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ISOC1 promotes the proliferation of gastric cancer cells by positively regulating CDK19

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Abstract. – OBJECTIVE: To explore the role and potential mechanism of isochorismatase domain-containing 1 (ISOC1) in gastric cancer.

PATIENTS AND METHODS: The expression levels of ISOC1 in gastric cancer (GC) tissues as well as corresponding cell lines, was evaluated by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). A cell line stably expressing ISOC1 was constructed by vector construction and cell transfection, and the proliferation ability of the stably transfected cells was examined. Subsequently, the ISOC1 target database was screened, which suggested that CDK19 may be the potential target. The correlation between ISOC1 and CDK19 mRNA and protein expressions in clinical tissue specimens and cell lines was evaluated by qRT-PCR and Western blot, and the Luciferase reporter gene experiment was applied to verify the regulatory effect of ISOC1 on CDK19.

RESULTS: ISOC1 was shown to be markedly increased in GC tissues compared to adjacent cancer tissues by qRT-PCR. In addition, compared with patients with low ISOC1 expression, the pathological stage and tumor size of gastric cancer patients with high ISOC1 expression were remarkably larger. Then, the ISOC1 knockdown cell line was established, and it was found through cell proliferation function experiments that the proliferation rate of gastric cancer cells was remarkably slower than the control group after knocking down ISOC1. Subsequently, bioinformatics and Luciferase reporter gene experiments suggested that ISOC1 had a direct regulatory effect on CDK19. In addition, recovery experiments also demonstrated that CDK19 overexpression could reverse the effect of ISOC1 silencing on cell proliferation.

CONCLUSIONS: ISOC1 was markedly upregulated in GC tissues. It could positively regulate its downstream target CDK19, which in turn promoted the proliferation of GC cells. Therefore, our study may provide new ideas for understanding the progression of GC.

Key Words:
ISOC1, CDK19, Gastric cancer, Proliferation.

Introduction

Gastric cancer (GC) is one of the malignant tumors with extremely high morbidity and lethality. Its incidence ranks fourth among all malignant tumors, and its lethality ranks second. The study shows that the annual increase of GC cases in China accounts for about 40% of the world[1-3]. At present, the clinical lack of effective and sensitive diagnostic methods or markers for early GC has led to a low clinical diagnosis rate of early GC. Therefore, there is still a long way to go on basic research and clinical diagnosis and treatment of GC[4,5]. Tumorigenesis of GC is a very complicated pathological process and the patients may undergo a variety of pathological changes, which is caused by the interaction and effects of environmental factors, genes and *H. pylori* infection[6,7]. Genetic factors may affect the pathogenesis of GC[8,9], which are involved in oxidative damage DNA protection, mucosal protection and immunity and inflammation after *H. Pylori* infection. Therefore, it is imperative to study the molecular mechanisms involved in tumorigenesis of GC[10,11]. The deep understanding of GC tumorigenesis will supply clinical diagnostic and therapeutic values for improving diagnosis and treatment of GC[12,13].

Isochorismatase domain-containing 1 (ISOC1) is a coding gene containing an allele base domain[14]. The exact function of ISOC1 in humans has not been elucidated; however, research has speculated that it may be related to several metabolic pathways[14]. ISOC1 may function as a

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cancer-promoting gene in some human tumors, but the specific mechanism and its role in GC is unknown[14,15]. Here, we proved that ISOC1 was upregulated in GC tissues. Besides, bioinformatics prediction suggested that ISOC1 may regulate CDK19, which could promote cell proliferation in various tumors. The expressions of ISOC1 and CDK19 were positively correlated in tumor cell lines and clinical specimens, which suggested that ISOC1 might promote gastric carcinogenesis by regulating CDK19[16]. This may provide new ideas for further understanding the progress of GC and improve the development of genetic diagnostics and therapeutics of GC.

Patients and Methods

Patients and GC Samples

The cancer tissues and the normal tissue specimens of 56 pairs of GC patients treated by our hospital for general surgery were collected and were stored in a liquid nitrogen tank. In this study, tumor staging was assessed based on the guideline proposed by the Union for International Cancer Control (UICC). The collection and use of clinical specimens were approved by the Ethics Committee of Hebei Medical University. All participating patients had signed informed consent.

Cell Lines and Reagents

Human GC cell lines AGS, SGC-7901, BGC-823, and immortalized normal gastric mucosal epithelial cells GES-1 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high-glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL). All cells were cultured in a 37°C, 5% CO2 incubator. When the cells grew to 80% -90% confluence, they were digested and passaged with 1 × trypsin + EDTA (ethylenediaminetetraacetic acid).

Transfection

The control group (sh-NC) and ISOC1 knockdown lentivirus (sh-ISOC1) were purchased from Shanghai Gima Company (Shanghai, China). After cell density reached 30-40%, transfection was performed as suggested.

Cell Proliferation Assays

After transfection, 2000 cells were seeded into 96-well plates. After incubation for 24 h, 48 h, 72 h, and 96 h, cells were added with Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Kumamoto, Japan), and after incubated for another 2 hours, the optical density (OD) value of each well were measure at 490 nm in a microplate reader.

Colony Formation Assay

After transfection, 800 cells/well were seeded into a 6-well plate and cultured for 2 weeks. The medium should not be changed in the previous week to improve adhesion. The clones would form after 2 weeks, which were then stained with 0.1% crystal violet staining solution (Beyotime, Shanghai, China). Then, cells were washed and observed under the light scope for counting.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

The cells were incubated with 50 μm EdU (Sigma-Aldrich, St. Louis, MO, USA) for 2 hours, then, stained with Apollo and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA), and the number of EdU-positive cells was detected by fluorescence microscopy. The display rate of EdU-positive was shown as the ratio of the number of EdU-positive cells to the total DAPI-colored cells (blue cells).

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

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA). RNA reverse transcription was performed with Prime Script reverse transcription kit, then, qRT-PCR was performed using the SYBR® Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan) by the StepOne Plus Real-time PCR system. The primers were listed below: ISOC1: F: 5'-CGACATGCACCGCAAATTCG-3 ', R: 5'-TGAGCTGGATCTGCAACGG-3'; CDK19: F: 5'-AAGCCAAAGCCTGGGGATAC-3', R: 5'-AAGCCCTCCTGGAATCTTGC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-TGACTTCAACAGCGACACCCA-3', R: 5'-CACCCTGTTGCTGTAGCCAAA-3'. GAPDH was used as internal reference, and gene expression was calculated by $2^{-\Delta\Delta Ct}$ method.

Western Blot

The proteins were lysed by PRO-PREPTM protein lysate. Total protein concentration was

calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). ISOC1 and CDK19 rabbit anti-human monoclonal antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA, USA); horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody was obtained from GenScript, and the internal control was GAPDH. The images were analyzed semi-quantitatively using alpha SP image analysis software.

Dual-Luciferase Reporter Assay

The wild-type and mutant ISOC1 were cloned into the pMIR Luciferase reporter vectors and co-transfected into cells with CDK19 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The Dual-Luciferase reporter assay was performed with Promega kit (Madison, WI, USA).

Statistically Analysis

GraphPad Prism 5 V5.01 software (La Jolla, CA, USA) was used for statistical analysis. The Student's *t*-test and one-way analysis of variance (ANOVA) followed by post-hoc test (Least Significant Difference) were used to analyze the statistical differences between the two groups and multiple groups. Each experiment was repeated at least three independent experiments, and the data were expressed as mean ± standard deviation ($\bar{x} \pm s$). $p<0.05$ was considered statistically significant.

Results

ISOC1 was Upregulated in GC Tissues and Cell Lines

To determine the role of ISOC1 in GC, a total of 56 GC tumor tissues and adjacent tissues were collected, and the ISOC1 expression was detected by qRT-PCR, which revealed that ISOC1 was upregulated in GC tissues (Figure 1A). In addition, ISOC1 was markedly higher expressed in GC cell lines, which suggested that ISOC1 may function as an oncogene in GC (Figure 1B).

According to the mRNA results, the 56 GC patients were divided into two groups: relative high group and relative low group. The analysis of ISOC1 expression and the age, sex, pathological stage, tumor size, lymph node metastasis and

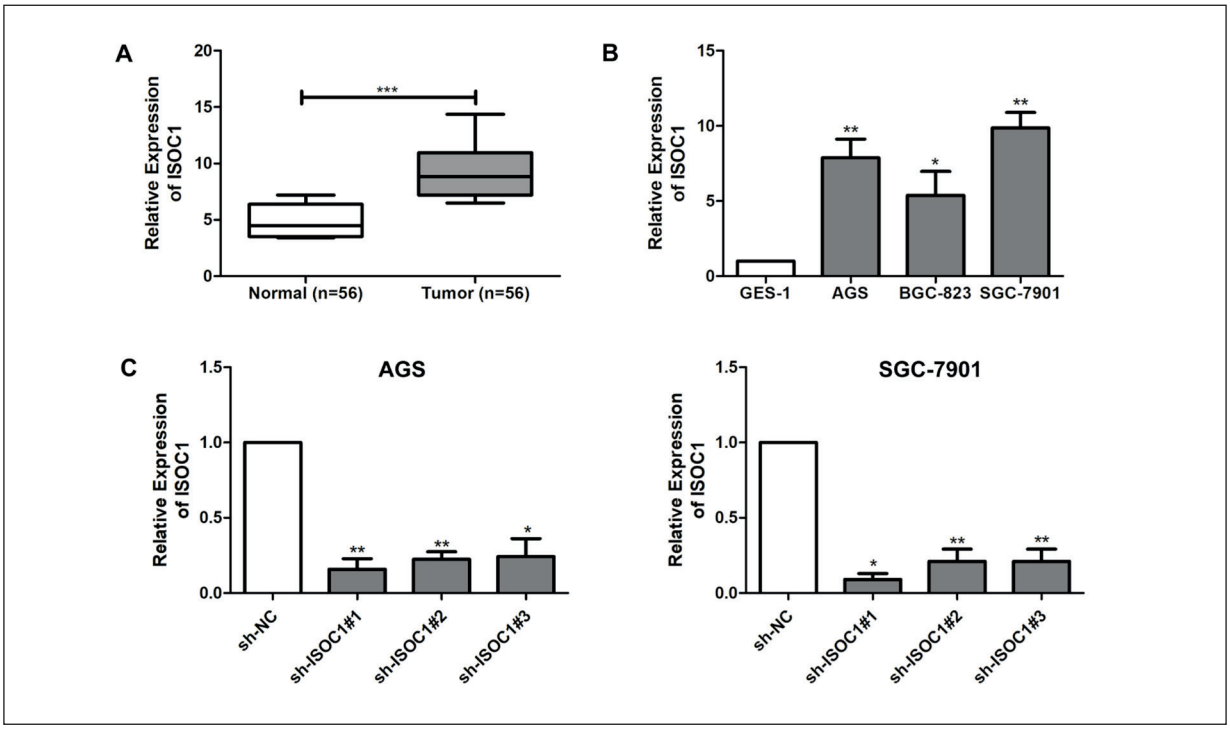

Figure 1. ISOC1 is highly expressed in gastric cancer tissues and cell lines. **A,** qRT-PCR was used to detect the expression of ISOC1 in gastric cancer tumor tissues and adjacent tissues. **B,** qRT-PCR was used to detect the expression of ISOC1 in gastric cancer cell lines. **C,** qRT-PCR was used to verify the interference efficiency of ISOC1in gastric cancer cell lines AGS and SGC after transfection of ISOC1 knockdown vector in. Data were average ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of ISOC1 expression with clinicopathologic characteristics of gastric cancer.

Parameters	Number of cases	ISOC1 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.174
< 60	23	14	9	
≥ 60	33	14	19	
Gender				0.285
Male	28	16	12	
Female	28	12	16	
T stage				0.003
T1-T2	33	22	11	
T3-T4	23	6	17	
Tumor size (cm)				0.005
< 4	36	23	13	
≥ 4	20	5	15	
Lymph node metastasis				0.592
No	30	14	16	
Yes	26	14	12	
Distance metastasis				0.174
No	33	19	14	
Yes	23	9	14	

distantly shifted relationships. As indicated in Table I, high expression of ISOC1 was positively in correlation with GC pathological stage and tumor size, but not with age, gender, lymph node metastasis, and distant metastasis.

Knockdown of ISOC1 Inhibited Cell Proliferation in GC

To further study the effect of ISOC1 on GC, an ISOC1 knockdown cell model was constructed. Then, qRT-PCR experiments were performed to verify the interference efficiency (Figure 1C). CCK-8 cell proliferation experiments revealed that the proliferation rate of GC cells decreased significantly after knocking down ISOC1 (Figure 2A). Moreover, plate cloning and EdU assays demonstrated that the proliferation of GC cells was significantly higher after knocking down ISOC1 (Figure 2B). In addition, the EdU experiment also reflected a remarkable decrease in the number of GC-positive proliferating cells after knocking down ISOC1 (Figure 2C).

ISOC1 Was Bound to CDK19

Bioinformatics suggested that ISOC1 had a direct regulatory effect on CDK19. Subsequently, a Luciferase reporter experiment showed that ISOC1 can directly bind to CDK19 (Figure 3A). In order to further explore the ways in which ISOC1 promoted the malignant progression of GC, the expression level of CDK19 in GC cells after silencing ISOC1 was detected by Western blot, which suggested that silencing of ISOC1 could downregulate the expression of CDK19, thereby inhibiting tumorigenesis of GC (Figure 3B). Furthermore, the level of CDK19 was lower in GC tissues (Figure 3C). Additionally, the expressions of ISOC1 and CDK19 were significantly positively correlated (Figure 3D). Hence, ISOC1 could bind to CDK19.

ISOC1 Modulated CDK19 in GC Cells

To further evaluate the interaction between ISOC1 and CDK19, ISOC1 knockdown vector and CDK19 overexpression vector were transfected in GC cell lines. Western Blot assays suggested that the co-transfection of ISOC1 knockdown vector and CDK19 overexpression vector significantly increased the expression levels of ISOC1 and CDK19 after single transfection of ISOC1 knockdown vector (Figure 4A). Subsequently, through CCK-8 cell proliferation experiments, it was found that overexpressing CDK19 can reverse the effect of ISOC1silencing on GC cell proliferation ability (Figure 4B). In addition, plate cloning experiments and EdU experiments demonstrated that CDK19 overexpression reversed the inhibitory effect of knocking down ISOC1 on the number of GC-positive proliferating cells (Figure 4C and 4D). These data indicated that ISOC1 could indeed modulate CDK19 in human GC cells.

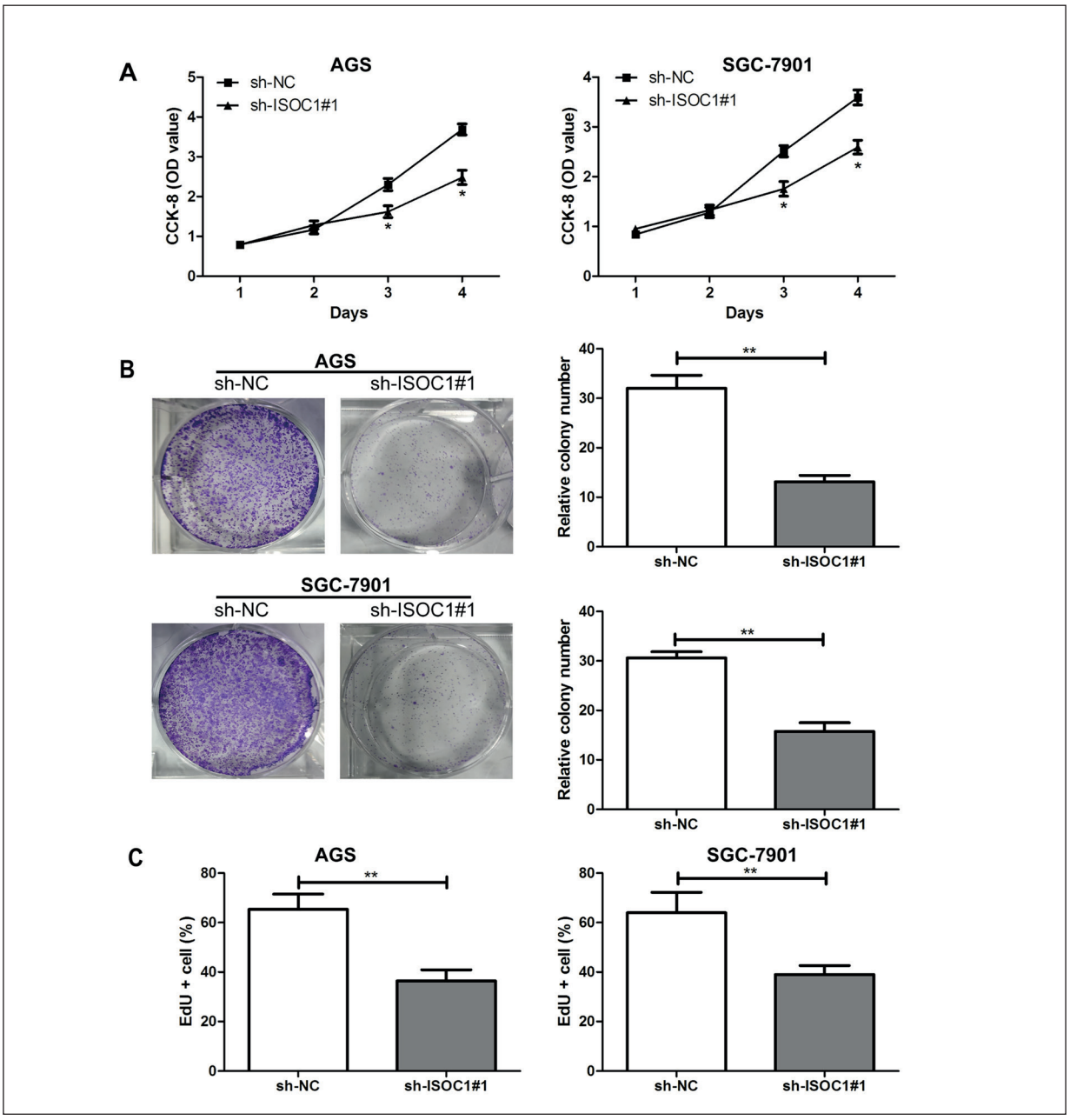

Figure 2. Silencing ISOC1 inhibits gastric cancer cell proliferation. **A,** CCK-8 cell proliferation test was used to detect the effect of gastric cancer cell proliferation ability after knocking down ISOC1 in gastric cancer cell lines AGS and SGC-7901. **B,** Plate cloning test was used to detect the colony forming ability of gastric cancer cells after ISOC1 knockdown in gastric cancer cell lines AGS and SGC-7901 (magnification: 10 ×). **C,** EdU experiments were performed to detect the number of gastric cancer positive proliferating cells after knocking down ISOC1 in gastric cancer cell lines AGS and SGC-7901. Data are average ± SD, *p<0.05, **p<0.01.

Discussion

Gastric cancer is a malignant tumor that threatens the health of residents in China. Although GC treatment and diagnosis have achieved certain results, its morbidity and mortality are still at a high level, which affects the normal life and quality of life of patients[1-3]. The tumorigenesis of tumors are the result of the interaction of multiple factors. Even at different stages of tumor development, the same factors play different roles[6,7]. Environmental risk factors could lead to abnormal gene transcription

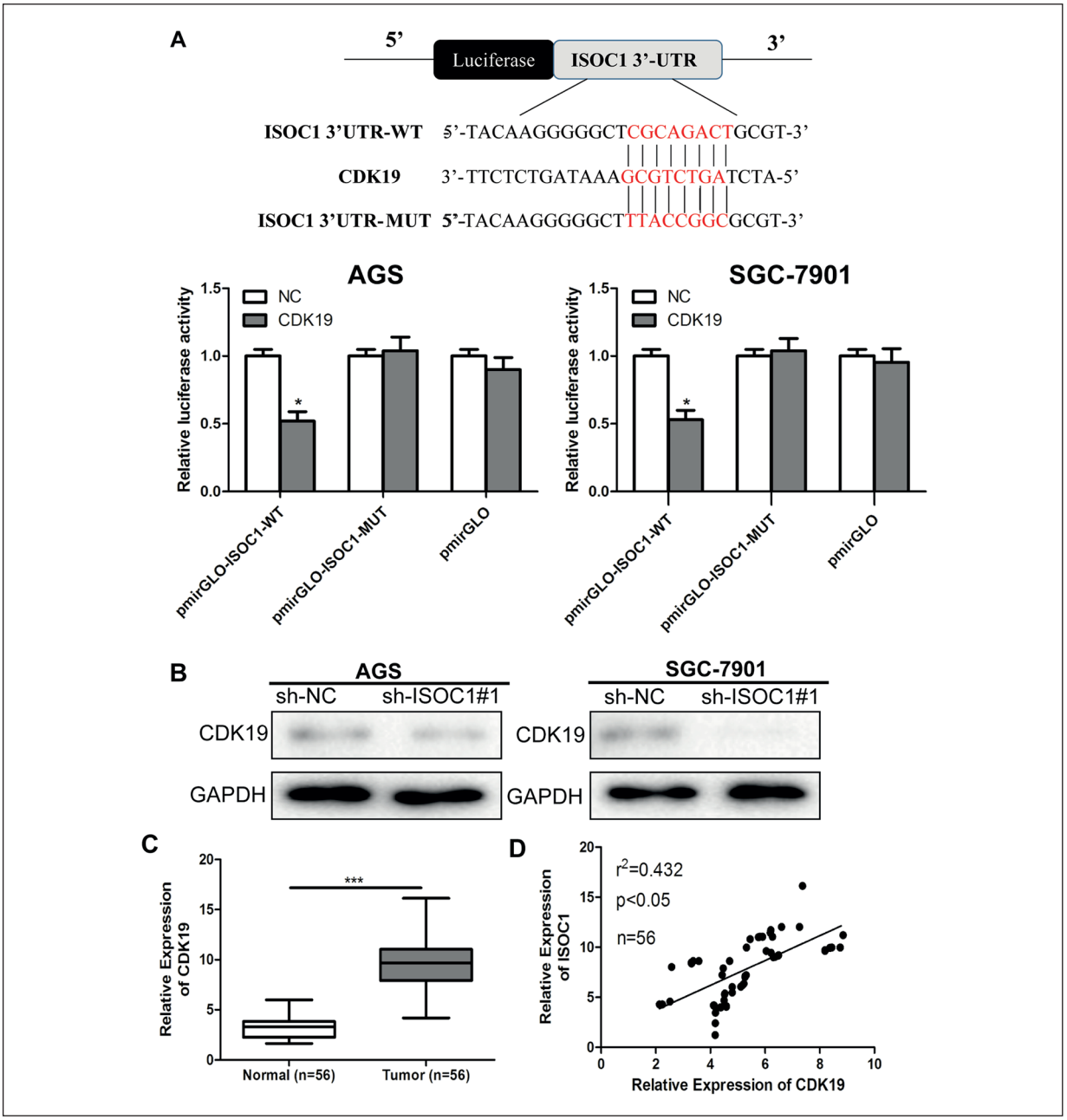

Figure 3. ISOC1 and CDK19 are directly combined. **A,** Bioinformatics and luciferase reporter gene experiments suggested that ISOC1 can directly bind to CDK19. **B,** Western bolt was used to verify the expression level of CDK19 in gastric cancer cells after knocking down ISOC1 in gastric cancer cell lines AGS and SGC-7901. **C,** qRT-PCR was used to detect the expression of CDK19 in gastric cancer and adjacent tissues. **D,** qRT-PCR was used to reveal the correlation between ISOC1 and CDK19 in gastric cancer. The data are average ± SD, *p<0.05, ***p<0.001.

and expression and cause significant changes in various biological functions and even pathological changes in the body[7-9]. At present, many reports have shown that gene expression, transcription, and protein expression levels function in the pathogenesis of GC. Research and regulation of these genes has a very important role for understanding the tumorigenesis of GC. At the same time, these regulatory genes may also be potential therapeutic targets, thus providing theoretical support for GC[10-13].

It has been suggested that ISOC1 is closely related to the tumorigenesis of GC. A variety of dysregulated expressions of ISOC1 have been found in tissue specimens and plasma specimens

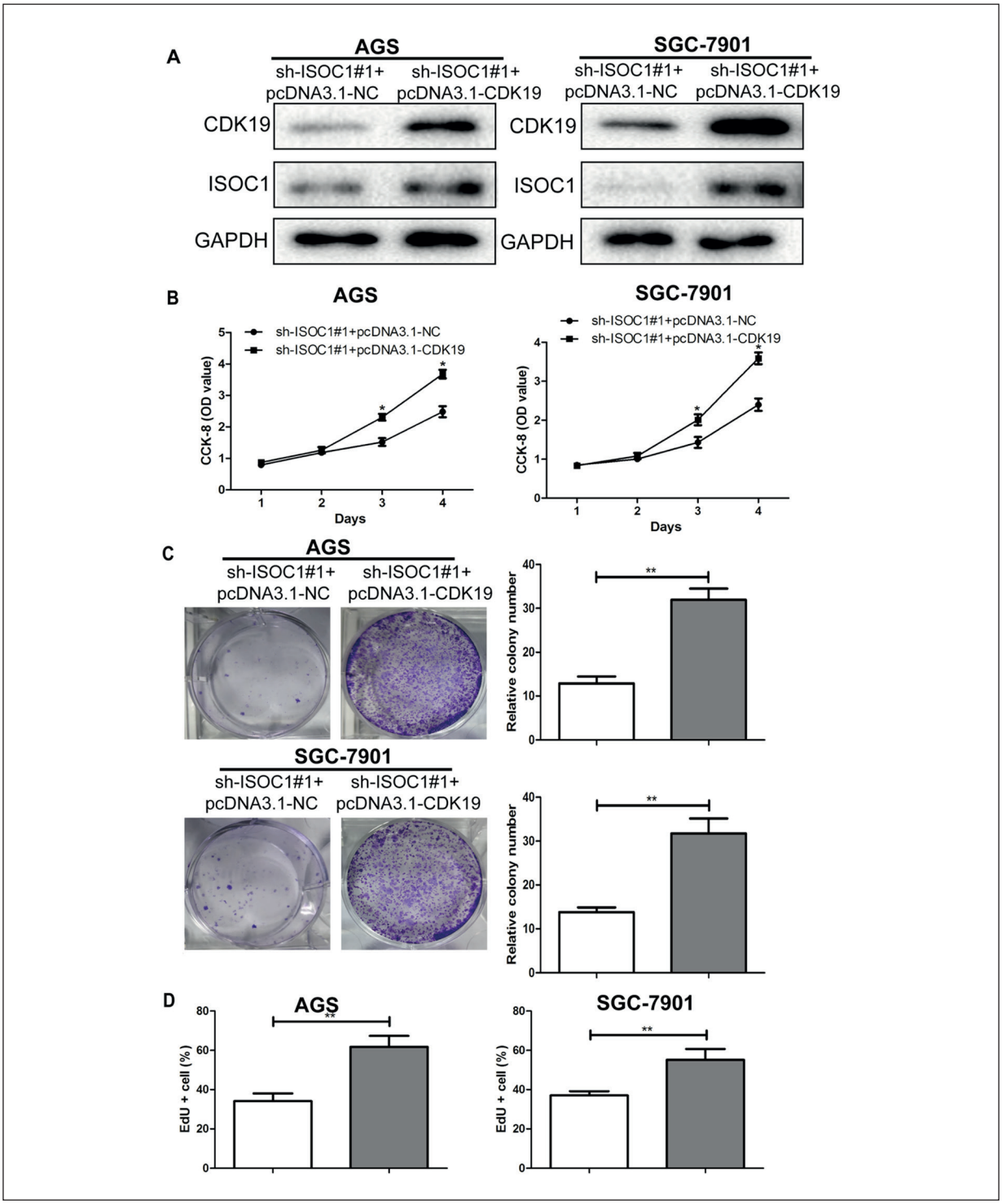

Figure 4. ISOC1 regulates CDK19 to promote gastric cancer cell proliferation. **A,** Western bolt was used to detect the expression levels of ISOC1 and CDK19 after transfection of ISOC1 knockdown vector and CDK19 overexpression vector in gastric cancer cell lines AGS and SGC-7901, respectively. **B,** CCK-8 cell proliferation assay was used to assess the cell proliferation ability after transfection with ISOC1 knockdown vector and CDK19 overexpression vector in SGC-7901 and SGC-7901 respectively. **C,** Plate cloning test was used to measure the cell colony forming ability after transfection of ISOC1 knockdown vector and CDK19 overexpression vector in gastric cancer cell lines AGS and SGC-7901 (magnification: 10 ×). **D,** EdU test was used to show the number of gastric cancer positive proliferating cells after transfection of ISOC1 knockdown vector and CDK19 overexpression vector in gastric cancer cell lines AGS and SGC-7901, respectively. Data are average ± SD, *$p<0.05$, **$p<0.01$.

of various tumor patients[14,15]. The isoenzyme domain of ISOC1 may have putative allomerase activity, which is catalyzed by hydrolysis to convert the isomer to 2,3-dihydroxy-2,3-dihydroxybenzoate and pyruvate[14]. The specific function of ISOC1 in human tumors that not been fully elucidated except for its putative allele activity[15]. However, there are few reports that ISOC1 is related to the malignant progress of GC. Therefore, this objective of this study was firstly to elucidate the oncogenic role of ISOC1 in the progression of GC, as well as the specific mechanism of ISOC1 regulating CDK19. Here, we proved that ISOC1 was remarkably increased in GC tissues, and overexpression of ISOC1 was positively in correlation with the pathological stage and tumor size of GC patients, which was shown that upregulation of ISOC1 expression could promote malignant progression of GC. Moreover, *in vitro* experiments demonstrated that the cell proliferation and colony-forming ability of GC cell lines were significantly weakened after ISOC1 knockdown. Bioinformatics suggested that ISOC1 had a direct regulatory effect on CDK19. Subsequently, Luciferase reporter gene experiments suggested that ISOC1 can directly bind to CDK19. In addition, qRT-PCR experiments showed that ISOC1 and CDK19 were significantly positively correlated in GC tissues. Furthermore, ISOC1 silencing can markedly downregulate CDK19 level in GC cells, thereby inhibiting the development of GC. Cell recovery experiments suggested that CDK19 overexpression can reverse the effect of ISOC1 silencing on GC cell proliferation. This evidence indicates that ISOC1 can promote cell proliferation in GC by modulating CDK19.

Conclusions

The expression of ISOC1 is highly expressed in GC tissues. ISOC1 could positively regulate its downstream CDK19, which in turn promotes the GC cell proliferation. Our study will provide new ideas and therapeutics for GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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