

# Inhibition of NLRC5 attenuates the malignant growth and enhances the sensitivity of gastric cancer cells to 5-FU chemotherapy by blocking the carcinogenic effect of YY1

SHAN LIANG<sup>1,2</sup>, TINGTING XIANG<sup>1</sup>, SHIYU LIU<sup>1</sup> and WEI XIANG<sup>1</sup>

<sup>1</sup>College of Modern Agriculture and Bioengineering, Yangtze Normal University, Chongqing 408000;

<sup>2</sup>Institute of Sericulture and Systems Biology, Southwest University, Chongqing 400700, P.R. China

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**Abstract.** Gastric cancer (GC) is one of the commonest malignant tumors of the digestive system, characterized by high morbidity and mortality rates. It has been reported that NOD like receptor (NLR) family, CARD domain containing 5 (NLRC5) serves an important role in the occurrence and development of GC. Therefore, the current study aimed to investigate the role of NLRC5 in GC. The mRNA and protein expression levels of NLRC5 in GC cell lines were determined by reverse transcription-quantitative PCR and western blot analysis, respectively. Additionally, following NLRC5 knockdown, cell proliferation, invasion and migration were evaluated using Cell Counting Kit 8, colony formation, wound healing and Transwell assays, and western blot analysis. The NLRC and Yin Yang 1 (YY1) expression in the AGS cells with 5-FU resistance were detected by western blotting. The sensitivity of GC cells to 5-fluorouracil (5-FU) was detected by flow cytometry and western blot analysis. Additionally, the binding capacity of YY1 on NLRC5 promoter was predicted using JASPAR database and it was further verified by chromatin immunoprecipitation and luciferase reporter assays. Finally, to elucidate the mechanism underlying the effect of NLRC5 on GC, YY1 was overexpressed and NLRC5 was silenced in GC cell lines. The results showed that NLRC5 was abnormally upregulated in GC cells. In addition, NLRC5 knockdown significantly attenuated the proliferation, invasion and migration abilities of GC cells, while it enhanced the sensitivity of GC cells to 5-FU. The above effects were regulated by the YY1 transcription factor. Overall, the results of the present study indicated that NLRC5 silencing could reduce the malignant growth and enhance the

sensitivity of GC cells to 5-FU chemotherapy via inhibiting the carcinogenic effect of YY1.

## Introduction

Gastric cancer (GC) is one of the commonest malignant tumors of the digestive system, ranking fifth in incidence and third in mortality worldwide (1). Cell invasion and metastasis are considered as important causes leading to postoperative recurrence and mortality in patients with GC. Therefore, the early identification of factors involved in cell invasion and metastasis could improve the cure rate and prolong the survival rate of patients with GC (2). Among tens of thousands genes in the tumor tissues, genes associated with tumor development are called driver genes (3). Changes in the expression of driver genes may result in changes in the incidence of tumors. Therefore, selecting the appropriate molecular targeted drugs for each driving gene could improve personalized therapy for the recovery of patients with GC.

NOD like receptor (NLR) family, CARD domain containing 5 (NLRC5) is a member of the NLR family. It has been reported that the short-term effects of NLRC5 are associated with immune responses and inflammation, while the long-term ones may lead to the dysregulation of the immune system (4). The above findings indicate that NLRC5 could be involved in the development of cancer. A previous study demonstrated that NLRC5 is upregulated and promoted cell proliferation, migration and invasion in clear cell renal carcinoma by activating the Wnt/catenin signaling pathway (5). Additionally, NLRC5 can promote cell proliferation in hepatocellular carcinoma by regulating the AKT/VEGF-A signaling pathway (6). NLRC5 is upregulated in GC tissues, while increased NLRC5 expression is associated with worse prognosis (7). However, the specific regulatory mechanism of NLRC5 in GC has not been previously reported. GC is characterized by strong heterogeneity, poor sensitivity to chemotherapy and poor prognosis (8). Therefore, GC is considered as a tumor with high requirements for individualized therapy (9). A previous study suggests that regulatory NLRC5 variation can affect the survival of patients with colorectal cancer and their response to 5-fluorouracil (5-FU) chemotherapy (10). Therefore, the effect of NLRC5 regulation

*Correspondence to:* Dr Wei Xiang, College of Modern Agriculture and Bioengineering, Yangtze Normal University, 16 Juxian Dadao, Fuling, Chongqing 408000, P.R. China  
E-mail: xiangwei2027@163.com

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on the sensitivity of GC cells to chemotherapy remains to be elucidated.

Yin Yang 1 (YY1) is a transcriptional protein involved in a variety of biological functions, including cell cycle progression, cell proliferation, differentiation and apoptosis (11). A number of studies have shown that abnormal expression of YY1 can serve a regulatory role in tumor proliferation and metastasis through interaction with different protein cofactors (12,13). YY1 is upregulated in GC cell lines and primary GC (14). Moreover, YY1 has extensive carcinogenic effect in GC (15,16). YY1 is predicted to start NLRC5 by ALGGEN-PROMO, and YY1 predicted to bind to NLRC5 promoter by JASPAR database. Therefore, it was hypothesized that NLRC5 could be activated by YY1 transcription factor to participate in the malignant process of GC cells.

The present study aimed to investigate the regulation and underlying mechanism of NLRC5 on the proliferation, invasion and migration of GC cells and their sensitivity to chemotherapy, thus providing a new theoretical basis for the targeted therapy of GC.

## Materials and methods

**Databases.** The ALGGEN-PROMO (17) and JASPAR databases (18) (<http://jaspar.genereg.net>) were used to predict the binding capacity of YY1 on NLRC5 promoter.

**Cell culture.** The human gastric mucosa cell line GES-1 and the GC cell lines AGS, MKN-45, KATO III and NCI-87, procured from Bena Culture Collection, were cultured in DMEM supplemented with 10% FBS, 1% penicillin and 1% streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO<sub>2</sub>. Cells were treated with 5 µg/ml 5-FU (Beyotime Institute of Biotechnology) at 37°C for 48 h.

**Cell viability assay.** A Cell Counting Kit 8 (CCK-8; Nanjing Jiancheng Bioengineering Institute) assay was used to evaluate cell viability. Briefly, cells were seeded into a 96-well plate at a density of 1×10<sup>4</sup> cells/well. Following treatment with the appropriate compounds, cells in each well were supplemented with 10 µl CCK-8 solution and incubated at 37°C for an additional 4 h. Finally, the absorbance at a wavelength of 450 nm was measured in each well using a microplate spectrophotometer reader (BioTek Instruments, Inc.).

**Reverse transcription-quantitative (RT-q)PCR.** Total RNA was extracted from cells (1.5×10<sup>6</sup>) using a TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Subsequently, RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocols. The expression levels of the target gene were quantified with real-time PCR using the SYBR-Green PCR Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed on the Applied Biosystems real time PCR system (Thermo Fisher Scientific, Inc.) and the gene expression levels were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method (19). The following thermocycling conditions for qPCR were used: Initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 10 sec, and annealing and extension at 60°C

for 45 sec. The following primers were used: NLRC5 forward, 5'-TGAGGGAGTCTGCACTATGGA-3' and reverse, 5'-TCCGATTCAGGGCTCAGGTA-3'; YY1 forward, 5'-TCAGACAAGTCACGTCAGGC-3' and reverse, 5'-CTCCATGTGTCACTCCAC-3'; and GAPDH forward, 5'-CGGAGTCAACGGATTGTCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

**Western blot analysis.** AGS cells were lysed using a protein extraction solution (Beyotime Institute of Biotechnology) and the protein concentration was measured using a BCA kit (SinoBio Biotech). Proteins (50 µg) were uploaded and were then transferred onto a PVDF membrane (MilliporeSigma). Following blocking with 5% skimmed milk powder in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 1 h, the membrane was incubated with primary antibodies (dilution, 1:1,000) at 4°C overnight. Subsequently, the membrane was incubated with the corresponding HRP-conjugated secondary antibodies (dilution, 1:5,000; Abcam) at 37°C for 1 h. The protein bands were visualized using an ECL reagent (ECL-plus; Thermo Fisher Scientific, Inc.) and analyzed using ImageJ software (version 1.8.0; National Institutes of Health). Primary antibodies used in this study were as follows: NLRC5 (cat. no. GTX85160; GeneTex, Inc.), YY1 (cat. no. ab109237; Abcam), MMP2 (cat. no. ab92536; Abcam), MMP9 (cat. no. ab76003; Abcam), cleaved caspase 3 (cat. no. ab32042; Abcam), p53 (cat. no. ab32389; Abcam) and GAPDH (cat. no. ab9485; Abcam).

**Cell transfection.** Small interfering (si)RNAs targeting NLRC5 (si-NLRC5-1 and si-NLRC5-2) and YY1 (si-YY1), their corresponding blank controls (si-NC), YY1 overexpression (ov) plasmid (ov-YY1) and ov-NC were synthesized by Guangzhou RiboBio Co., Ltd. Cell transfection was performed using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 24 h according to the manufacturer's instructions. siRNA-NLRC5-1-sense: 5'-AAGAACGAGAGACUCUGCCAACUGCdTdT-3', siRNA-NLRC5-1-antisense: 5'-GCAGUUGGCAGAGUCUCUCGUUCUdTdT-3', siRNA-NLRC5-2-sense: 5'-GGGACTGAGAGCTTTGTAT-3', siRNA-NLRC5-2-antisense: 5'-CGCACCCCTAGACTGAAA-3', scrambled-RNAi-sense: 5'-UUCUCCGAA CGUGUCACGUTT-3', and scrambled-RNAi-antisense: 5'-ACGUGACACGUUCGGAGAATT-3'. At 48 h post-transfection, subsequent experiments were conducted.

**Experimental groups.** To determine the impact of NLRC5 on the sensitivity of GC cells to 5-FU, cells were divided into the control, 5-FU, 5-FU + si-NC and 5-FU + si-NLRC5 groups. To substantiate the impact of the YY1/NLRC5 axis on the sensitivity of GC cells to 5-FU, GC cells were divided into the following five groups: The control group, the ov-NC group, the ov-YY1 group, the ov-YY1 + si-NC group and the ov-YY1 + si-NLRC5 group.

**Colony formation assay.** Cells were seeded into 6-well plates at a density of 10<sup>3</sup> cells/well and incubated at 37°C in humidified 5% CO<sub>2</sub> incubator for 15 days. Subsequently, the formed colonies (>50 cells/colony) were stained with 0.5% crystal violet for 30 min at room temperature. Images were captured

and the number of the colonies (>50 cells/colony) in three fields of view was counted with the naked eye under a light microscope (magnification, x10).

**Wound healing assay.** For wound healing assays, AGS cells were seeded into a 6-well plate at a density of  $10^5$  cells/well overnight at 37°C. When cells grown in complete medium reached 75% confluency, a wound was created using a sterile pipette tip. Subsequently, cells were washed with PBS several times to remove cell debris and incubated at 37°C for an additional 48 h in serum-free medium. Images of the wound were captured under an inverted fluorescence microscope and the wound closure rate was then assessed.

**Transwell assay.** Transwell chambers (Corning Life Sciences) with 8- $\mu$ m pore inserts coated with Matrigel at 37°C for 30 min were used to evaluate the invasion ability of AGS cells. Briefly, transfected cells with the presence or absence of 5-FU treatment in serum-free DMEM were added to the upper chamber of the Transwell insert at a density of  $5 \times 10^5$  cells/ml. The lower chamber was supplemented with 500  $\mu$ l complete medium as chemoattractant. Following incubation for 24 h at 37°C, cells on the upper surface were removed, while cells invaded onto the bottom of the membrane were fixed and stained with 0.1% crystal violet for 10 min at room temperature. The invaded cells were counted in five randomly selected fields under a light microscope (Olympus Corporation).

**Flow cytometric assay.** Cell apoptosis was assessed using an Annexin V-FITC apoptosis detection kit (Thermo Fisher Scientific, Inc.). Briefly,  $2 \times 10^5$  cells were re-suspended in 300  $\mu$ l binding buffer, mixed gently with 5  $\mu$ l Annexin V-FITC reagent at 4°C for 15 min in the dark prior to 10  $\mu$ l PI staining fluid being added at 4°C for 5 min. Finally, cell apoptosis was assessed using a flow cytometer (FACSAria™; BD Biosciences) and FlowJo software (version 10.0.7; Tree Star, Inc.). The total apoptosis rate is equal to the early apoptotic rate plus the dead cell rate.

**Chromatin immunoprecipitation (ChIP).** The ChIP assay was performed using the Imprint ChIP kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. A total of  $1 \times 10^7$  cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cell lysates were sonicated using a 10 sec on and 10 sec off mode for 12 cycles, on ice, to obtain chromatin fragments. DNA (8–40  $\mu$ g) was diluted with DNase-free water (Beyotime Institute of Biotechnology) and incubated with the primary antibody at 4°C overnight. The primary antibody used was an anti-YY1 antibody (1:200, cat. no. ab109237; Abcam). Next, the DNA that had bound to YY1 was collected using DNA extraction buffer of the kit in DNase-free water and amplified using qPCR to detect NLRC5. PCR products were separated by 1% gel electrophoresis using agarose gels prestained with ethidium bromide. Bands were analyzed using ImageJ software (version 1.8.0; National Institutes of Health).

**Luciferase reporter assay.** Cell were plated in 6-well plates and after the cells had adhered, 0.5  $\mu$ g vectors containing the 3'-untranslated region (UTR) of wild-type NLRC5 or mutant

3'-UTR NLRC5, with control vector or YY1 overexpression vector and pMIR-*Renilla* vector (Shanghai GeneChem Co., Ltd) were co-transfected with the transfection kit (Polybrene; Shanghai GeneChem, Co., Ltd.) into the cells ( $1 \times 10^6$  cells/well) and cells were incubated for 48 h at 37°C. Cells were collected 24 h after transfection. Finally, the luciferase activity was detected using a *Renilla*-Glo Luciferase Assay System (cat. no. E2710; Promega Corporation) at room temperature and a spectrophotometer at 490 nm (Thermo Fisher Scientific, Inc.). *Renilla* luciferase activity was used to normalize the firefly luciferase activity.

**Statistical analysis.** Data are expressed as the mean  $\pm$  SD. All results were analyzed using SPSS 18.0 software (SPSS, Inc.). The differences in the present study except for the CCK8 results among multiple groups were compared with one-way ANOVA followed by Tukey's post hoc test. CCK8 results were analyzed by Two-way ANOVA followed by Tukey's post hoc test. Each experiment was performed at least three times.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**NLRC5 knockdown attenuates the proliferation, invasion and migration of GC cells.** The expression levels of NLRC5 in GC cells were detected by RT-qPCR and western blot analysis. The results showed that NLRC5 was significantly upregulated in AGS, MKN-45, KATO III and NCI-87 cells compared with GES-1 cells (Fig. 1A and B). The expression of NLRC5 was notably higher in AGS cells compared with the other GC cell lines. Therefore, AGS cells were selected for the follow-up experiments. Subsequently, GC cells were transfected with si-NLRC5-1 or si-NLRC5-2 clones using the cell transfection technology. The silencing activity of si-NLRC5-1 was more potent compared with that of si-NLRC5-2 and it was therefore selected for subsequent experiments (Fig. 1C and D). Furthermore, cells were divided into the control, si-NC and si-NLRC5 groups. CCK-8 assays revealed that compared with the si-NC group, the cell viability was significantly decreased in the si-NLRC5 group in a time-dependent manner (Fig. 1E). Additionally, the colony formation assay showed that the proliferation ability of GC cells was notably reduced in the si-NLRC5 group (Fig. 1F). Furthermore, wound healing and Transwell assays demonstrated that the invasion and migration abilities of GC cells were significantly attenuated in the si-NLRC5 group compared with the si-NC group (Fig. 2A and B). Finally, western blot analysis revealed that the expression levels of the migration-related proteins MMP2 and MMP9 were markedly reduced in NLRC5-depleted GC cells (Fig. 2C).

**NLRC5 silencing enhances the sensitivity of GC cells to 5-FU.** The aforementioned findings indicated that NLRC5 silencing could inhibit the proliferation, invasion and migration of GC cells. However, whether NLRC5 is associated with the prognosis of GC remains elusive. Therefore, 5-FU, a commonly used postoperative chemotherapy drug, was selected to assess whether NLRC5 knockdown could enhance the sensitivity of GC cells to chemotherapy. It was found that the expression

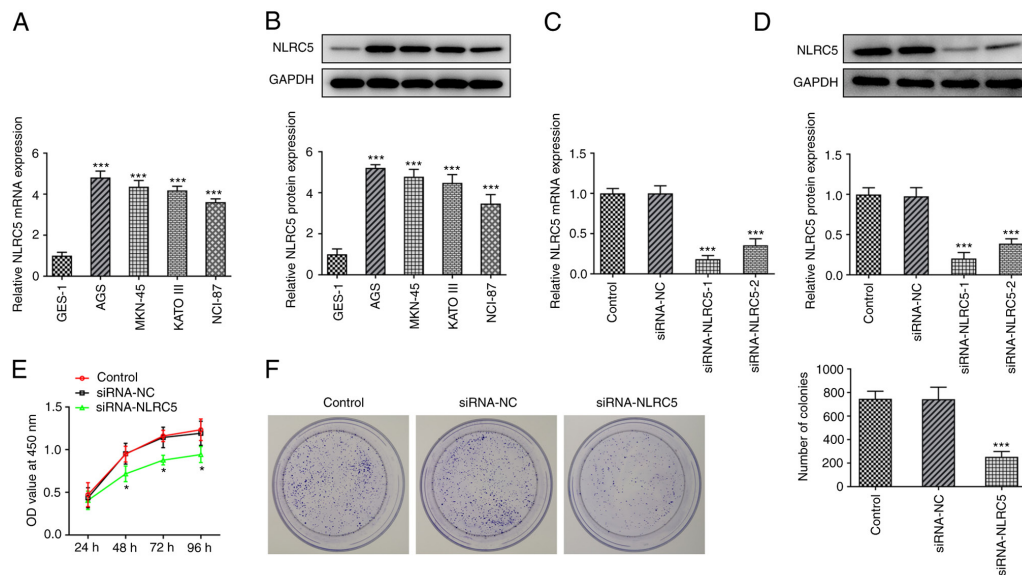


Figure 1. Inhibition of NLRC5 reduces proliferation of GC cells. (A) RT-qPCR detected the expression of NLRC5 in difference cell lines. (B) Western blotting detected the expression of NLRC5 in difference cell lines. \*\*\* $P < 0.001$  vs. GES-1. (C) RT-qPCR detected the expression of NLRC5 after cell transfection. (D) Western blot detected the expression of NLRC5 after cell transfection. \*\*\* $P < 0.001$  vs. siRNA-NC. (E) CCK-8 detected the cell viability. (F) Colony formation assay was used to detected the cell proliferation. \* $P < 0.05$ , \*\*\* $P < 0.001$  vs. shRNA-NC. NLRC5, NOD like receptor family, CARD domain containing 5; GC, gastric cancer; RT-qPCR, Reverse transcription-quantitative PCR; si, short interfering; NC, negative control.

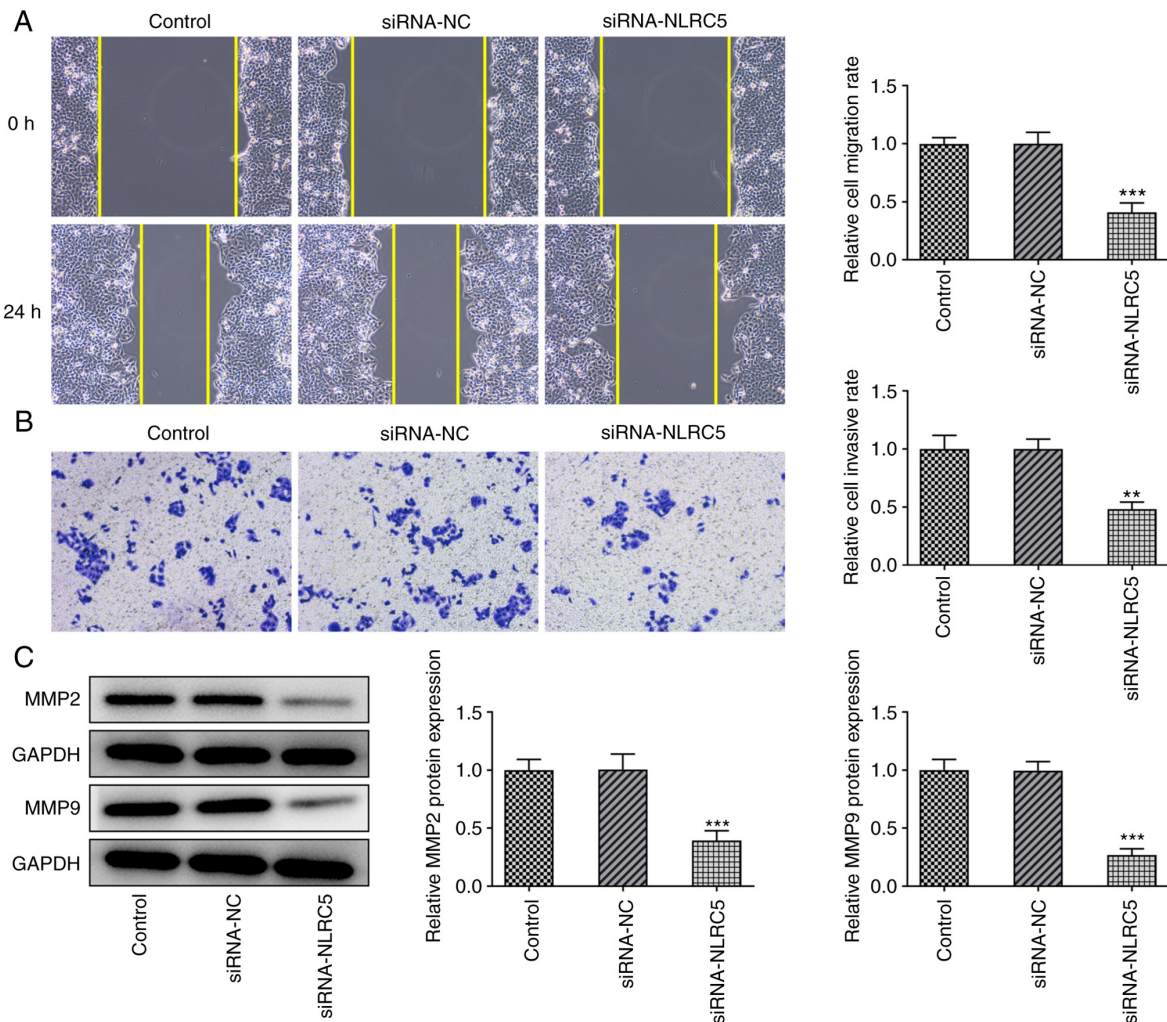


Figure 2. Inhibition of NLRC5 reduces invasion and migration of GC cells. (A) Wound healing detected the ability of cell migration (magnification, x100). (B) Transwell detected the ability of cell invasion (magnification, x100). (C) Western blotting detected the expression of MMP2 and MMP9. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. siRNA-NC. NLRC5, NOD like receptor family, CARD domain containing 5; GC, gastric cancer; si, short interfering; NC, negative control.



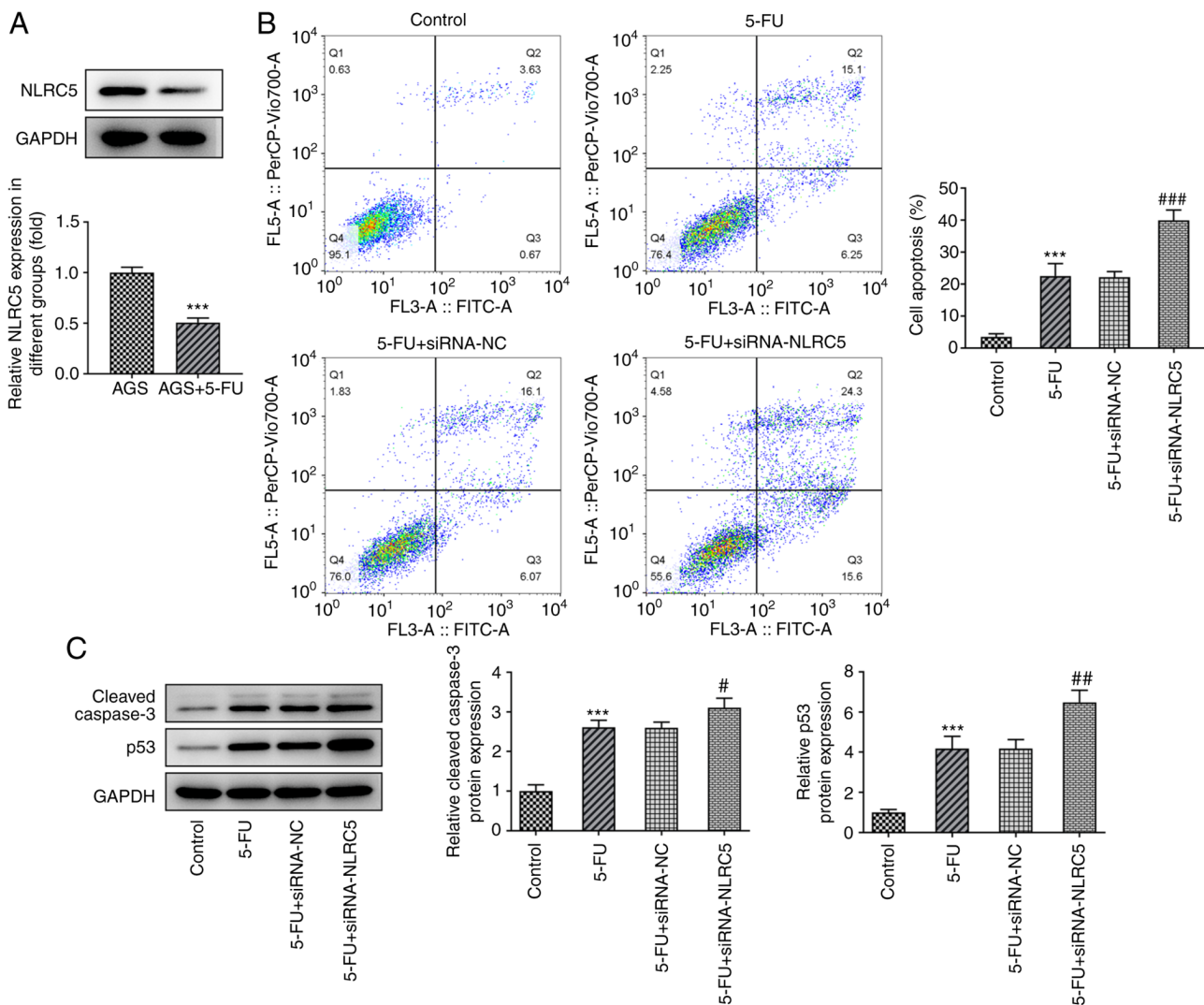


Figure 3. Inhibition of NLRC5 increases the sensitivity of GC cells to 5-FU. (A) The expression of NLRC5 was detected by western blotting. \*\*\* $P < 0.001$  vs. AGS. (B) Cell apoptosis was detected by flow cytometry. (C) Western blotting detected the expression of apoptosis-related proteins. \*\*\* $P < 0.001$  vs. control, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. 5-FU + siRNA-NC. NLRC5, NOD like receptor family, CARD domain containing 5; GC, gastric cancer; 5-FU, 5-fluorouracil.

of NLRC5 in AGS cells with 5-Fu resistance was decreased compared with that in AGS cells (Fig. 3A). Cells were divided into the control, 5-FU, 5-FU + si-NC and 5-FU + si-NLRC5 groups. Cell apoptosis was assessed by flow cytometry and the results showed that 5-FU promoted AGS cell apoptosis. Accordingly, cell treatment with 5-FU notably upregulated the expression of cleaved caspase 3 and p53. However, cell apoptosis and the expression levels of cleaved caspase 3 and p53 were further increased in the 5-FU + si-NLRC5 group, compared with the 5-FU + si-NC group (Fig. 3B and C). The above results suggested that NLRC5 silencing could increase the sensitivity of GC cells to 5-FU.

**YY1 binds to NLRC5 promoter.** Bioinformatics analysis using the JASPAR database predicted that YY1 could bind to NLRC5 promoter (Fig. 4A). The above finding was verified by overexpression and silencing experiments. Therefore, GC cells were transfected with YY1 overexpression or silencing constructs and the transfection efficiency was then evaluated. The mRNA and protein expression levels of YY1 in YY1 overexpressing or depleted GC cells were assessed by RT-qPCR

and western blot analysis, respectively (Fig. 4B and C). The results showed that YY1 overexpression in GC cells significantly upregulated NLRC5. By contrast, YY1 knockdown notably inhibited NLRC5 expression (Fig. 4D and E). These findings indicated that YY1 could regulate NLRC5 expression. Furthermore, luciferase reporter assay was carried out to measure NLRC5 promoter activity in cells overexpressing YY1. The results demonstrated that NLRC5 promoter activity was significantly enhanced in GC cells transfected with YY1 overexpression plasmid (Fig. 4F). Accordingly, the binding capacity of YY1 on NLRC5 promoter was further verified by chromatin immunoprecipitation assay (Fig. 4G).

**NLRC5 knockdown inhibits the promotive effect of YY1 on GC cell proliferation, invasion and migration.** To further investigate the regulatory mechanism of NLRC5 on GC cell invasion, migration and sensitivity to 5-FU chemotherapy, GC cells were divided into the following five groups: The control group; the ov-NC group; the ov-YY1 group; the ov-YY1 + si-NC group; and the ov-YY1 + si-NLRC5 group. CCK-8 and colony formation assays showed that the proliferation ability of GC cells

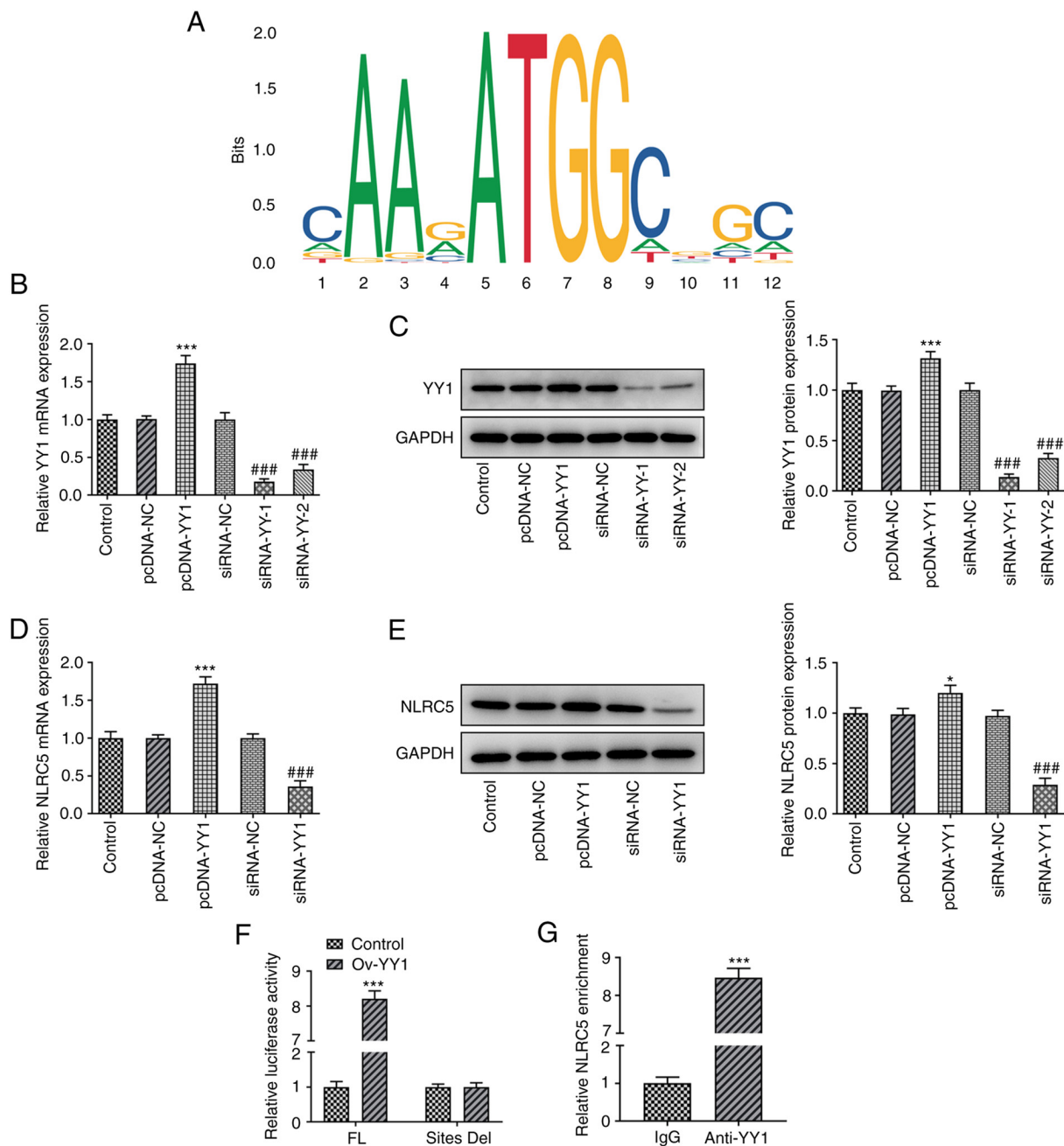


Figure 4. YY1 binds to the promoter of NLRC5. (A) JASPAR website was used to predict the binding between YY1 and NLRC5. (B) RT-qPCR was used to detect the expression of YY1. (C) Western blotting was used to detect the expression of YY1. (D) RT-qPCR was used to detect the expression of NLRC5 following cell transfection. (E) Western blotting was used to detect the expression of NLRC5 following cell transfection. \* $P < 0.05$ , \*\*\* $P < 0.001$  vs. pc-DNA-NC. \*\*\* $P < 0.001$  vs. siRNA-NC. (F) Promoter activity of NLRC5 was detected by luciferase. \*\*\* $P < 0.001$  vs. Ov-NC. (G) Chromatin immunoprecipitation detected the binding ability of YY1 and NLRC5 promoter. \*\*\* $P < 0.001$  vs. IgG. YY1, Yin Yang 1; NLRC5, NOD like receptor family, CARD domain containing 5; RT-qPCR, reverse transcription-quantitative PCR; si, short interfering; NC, negative control; Ov, overexpression; FL, full length.

in the ov-YY1 group was significantly increased compared with that in the ov-NC group. Additionally, compared with the ov-YY1 + si-NC group, the cell proliferation ability was notably decreased in the ov-YY1 + si-NLRC5 group (Fig. 5A and B). Furthermore, wound healing and Transwell assays revealed that compared with the ov-NC group, the migration and invasion abilities of GC cells were markedly enhanced in the ov-YY1 group. However, the cell migration and invasion was significantly reduced in the ov-YY1 + si-NLRC5 group compared with the ov-YY1 + si-NC group (Fig. 5C and D). Finally, western blotting results also showed that MMP2 and

MMP9 were notably upregulated in YY1 overexpressing cells, while the expression levels of both molecules were restored in the ov-YY1 + si-NLRC5 group compared with the ov-YY1 + si-NC group (Fig. 5E).

*NLRC5 silencing attenuates the enhancing effect of YY1 on the sensitivity of GC cells to 5-FU.* It was then found that the expression of YY1 in AGS cells with 5-Fu resistance was decreased compared with that in AGS cells (Fig. 6A). Subsequently, cell apoptosis was assessed by flow cytometry and western blot analysis. The results showed that AGS cell

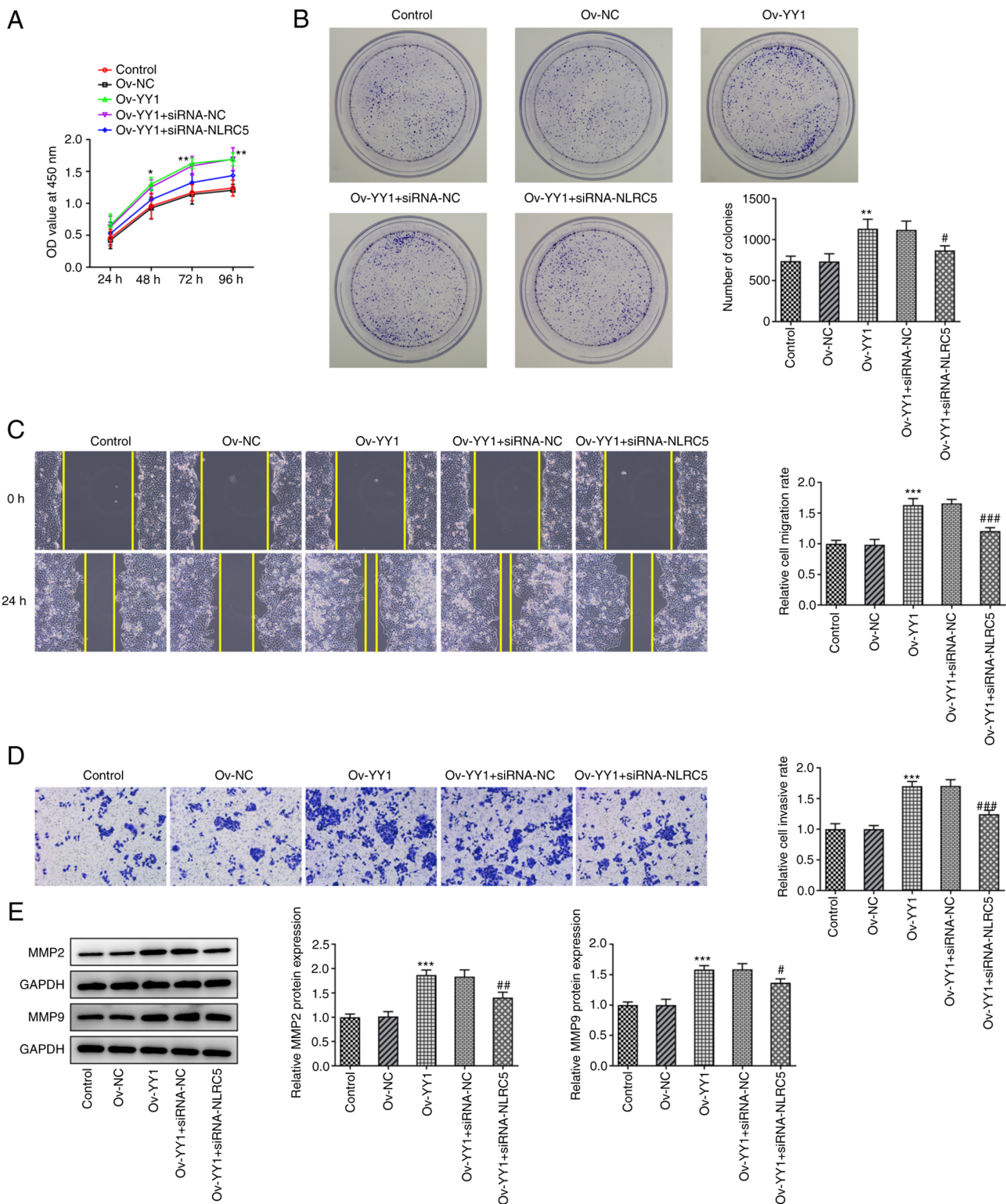


Figure 5. Inhibition of NLRC5 blocks the promotion of YY1 on proliferation, invasion and migration. (A) CCK-8 detected the cell viability. (B) Colony formation assay was used to detected the cell proliferation. (C) Wound Healing detected the ability of cell migration (magnification, x100). (D) Transwell detected the ability of cell invasion (magnification, x100). (E) Western blotting detected the expression of MMP2 and MMP9. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. Ov-NC. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs. Ov-YY1+siRNA-NC. NLRC5, NOD like receptor family, CARD domain containing 5; YY1, Yin Yang 1; Ov, overexpression; NC, negative control; si, short interfering.

apoptosis and the protein expression levels of cleaved caspase 3 and p53 were notably increased following cell exposure to 5-FU. However, following YY1 overexpression, cell apoptosis and the expression levels of cleaved caspase 3 and p53 were markedly reduced compared with the 5-FU + ov-NC

group. Additionally, compared with the 5-FU + ov-YY1 + si-NC group, cell apoptosis and the expression of both apoptosis-related proteins were significantly enhanced in the 5-FU + ov-YY1 + si-NLRC5 group (Fig. 6B and C). These findings suggested that NLRC5 knockdown abrogated the



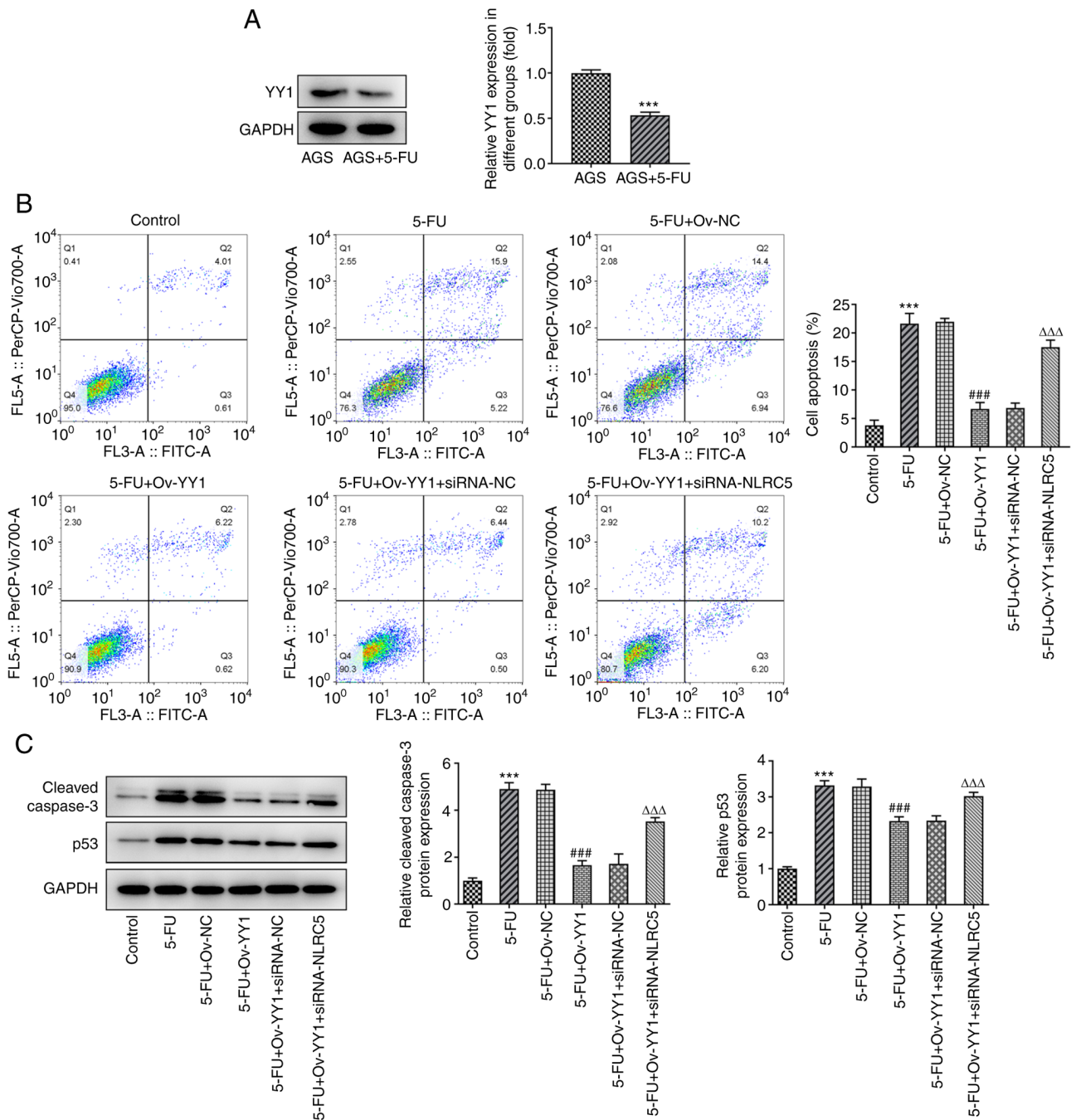


Figure 6. Inhibition of NLRC5 blocks the increasing effect of YY1 on the sensitivity of GC cells to 5-FU. (A) The expression of NLRC5 was detected by western blotting. \*\*\* $P < 0.001$  vs. AGS. (B) Cell apoptosis was detected by flow cytometry. (C) Western blot detected the expression of apoptosis-related proteins. \*\*\* $P < 0.001$  vs. Control. ### $P < 0.001$  vs. 5-FU+Ov-NC. ΔΔΔ $P < 0.001$  vs. 5-FU +Ov-YY1+siRNA-NC. NLRC5, NOD like receptor family, CARD domain containing 5; YY1, Yin Yang 1; GC, gastric cancer; 5-FU, 5-fluorouracil.

enhancing effect of YY1 on the sensitivity of GC cells to 5-FU.

## Discussion

GC is a common tumor of the gastrointestinal system. Although the pathogenesis of GC has not been fully elucidated, its occurrence and development is associated with a series of molecular changes, including the activation of several major signal pathways, mutations, and abnormal expression and regulation of related genes (20,21). The present study revealed that NLRC5

expression was increased in several GC cell lines. A previous study showed that NLRC5 is significantly upregulated in cells infected with *Helicobacter pylori* (22). In addition, another study demonstrated that NLRC5 is upregulated in mucosal organs such as the stomach (23). These findings were consistent with the results of the current study demonstrating that the expression levels of NLRC5 were significantly elevated in the GC cell lines AGS, MKN-45, KATO III and NCL-87.

It has been previously reported that NLRC5 regulates the proliferation, invasion and migration of tumor cells (5,6). Therefore, NLRC5 knockdown can significantly inhibit the



malignant biological behavior of glioma cells by attenuating the activation of the Wnt/ $\beta$ -catenin signaling pathway (24). In endometrial cancer, NLRC5 can promote the migration and invasion of endometrial cancer cell by activating the PI3K/AKT signaling pathway (25). Another study revealed that the increased expression levels of NLRC5 in GC tissues are associated with worse prognosis (7). However, the effect of NLRC5 on regulating the proliferation, invasion and migration of GC cells has not been previously investigated. Therefore, the results of the present study demonstrated that NLRC5 knockdown in the GC cell line AGS markedly attenuated the proliferation, migration and invasion of these cells.

Gene heterogeneity, differences in protein expression levels and the clinicopathological characteristics of patients with GC may result in huge differences in the response of patients to chemotherapeutic and targeted therapy drugs. Therefore, a previous study revealed that NLRC5 was a poor prognostic indicator in patients with non-small cell lung cancer (26). Additionally, increased NLRC5 expression was associated with advanced stage and poor prognosis in patients with renal clear cell carcinoma (5). However, the effect of NLRC5 on the prognosis of GC has not been previously reported. 5-FU combined with platinum is the recommended first-line drug regimen for postoperative systemic chemotherapy, with an effective rate of 30-50% (27). Therefore, 5-FU was selected to explore whether NLRC5 silencing could enhance the sensitivity of AGS cells to chemotherapy. The results showed that NLRC5 knockdown enhanced the sensitivity of GC cells to 5-FU and upregulated the expression of apoptosis-related proteins.

To further investigate the regulatory mechanism of NLRC5 in GC cell proliferation, invasion, migration and sensitivity to chemotherapeutic drugs, the binding capacity of YY1 on NLRC5 promoter was evaluated. Bioinformatics analysis using the ALGGEN-PROMO and JASPAR databases predicted that YY1 could activate NLRC5 by binding to its promoter region. This finding was consistent with the results obtained by Guo *et al* (28). YY1 serves a significant role in the occurrence and development of GC. A previous study demonstrated that YY1 can regulate the coiled-coil domain containing 43/adhesion regulating molecule 1 axis to promote the proliferation and metastasis of GC cells (15). Zhang *et al* (16) also found that YY1 is significantly upregulated in tumor tissues and serum from patients with GC. Therefore, it was hypothesized that NLRC5 could be activated by the transcription factor YY1 to regulate the proliferation, invasion and migration of GC cells. The role of YY1 in the above processes and sensitivity of GC cells to 5-FU chemotherapy was therefore explored. The results showed that NLRC5 knockdown could inhibit the effects of YY1 on promoting GC cell proliferation, invasion and migration. Additionally, NLRC5 silencing attenuated the effect of YY1 on enhancing the sensitivity of GC cells to 5-FU.

The present study has some limitations. First it only discussed cell experiments, which were not verified in animal experiments or other cell lines and the expression of NLRC5 in GC patients cannot be detected. The experimental results of the present study will be further verified in animal experiments and clinical experiments in the future. Moreover, the present study discussed only one of the mechanisms by which NLRC5 regulates the malignant progression of gastric cancer cells. NLRC5 can also serve a role in regulating the malignant progression of gastric

cancer by regulating other signaling pathways or cytokines. Future experiments will also explore the regulatory mechanism of NLRC5 in gastric cancer from more perspectives.

In brief, the present study indicated that NLRC5 knockdown could reduce the malignant growth and enhance the sensitivity of GC cells to 5-FU chemotherapy by inhibiting the carcinogenic effect of YY1. These findings could provide a theoretical basis for the treatment of GC via enhancing the sensitivity of GC cells to chemotherapeutic drugs.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

SLia performed the experiments. TX and SLiu analyzed the data. WX designed the experiments, interpreted the data and wrote the manuscript. SLiu and WX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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