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Molecular mechanisms of MCM3AP-AS1 targeted the regulation of mir-708-5p on cell proliferation and apoptosis in gastric cancer cells

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Abstract. – OBJECTIVE: Gastric cancer (GC) is a common malignancy of the digestive tract. Accumulated studies proved that long non-coding RNA MCM3AP-AS1 (MCM3AP-AS1) m... the mechanism of the progression of G... ever, the molecular mechanism is still g...er elusive. Hence, we aimed to explore the m... ular mechanism of MCM3AP-AS1 targeting ... regulation of microRNA-708-5p on cell prolife... tion and apoptosis in GC cells.

MATERIALS AND METH... expression levels of MCM3AP-AS1 (MCM3A... tisense RNA 1) in gastric mucosa... ls GES... gastric cancer cell lines of ...03 a... cells were detected b... gR... reover, the protein levels of Cy... 01, P2... and Bcl-2 in MGc-803 and SG... 01 cells a... sfection were detected b... tern blot. MT... y was performed to ... proliferation and flow cytometry w... arried ... determine GC cell apoptosis *in vitro*. In the ... int, the targeting relations... etween MCM3... and microRNA-708... was detected by Du...-Luciferase reporte... ay.

R... LTS: T... evel of MCM3AP-AS1 was significa... re... ed in G... ell lines. Knockdown of MCM... curb... ell proliferation and enh... ed ap... is in ...-803 and SGC-7901 cells. ...more, ... of the downregulation of MC... P-AS1 o... proliferation and apoptosis was ... ersed by knockdown of miR-708-5p, which wa... MCM3AP-AS1 *in vitro*.

... NCLU... NS: MCM3AP-AS1 regulates the ...liferation and apoptosis of gastric cancer ... by targeting the expression of microRN... 5p. The study may be useful to the therapy t... get of human GC.

K... ords:
...R-708-5p, GC cell lines, Proliferation, A...

...bbr...ations
GC = Gastric Cancer, MCM3AP-AS1 = MCM3AP antisense RNA 1, lnc RNA = long non-coding RNA.

Introduction

Gastric cancer (GC) is a common malignant tumor of the digestive tract[1] and the second cancer for mortality rates worldwide[2]. At present, the main means of treating gastric cancer are surgery and chemotherapy, but the severe side effects and drug resistance caused by chemotherapy seriously affect the prognosis of patients[3]. Although researches revealed the partial mechanism of pathogenesis in human GC, further investigations to discover the potential regulatory mechanism in GC is needed.

Long non-coding RNA (lnc RNA) played an important role in cell proliferation, metastasis, invasion and apoptosis of various tumors[4], including GC[5]. Such as lncRNA FOXD2-AS1, a tumor promoter in colorectal cancer[6] and involved in the poor prognosis of GC[7]. LncRNA H19 and miR-141 regulated cell proliferation and migration in GC cells[8]. What's more, MCM3AP-AS1 was also proved to enhance hepatocellular car-

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cinoma growth[9]. Studies have applied bioinformatics methods to screen gastric cancer and its adjacent non-cancerous tissues and found that MCM3AP-AS1 was up-regulated in gastric cancer. All evidence suggested that MCM3AP-AS1 may participate in the pathogenesis in human GC. However, the effects of MCM3AP-AS1 on biological behaviors such as cell proliferation and apoptosis of GC have not been studied completely. The study aimed to discover the role of MCM3AP-AS1 on cell proliferation and apoptosis in GC cell lines *in vitro*.

Over the past decades, microRNA (miRNA) has been proved to be involved in tumorigenesis[10,11]. The expression of miR-181a-5p effected on cell proliferation in GC[12]. Meanwhile, miR-708-5p regulated prostate cancer cell apoptosis[13]; overexpression of miR-708-5p had a certain inhibitory effect on cell proliferation[14]. MiR-708-5p referred to the process of GC. The study demonstrated miR-708-5p expression was downregulated in GC cell lines. However, it has not been reported whether MCM3AP-AS1 targeted and regulated the biological behavior of miR-708-5p regulating GC cells *in vitro*.

In this study, bioinformatics methods and molecular biology techniques were used to investigate whether MCM3AP-AS1 targeted miR-708-5p to regulate the proliferation and apoptosis of gastric cancer cells, in order to find new therapeutic targets for clinical.

Materials and Methods

Cell Culture and Transient Transfection

Normal gastric mucosal cells GES-1 and gastric cancer cells MGc-803 and SGC-7901 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Of which GES-1, MGc-803 and SGC-7901 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) medium containing 10% (volume fraction) fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and double antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mg) (Gibco, Rockville, MD, USA) at a 37°C, 5% CO_2 in cell incubator.

The vectors of MCM3AP-AS1 overexpression (si-MCM3AP-AS1), overexpression empty control (pcDNA3.1), MCM3AP-AS1 knockdown (si- MCM3AP-AS1), knockdown negative control (si-NC), miR-708-5p inhibitor (anti-miR-708-5p) and inhibitor negative control (anti-miR-NC) were purchased from Sangon Biotech (Shanghai, China). Subsequently, 24 h after cell passage, the cells were in logarithmic growth; next, Lipofectamine 2000 (Thermo Fisher Scientific) was carried out to transfect vectors into cells according to reagent manufacturer's manual.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The logarithmic growth phase and transfected 48 h cells were collected, total RNA was extracted by Trizol (Thermo Fisher Scientific). SuperScript First-Strand Synthesis System (Thermo Fisher Scientific) was used to reverse transcription into cDNA for qRT-PCR, reacting solutions were mixed using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), U6 and GAPDH were performed as internal reference genes. The process was as follow: 95°C for 3 minutes, 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 20 s, and then signals were collected by QuantStudio 3 QS3 (Thermo Fisher Scientific). Special primers were as follows: miR-708-5p: (Forward: 5'-GGC GCG CAA GGA TCT TAC AAT C-3', Reverse: 5'-GTG CAG GGT CCG AGG TAT-3'); U6: (Forward: 5'-GAG CCA TTG CGG AAC G-3', Reverse: 5'-CTA TCC CCA CAT AGT CCA GG-3'); MCM3AP-AS1: (Forward: 5'-GCT GCT AAT GGC AAC ACT GGA-3', Reverse: 5'-AGG TGC TGT CTG GTG GAG AT-3'); GAPDH: (Forward 5'- GGT GAA GGT CGG AGT CAA CG-3', Reverse: 5'-CAA AGT TGT CAT GGA TGA ACC-3')

Western Blot

The total protein was extracted from MGc-803 and SGC-7901 cells condition in the logarithmic growth phase after transfecting for 48 h. The total protein concentration was determined by BCA Protein Assay Kit (Pierce, Waltham, MA, USA). SDS-polyacrylamide gel was prepared, 50 μg denatured protein was added per well, and electrophoresis was run. The isolated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland); the voltage was controlled at 100 mV for 70 minutes. Then skim milk was used (Sangon Biotech) to block PVDF for 60 min; the diluted primary antibody was incubated at 4°C overnight. Tris Buffered Saline Tween (TBST) (Sangon Biotech) was washed three times for 5 min each. The diluted secondary antibody was incubated at room temperature for 60 minutes in the shaker, TBST

was washed three times for 5 min each. In the end point, protein signals were identified by using SuperSignal Chemiluminescent Substrates (Thermo Fisher Scientific) through Image Lab software (Bio-Rad). The special antibodies used in the study are listed as follows: the primary antibodies of Cyclin D1 (ab16663, 1:100), P21 (ab109520, 1:5000), Bax (ab32503, 1:5000), Bcl-2 (ab182858, 1:2000) and GAPDH (ab9485, 1:2500) were purchased from Abcam (Cambridge, MA, USA).

MTT Assay

2.5% trypsin (Gibco) was carried out to digest MGc-803 and SGC-7901 cells, the cell concentration was adjusted to 1 ´ 10^5/ml and 100 µl per well were added in 96-well plates. Whereafter, they were mixed with 20 µl MTT reagent (Beyotime, Shanghai, China) and incubated for 4 hours before detection. Finally, the absorbance was measured at 490 nm (OD) by SpectraMax®iD3 (Molecular Devices, San Jose, CA, USA).

Flow Cytometry

After transfection, MGc-803 and SGC-7901 cells were cultured for 48 h, then the m m was discarded and washed twice with PB phate-buffered saline) (HyClone). Subseq ly, 500 ul 1×Binding Buffer, 5 ul Annexin V- (Biosea Biotechnology, Beijing, China) were a ed in solutions and incubated for 15 minutes the dark place; 2.5 µl PI (Pro ide) was added and incubated for 5 n tes, a low cytometry was performed t tect ce optosis *in vitro*.

Dual-Luciferase Reporter A

Constructed rs: wildtyp VT-MC-M3AP-AS1 3'U mutant (M -pGL3-MCM3AP-AS UTR pression vectors of MCM3AP-AS1, renilla s (pGL3) were purchase m Promega (M n, WI, USA). Subseq ly, they were trans ected with one of th nto M 803 and SGC-7901 cells by usi ofect ne™ 20 and cultured for 48 hours. ual-L rase assay kit (Promega) was a trate lyse cells and stimulate se si signals were captured by Spe Max®iD olecular Devices, San Jose, CA A).

tistical Analysis

PSS 22.0 (SPSS Inc., IBM, Armonk, NY, U as used for data analysis. The Student's *t*-test as used for comparison between the two groups. One-way analysis of variance with post-hoc test (ANOVA) was followed by T for multiple comparisons of grou raphi Prism (San Diego, CA, USA) w erformed to plot images. $p<0.05$ indicates t e difference was statistically significant.

Results

The Level of MC -A as Strikingly Inc sed Cell l s

GC cell lin nd GE s w cultured for 48 hour 7°C, 5% C RNA was extracted evel of MC AP-AS1 was measure qR The results showed that it was notably induced c-803 and SGC-7901 cell e 1).

ockdown of MCM3AP-AS1 ikingly Sup ssed Cell liferation i C Cell Lines

ll inter g RNA (si-RNA) of MCM3 MCM3AP-AS1) and negative control (si-NC) were constructed and transfected into GC cell lines, respectively. The level 3AP-AS1 was strikingly decreased in MC 3AP-AS1 group (Figure 2A and 2D). What's more, MTT assay was performed to detect GC cell proliferation *in vitro*. Evidence revealed that knockdown of MCM3AP-AS1 curbed cell proliferation in GC cell lines (Figure 2B and 2E). Furthermore, the expression of

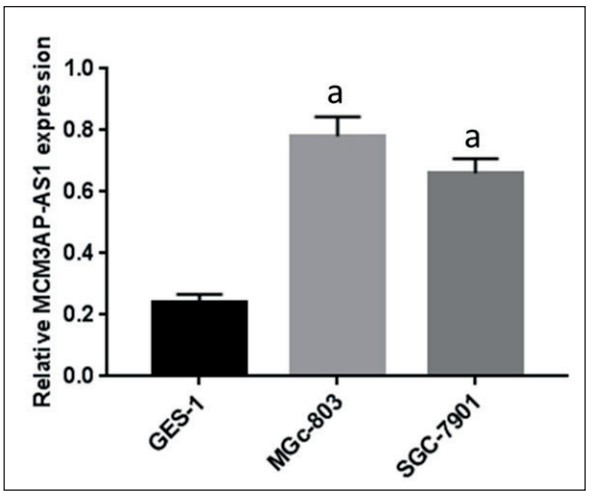

Figure 1. The level of MCM3AP-AS1 was strikingly increased in GC cell lines. The level of MCM3AP-AS1 in MGc-803 and SGC-7901 cells was measured by qRT-PCR. [a]$p<0.05$.

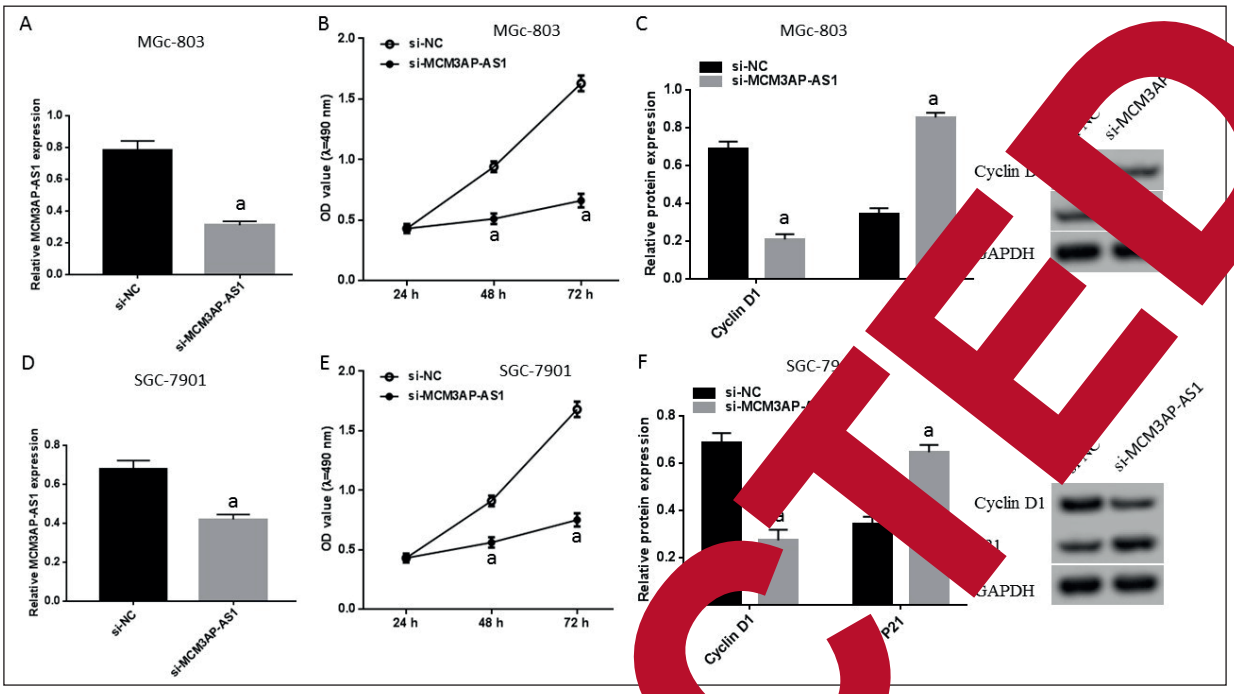

Figure 2. Knockdown of MCM3AP-AS1 strikingly suppressed c iferation i cell lines. Constructed knockdown vector of MCM3AP-AS1 (si-MCM3AP-AS1) and negative control (si- transfected into MGc-803 and SGC-7901 cells **(A-F)**, respectively. The level of MCM3AP-AS1 was measure 03 **(A)** and SGC-7901 **(D)** cells *via* qRT-PCR. What's more, MTT assay was carried out t e cell proliferation of MGc-803 **(B)** and SGC-7901 **(E)** cells. Finally, the expression of Cyclin D1 and P21 was blot *in vitro* **(C** and **F)**. $^{a}p<0.05$.

Cyclin D1 and P21, which was relative to cell cycles, was measured by Western blot a the effect of MCM3AP-AS1 d lation on cell proliferation *in vitro* re 2 nd 2F) was verified.

Downregulation MCM3 S1 Significantly P oted Cell Apoptosis in Lines

After trans ng NC or si-MCM3AP-AS1 into MGc-803 and SG 01 cells, cell apoptosis was yzed by flow cy y. The results suggest hat kn ckdown of M M3AP-AS1 enhanc ll apo s in GC cell lines (Figure 3A, 3B nd Meanwhile, the expression of Bax a as me d, which was referred t apopto Westo lot. It proved the result effect AP-AS1 on cell apoptosis *in v* (Figure nd 3F).

-708-5p Was a Target Gene of M3AP-AS1

formatics software was used to predict the relati hip between MCM3AP-AS1 and miR-708-5p and proved they had binding sites (Figure 4A). After transfection with WT-MCM3AP-AS1 or mut-MCM3AP-AS1, miR-708-5p was verified to be a target gene of MCM3AP-AS1 (Figure 4B). Moreover, the level of miR-708-5p was decreased by the upregulation of MCM3AP-AS1 while enhanced the expression through knockdown of MCM3AP-AS1 (Figure 4C).

Knockdown of miR-708-5p Rescued the Effect of MCM3AP-AS1 on MGc-803 and SGC-7901 Cell Proliferation

After transfection with si-NC, si-MCM3AP-AS1, si-MCM3AP-AS1 + anti-miR-NC or si-MCM3AP-AS1 + anti-miR-708-5p into GC cell lines, the level of miR-708-5p was promoted by si-MCM3AP-AS1 while rescued *via* knockdown of miR-708-5p *in vitro* (Figure 5A and 5D). In addition, the downregulation of miR-708-5p reversed the effect of si-MCM3AP-AS1 on cell proliferation in GC cell lines (Figure 5B and 5E). Cell cycles relative proteins of Cyclin D1 and P21 also verified the conclusion of GC cell proliferation *in vitro*.

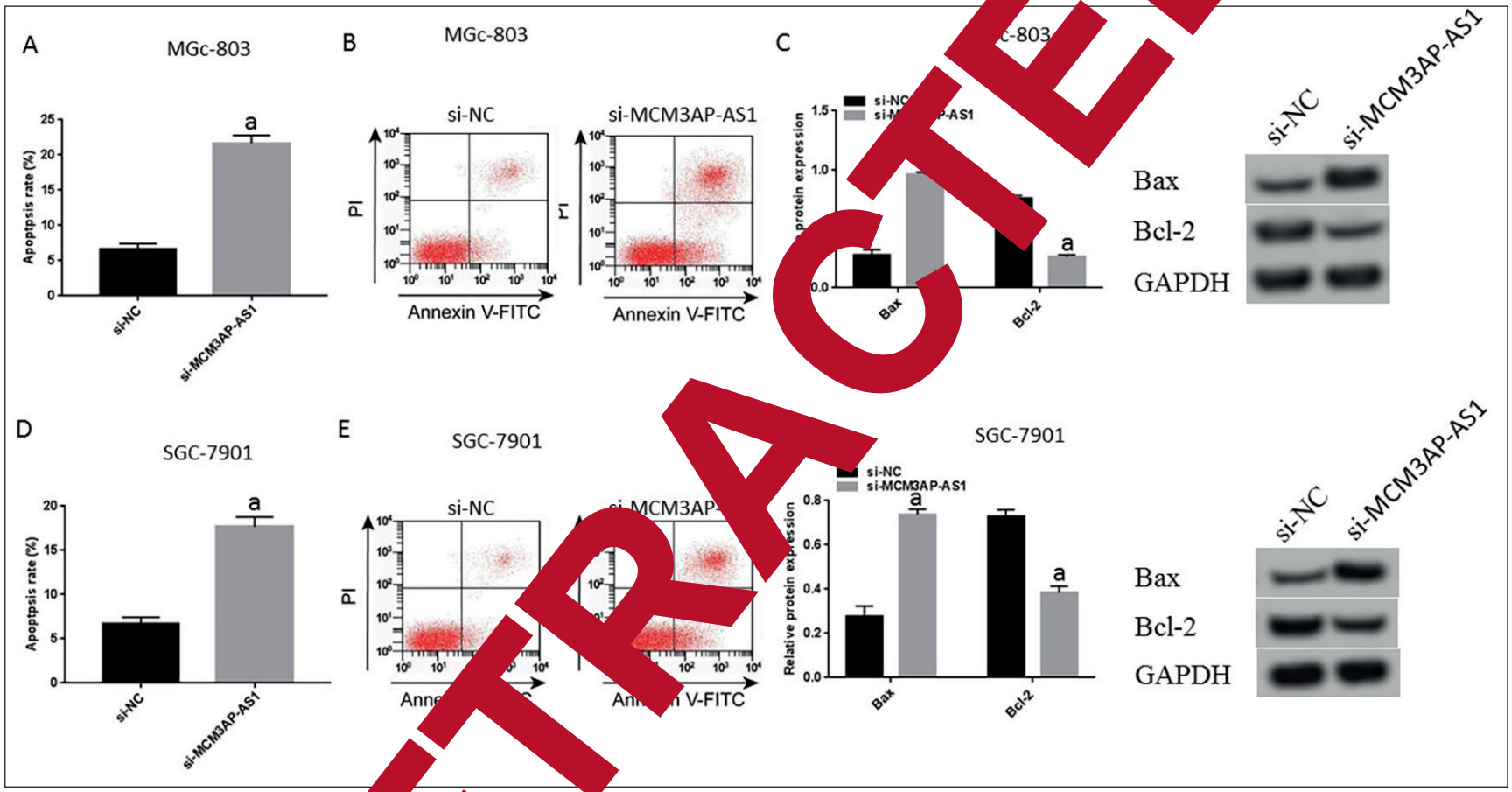

Figure 3. Downregulation of MCM3AP-AS1 significantly promoted cell apoptosis in GC cell lines. After transfecting with si-MCM3AP-AS1 into MGc-803 and SGC-7901 cells **(A-F)**, GC cell apoptosis was identified by flow cytometry **(A, B, D and E)**. The expression of apoptosis-relative proteins of Bax and Bcl2 was examined by Western blot **(C and F)**. $^{a}p<0.05$.

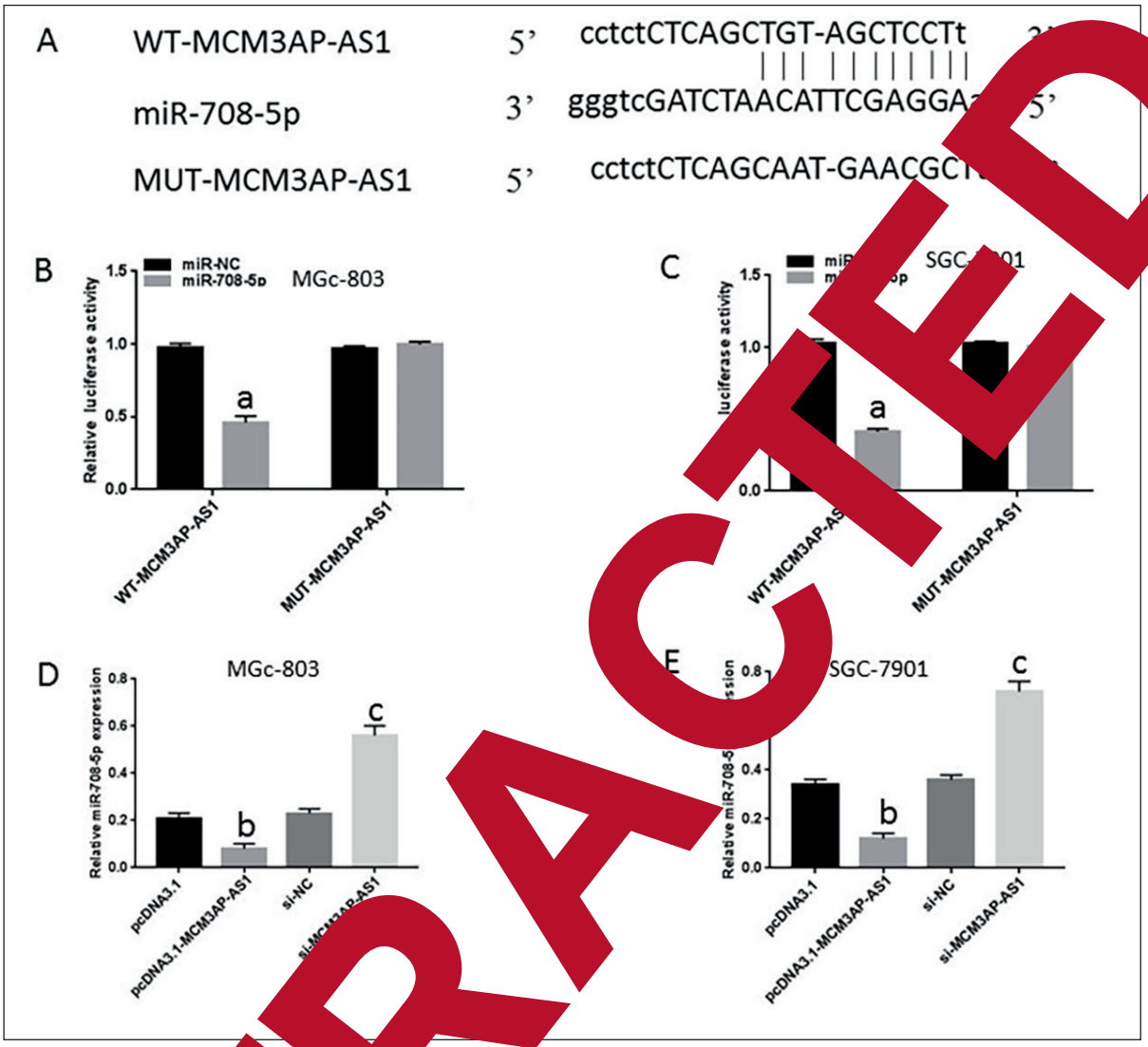

Figure 4. miR-708-5p ... target ge... CM3AP-AS1. The binding sites between MCM3AP-AS1 and miR-708-5p were predicted by bioinfor... software of ... **(A)**. Dual-luciferase reporter assay was used to verify the relationship of MCM3AP-AS1 and ...5p *in vitro* **(B)**. ...rmore, the level of miR-708-5p was measured after transfecting with up or down regulation ... 3AP-AS1 **(C)**. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$.

Down...ulation of miR-70...-5p Rest...d the ...ect of MCM3AP-AS1 on... Apo...sis in ...C Cell Lines

To ... investi... the role of MCM3AP-A... d mi...08-5p on GC progression-tra... was carried out with MCM3... AS1 and ...-708-5p vectors, including si-N... si-MCM3AP-AS1, si-MCM3AP-AS1 + an... si-MCM3AP-AS1 + anti-miR-...5p into MGc-803 and SGC-7901 cells. ...-expression of miR-708-5p regained the e... of si-MCM3AP-AS1 on cell apoptosis *in vi...* (Figure 6A and 6C). Meanwhile, the conclusion was also verified by measuring the expression of Bax and Bcl-2 in GC cell lines (Figure 6B and 6D).

Discussion

Gastric cancer is a malignant tumor that occurs in the gastric mucosa. Its incidence rate ranks first among all malignant tumors in China. Due to the different living habits and dietary structure in the north and the south, the incidence of gastric cancer in coastal areas and northwestern China

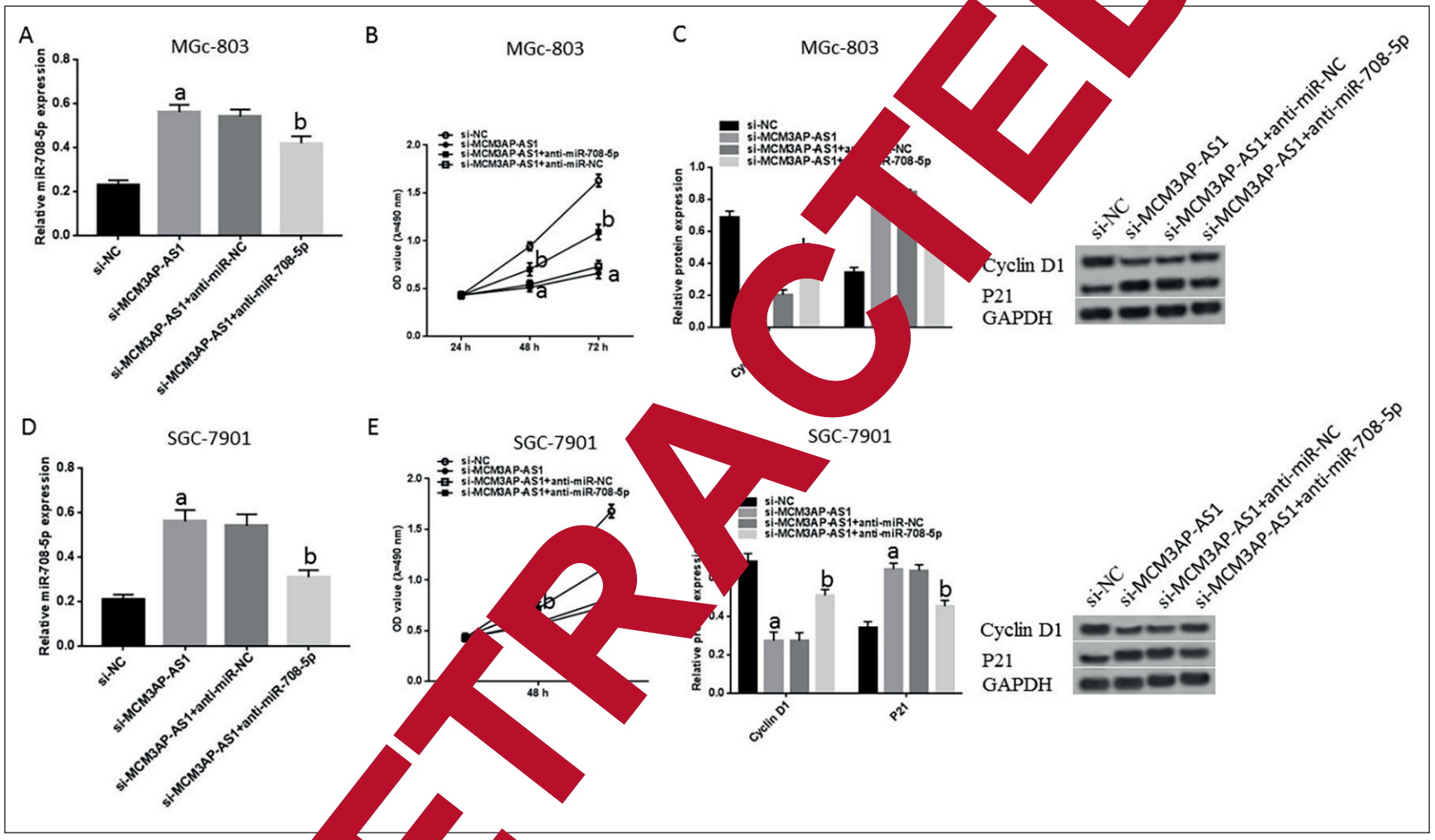

Figure 5. Knockdown of miR-708-5p r effect CM3AP-AS1 on MGc-803 and SGC-7901 cell proliferation. Transfected vectors of si-NC, si-MCM3AP-AS1, si-MCM3AP-AS1 + anti-miR-NC or si-MCM3 1 + a iR-708-5p into GC cell lines **(A-F)**. The level of miR-708-5p was measured by qRT-PCR **(A** and **D)**. Moreover, MTT was used to detect cell prolifera cell **E)**. Finally, the expression of apoptosis-relative proteins of Cyclin D1 and P21 was examined by Western blot **(C** and **F)**. $^{a}p<0.05$, $^{b}p<0.01$.

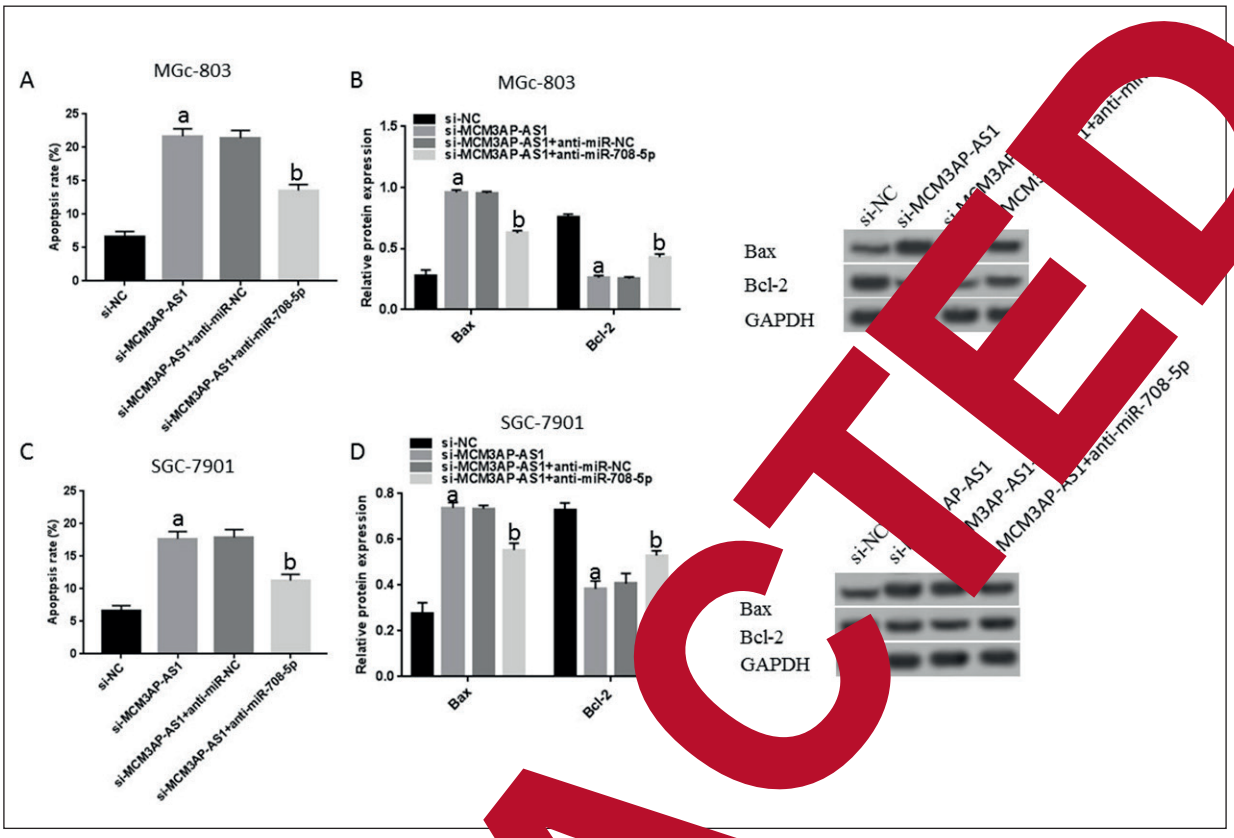

Figure 6. Downregulation of miR-708-5p restored [illegible]ffect [illegible]AS1 on cell apoptosis in GC cell lines. GC cell lines transfected with si-NC, si-MCM3AP-AS1, si-MC[illegible]P-[illegible] anti-miR-NC or si-MCM3AP-AS1 + anti-miR-708-5p into into MGc-803 and SGC-7901 cells **(A-D)**, respectively. [illegible]ptosis was measured by flow cytometry *in vitro* **(A** and **C)**. Meanwhile, Bax and Bcl-2 expression w[illegible]o identified [illegible]stern blot **(B** and **D)**. $^{a}p<0.05$, $^{b}p<0.01$.

is significantly higher tha[illegible]t in the [illegible]th. The recurrence and metasta[illegible] tumo[illegible] main reason for the di[illegible]sult[illegible] treatment. It is reported that t[illegible] metas[illegible]nd invasion are affected by v[illegible]s lncRNAs [illegible] the past decades. There[illegible]ole of lncRN[illegible] gastric cancer is of[illegible]t sig[illegible]nce for the targeted treatment of gastric cance[illegible]

LncRN[illegible]s a non-coding [illegible] that occupies 98% o[illegible]man gene transcript[illegible] and is greater than [illegible]nt in [illegible]gth[15]. In recent years, it has be[illegible]nd th[illegible]ncRNA[illegible]an regulate the activity o[illegible] protei[illegible]es at multiple levels, affecting [illegible]iolog[illegible] behaviors such as cell [illegible]ration, [illegible]ation and apoptosis of tumo[illegible]ls[16-18]. St[illegible]s have shown that lncRNA MC[illegible]P-AS1 expression in primary liver cance[illegible]higher than adjacent tissues and [illegible]al liver tissues[19]. Further, MCM3AP-AS1 [illegible] significantly up-regulated in glioblastoma, a[illegible]ibition of MCM3AP-AS1 expression inhibit[illegible]cell viability, migration, and angiogenesis in glioblastoma[20]. The qRT-PCR assay showed that the level of MCM3AP-AS1 in two GC cells was higher than that in normal GC cells, which was consistent with the above results, suggesting that MCM3AP-AS1 was a key factor in the development and progression of gastric cancer. Meanwhile, Wang et al[9] reported that MCM3AP-AS1 was elevated in hepatocellular carcinoma, which promoted the growth of hepatocellular carcinoma by targeting inhibited expression of mir-194-5p. To further investigate the role of MCM3AP-AS1 in GC, this study repressed the expression of MCM3AP-AS1 in GC cell lines and detected cell proliferation and apoptosis *in vitro*. The results showed that after inhibiting the expression of MCM3AP-AS1, GC cell proliferation was significantly decreased, and the cell apoptosis rate was enhanced. The levels of Cyclin D1 and Bcl-2 were significantly decreased, and the levels of P21 and Bax were notably increased, suggesting that MCM3AP-AS1 may promote cell proliferation and inhibit apoptosis. In addition, the expression

of miR-708-5p was significantly increased after inhibiting the expression of MCM3AP-AS1, suggesting that MCM3AP-AS1 may regulate the expression of miR-708-5p.

MiR-708-5p is a newly discovered microRNA that plays an important role in cell proliferation, migration, invasion, and apoptosis in tumor[13,21]. The miRcod bioinformatics software predicted that MCM3AP-AS1 and miR-708-5p had common fragment sequences, speculating that miR-708-5p may be the target gene of MCM3AP-AS1. Subsequently, Dual-Luciferase report assay and western blot were carried out to further verify MCM3AP- AS1 negatively regulated the expression of miR-708-5p by directly binding to the miR-708-5p 3'UTR region.

Studies have reported that miR-708-5p expression is decreased in human mesenchymal stem cells, and overexpression of miR-708-5p promoted cell proliferation and migration. The expression of miR-708-5p was down-regulated in renal cell carcinoma, and it could inhibit both cell apoptosis of renal cancer and the occurrence of renal cancer by inhibiting the oncogene zinc finger E-box binding to homologous box protein 2[22]. Furthermore, miR-708-5p inhibited migration of breast cancer by inhibiting endoplasmic reticulum membrane protein and reducing the activation of extracellular signal-regulated kinases[23]. Afterward, the effect of MCM3AP-AS1 targeting miR-708 GC cells was verified, anti-miR-708 was sfected into GC cells, which stab nockdo of MCM3AP-AS1. The results d tha inhibitor reversed the ffec ownregulation of MCM3AP-A on cel eration and apoptosis *in vitr* rthermore, pression of Cyclin D1, P and Bcl-2 furt erified the above co ion.

In sum ary, MCM3 1 targeted miR-708-5p to ulate cell prolif and apoptosis of GC . This study clarified the mechanism of M AP-A rgeting miR-708-5p and the tar ther or GC viding potential therapeut Howe further investigations in clinica ment uman GC are needed.

Conclusions

he level of MCM3AP-AS1 was notably enhanced in GC cell lines. Further, the downregu of MCM3AP-AS1 decreased cell proliferati and boosted apoptosis in MGc-803 and SGC-7901 cells. miR-708-5p was a target gene of MCM3AP-AS1; its knockdown re effect of the downregulation of M AP-A on cell proliferation and apopt in GC cel lines.

Conflict of Interest

The Authors declare that th ve no c flict of int

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