/5-FU cells, and the growth of resistant BEL-7024/5-FU cells can be inhibited and the apoptosis can be promoted by overexpression of miR-195. Whether miR-195 is involved in regulating the 5-FU resistance in gastric cancer has not been reported, so we aim to discover the functional mechanism of miR-195 in 5-FU resistance in gastric cancer.

In the present study, the expression level of miR-195 in AGS, AGS /5-FU, SGC-7901, and SGC-7901/5-FU cells was detected *via* qRT-PCR, and it was found that the expression level of miR-195 was significantly down-regulated in AGS/5-FU and SGC-7901/5-FU cells compared with that in their parental cells, which is consistent with the existing research results. Moreover, the expression level of miR-195 was successfully up-regulated in AGS/5-FU cells and inhibited in AGS cells, and it was showed that the up-regulation of miR-195 in AGS/5-FU cells could improve the sensitivity of AGS/5-FU cells to 5-FU, while the inhibition on miR-195 in AGS cells could reduce the sensitivity of AGS cells to 5-FU, indicating that the decline in the expression level of miR-195 can lead to resistance to 5-FU in gastric cancer. Zhang et al[15] showed that miR-195 can bind to HMGA1 3'UTR in prostate cancer cells in a targeted way, thereby inhibiting its expression. HMGA1 can be involved in various physiological activities of cells, including embryogenesis, cell cycle regulation, differentiation, senescence, and DNA repair, through the special protein-protein and protein-DNA interactions[16]. In recent years, it has been found that HMGA1 is lowly or not expressed in normal mature tissues, while it is highly expressed in the process of embryogenesis and tumor formation. There is increasingly more evidence that HMGA1 is widely overexpressed in invasive malignant tumors, and its high expression in some tumors often indicates the poor prognosis[17]. Based on the above research, the expression level of HMGA1 in gastric cancer cells was detected. Of note, it was found that the expression of HMGA1 in AGS/5-FU and SGC-7901/5-FU cells was remarkably increased compared with that in their parental cells, also indicating that HMGA1 plays an important role in drug resistance in gastric cancer. Then, the expression level of HMGA1 was determined after the expression of miR-195 was altered, and it was found that there were opposite changes in HMGA1 and miR-195, suggesting that miR-195 may also inhibit the HMGA1 expression level in a targeted way in gastric cancer cells. Furthermore, the dual-luciferase reporter gene assay proved that miR-195 could bind to HMGA1 3'UTR in a targeted way to inhibit its expression. Finally, the rescue experiment manifested that miR-195 promoted the formation of resistance to 5-FU in gastric cancer through targeting HMGA1.

Conclusions

We observed that the down-regulation of miR-195 is involved in inducing resistance to 5-FU in gastric cancer through promoting the expression of HMGA1, suggesting that the up-regulation of miR-195 may be used as a therapeutic method to reverse resistance to 5-FU in gastric cancer patients.

Conflict of Interests

The authors declare that they have no conflict of interest.

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