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KLF5 promotes proliferation in gastric cancer *via* regulating p21 and CDK4

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Abstract. – OBJECTIVE: This study aims to investigate the expression characteristics of Krüppel-like factor 5 (KLF5) in gastric cancer (GC) and its potential correlation to pathological indexes in GC patients. Molecular mechanisms underlying the regulatory effect of KLF5 on GC progression are explored.

PATIENTS AND METHODS: KLF5 expressions in GC tissues were analyzed through GEPIA dataset and those collected in our hospital. By analyzing the clinical data of GC patients, the correlation between KLF5 level and pathological characteristics of GC was determined. The regulatory effects of KLF5 on proliferative ability and cell cycle progression of SGC7901 and MGC803 cells were assessed by Cell Counting Kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU) assay, and flow cytometry. The regulation of KLF5 on expression levels of p21 and CDK4 in GC cells was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot.

RESULTS: KLF5 was markedly upregulated in GC tissues. KLF5 level was positively correlated to TNM staging, tumor size, and metastasis of GC. Meanwhile, high level of KLF5 predicted poor prognosis in GC patients. The knockdown of KLF5 in SGC7901 and MGC803 cells attenuated proliferative ability and arrested cell cycle in G0/G1 phase. Both mRNA and the protein levels of p21 were upregulated, and CDK4 levels were downregulated in SGC7901 and MGC803 cells transfected with si-KLF5.

CONCLUSIONS: KLF5 is upregulated in GC and closely linked to pathological characteristics of GC patients. High level of KLF5 indicates poor prognosis of GC. It is believed that KLF5 may be a potential therapeutic target for GC.

Key Words:
Gastric cancer, KLF5, Proliferation, p21, CDK4.

Introduction

Gastric cancer (GC) is an important public health problem that costs large amounts of medical resources. The occurrence and development of GC are complex pathological processes involving host factors, *Helicobacter pylori* infection, and environmental factors[1,2]. Normal gastric mucosa undergoes a progressive series of pathological injuries from gastritis, atrophy, intestinal metaplasia and dysplasia, eventually leading to the tumorigenesis[3,4]. Uncontrolled proliferation and metastasis of tumor cells are two vital features of GC[5]. Therefore, the suppression of these two phenotypes in tumor cells is able to effectively alleviate the tumor progression of GC[6]. It is necessary to clarify the mechanisms underlying the pathogenesis of GC.

Krüppel-like factors (KLFs) are subfamily of zinc finger proteins. Gene alteration of KLFs can inhibit the development of Drosophila embryos. KLFs are DNA-binding regulators with a DNA-binding region located at the carboxy terminus. This region contains 81 amino acids and is composed of three zinc finger structures. It is proved that a zinc finger structure is composed of highly conserved cysteine and histidine. Seven conserved amino acids are responsible for connecting the zinc finger structure, and such a structure participates in the protein-protein interaction[7]. The amino terminus of the KLFs contains a transcriptional activation or repression domain. A nuclear localization signal exists in this domain within or near the zinc finger structure[8]. KLF5, as a member of the KLF family, is involved in the cellular behavior regulation, organ injury repair,

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and tumor progression[9,10]. Previous authors[11-14] have demonstrated the role of KLF5 in many types of tumors.

In this study, KLF5 was found to be upregulated in GC tissues by analyzing GEPIA dataset. We further analyzed the regulatory effects of KLF5 on cellular behaviors of GC and the underlying mechanism.

Patients and Methods

GEPIA Database

Differential expressions of KLF5 in GC tissues and normal tissues were assessed by analyzing GEPIA database (http://GEPIA.cancer-pku.cn/index.html), an online database containing the gene sequencing data. Here, the dataset downloaded from GEPIA contained 408 GC tissues and 211 normal tissues.

Sample Collection

Paired GC tissues and adjacent normal tissues were surgically resected from 50 GC patients admitted in The First Affiliated Hospital of Zhengzhou University. All samples were pathologically confirmed and preserved in liquid nitrogen. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of The First Affiliated Hospital of Zhengzhou University. Baseline characteristics of the enrolled patients were listed in Table I.

Cell Culture

Gastric epithelial cells (GES1) and GC cells (AGS, BGC823, SGC7901, and MGC803) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. Medium was regularly replaced. Cell passage was conducted at 80-90% confluence.

Transfection

The cells were inoculated in a 6-well plate with 3×10^3 cells per well and cultured at 70% confluence. The cells were transfected using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, the cells were collected for functional experiments. KLF5 siRNAs were provided by GenePharma (Shanghai, China).

Table I. Correlation between KLF5 expression and clinicopathological characteristics of gastric cancer cohort (n=50).

Clinical parameter		KLF5 expression		χ^2-test *p*-value
		High (n = 36)	Low (n = 14)	
Gender				0.862
	Male	26	9	
	Female	10	5	
Age (years)				0.625
	< 50	12	4	
	> 50	24	10	
Tumor size				0.001
	< 5 cm	9	9	
	≥ 5 cm	27	5	
Invasion depth				0.005
	T1	2	4	
	T2	8	4	
	T3	13	3	
	T4	13	2	
Lymphatic metastasis				0.02
	Yes	28	7	
	No	8	7	
TNM stages				0.01
	I	5	6	
	II	7	5	
	III	24	3	

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The cells were lysed to harvest RNAs using TRIzol method (Invitrogen, Carlsbad, CA, USA), and the extracted RNAs were reversely transcribed following the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). Concentration and purity of RNA were determined using a spectrometer, and qualified RNA was applied for qRT-PCR using the SYBR Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. The relative level was calculated using $2^{-\Delta\Delta CT}$ method. The primer sequences were listed in Table II.

Cell Counting Kit-8 (CCK-8) Assay

The cells were seeded into 96-well plates with 3×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo, Molecular Technologies, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The cells were seeded into 96-well plates with 3×10^3 cells per well. The cells were labeled with 50 μM EdU for 2 h, dyed with AdoLo and Hoechst33342 in the dark for 30 min. EdU-positive rate was calculated as the ratio of EdU-positive cells (red) and Hoechst33342-stained cells (blue) under a fluorescent microscope.

Flow Cytometry

The cells were washed with phosphate-buffered saline (PBS) twice, digested, and prepared to cell suspension with 1×10^5/mL. The cells were incubated in 1 mL of pre-cold 70% ethanol at 4°C overnight. On the other day, the cells were washed with PBS twice and incubated with 100 μL of RNaseA at 37°C in the dark for 30-min water bath. Subsequently, the cells were dyed with 500 μL of Propidium Iodide (PI) at 4°C, in the dark for 15 min. At last, the cells in different cell cycle phases were determined at 488 nm wavelength.

Table II. The sequences related to the study.

Gene	Sequences
p21 F:	TGTCCGTCAGAACCCATGC
p21 R:	AAAGTCGAAGTTCCATCGCTC
KLF5 F:	ACACCAGACCGCAGCTCCA
KLF5 R:	TCCATTGCTGCTGTCTGATTTGTAG
GAPDH F:	AGCCACATCGCTCAGACAC
GAPDH	GCCCAATACGACCAAATCC
CDK4 F:	ATGGCTACCTCTCGATATGAGC
CDK4 R:	CATTGGGGACTCTCACACTCT

Western Blot

The cellular protein was extracted to determine the protein concentration. The protein sample was loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked in 5% skim milk for 1 hour. The membranes were reacted with primary and secondary antibodies. After washing with 1×Tris-Buffered Saline and Tween 20 (TBST) for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x}$ ± SD). Kaplan-Meier method was introduced for survival analysis. The data between the two groups were compared using the *t*-test. $p<0.05$ showed that the difference was statistically significant.

Results

KLF5 Was Upregulated in GC

By analyzing the dataset downloaded from GEPIA, KLF5 was found to be upregulated in GC tissues than that of normal ones (Figure 1A). Similarly, KLF5 was also highly expressed in GC tissues collected from our hospital relative to adjacent normal ones (Figure 1B). The above data indicated the potential involvement of KLF5 in GC progression.

Correlation Between KLF5 Level and Pathological Characteristics of GC

Pathological characteristics of the enrolled GC patients were collected. No significant difference in KLF5 level was identified in GC patients younger than 50 years and those older than 50 years (Figure 2A). Higher levels of KLF5 were observed in GC patients with larger

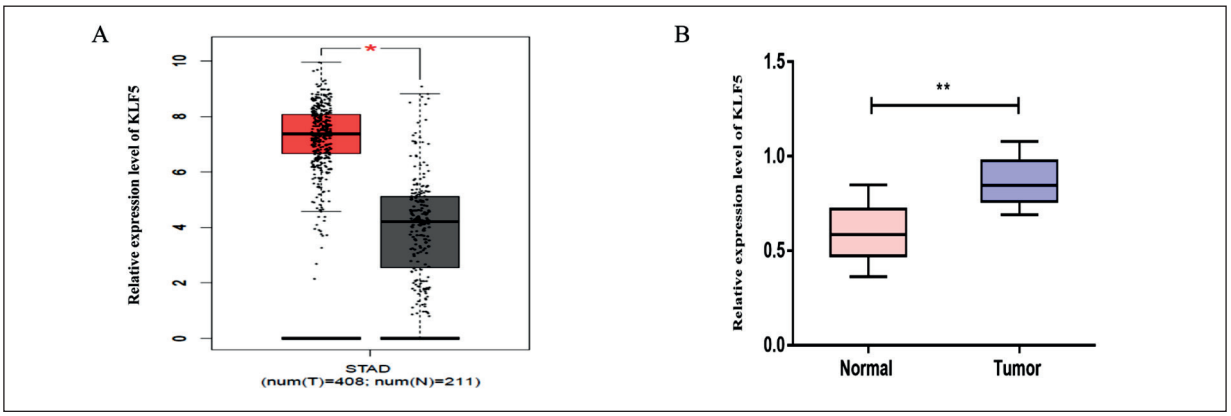

Figure 1. KLF5 was upregulated in GC. **A,** KLF5 levels in GC tissues (n=408) and normal tissues (n=211) analyzed in GEPIA dataset. **B,** KLF5 levels in GC tissues and adjacent normal ones collected in our hospital. **p*<0.05, ***p*<0.01.

tumor size (≥ 5 cm), TNM stage T3+T4, combination of metastasis foci or tumor grading III relative to those with smaller tumor size (<5 cm), T1+T2, no metastasis foci or tumor grading I+II (Figure 2B-2E). Furthermore, Kaplan-Meier curves depicted that the high level of KLF5 indicated worse prognosis in GC patients (Figure 2F).

Knockdown of KLF5 Alleviated Proliferative Ability and Arrested Cell Cycle in GC Cells

Subsequently, *in vitro* levels of KLF5 in the GC cell lines were detected. Compared with gastric epithelial cells, KLF5 was upregulated in GC cells (Figure 3A). Three KLF5 siRNAs were constructed (KLF5 siRNA-1, KLF5 siRNA-2, and KLF5

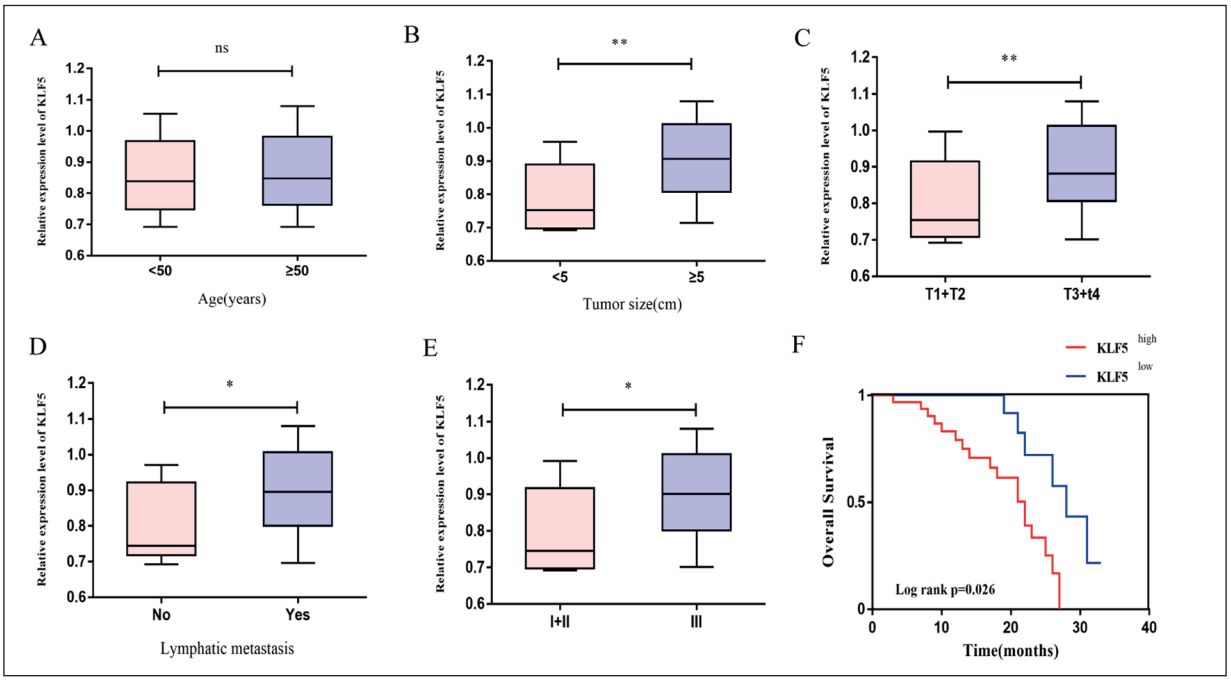

Figure 2. Correlation between KLF5 level and pathological characteristics of GC. **A,** KLF5 levels in GC patients younger or older than 50 years. **B,** KLF5 levels in GC tissues smaller or larger than 5 cm in tumor size. **C,** KLF5 levels in GC tissues with T1+T2 or T3+T4. **D,** KLF5 levels in GC patients either with lymphatic metastasis or not. **E,** KLF5 levels in GC tissues with tumor grading I+II or grading III. **F,** Kaplan-Meier curves indicated overall survival in GC patients with high or low level of KLF5. **p*<0.05, ***p*<0.01, ns: no significant difference.

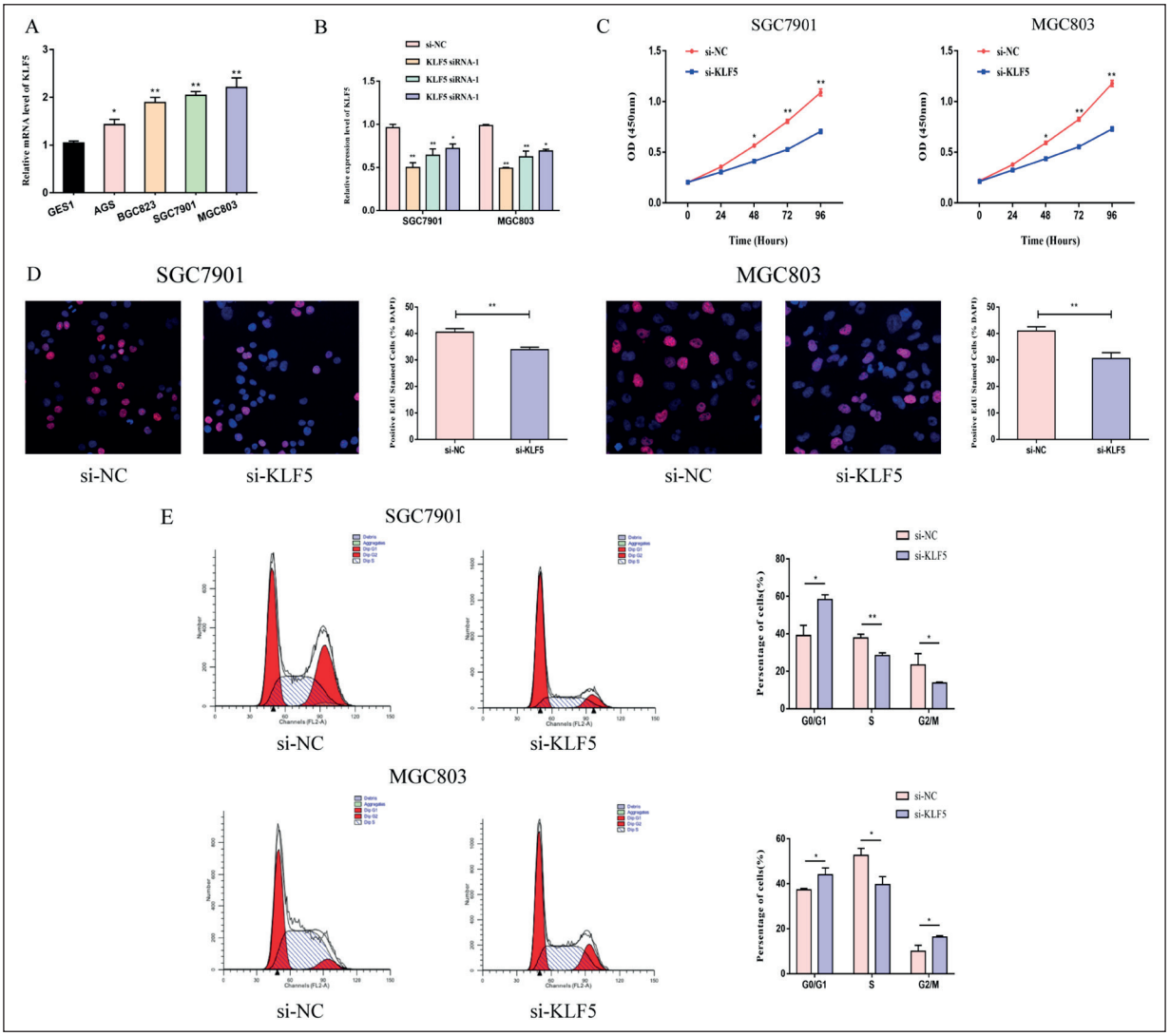

Figure 3. Knockdown of KLF5 alleviated proliferative ability and arrested cell cycle in GC cells. **A,** KLF5 levels in gastric epithelial cells (GES1) and GC cells (AGS, BGC823, SGC7901, and MGC803). **B,** Transfection efficacies of KLF5 siRNA-1, KLF5 siRNA-2 and KLF5 siRNA-3 in SGC7901 and MGC803 cells. **C,** Viability in SGC7901 and MGC803 cells transfected with si-NC or si-KLF5. **D,** EdU-positive ratio in SGC7901 and MGC803 cells transfected with si-NC or si-KLF5 (magnification: 100×). **E,** Cell ratio in G0/G1, S and G2/M phase in SGC7901 cells transfected with si-NC or si-KLF5. *p<0.05, **p<0.01.

siRNA-3), and transfection efficacy of KLF5 siRNA-1 was the mostly pronounced (Figure 3B). CCK-8 and EdU assay both revealed that the knockdown of KLF5 reduced the viability and EdU-positive ratio in SGC7901 and MGC803 cells (Figure 3C, 3D). Flow cytometry uncovered the elevated cell ratio in G0/G1 phase in SGC7901 cells transfected with KLF5 siRNA-1 (Figure 3E).

KLF5 Accelerated GC to Proliferate by Regulating Cell Cycle Genes

The cell cycle genes were detected after transfection of si-KLF5. Both mRNA and protein levels of p21 were upregulated in SGC7901 and MGC803 cells. Meanwhile, CDK4 was downregulated in GC cells with KLF5 knockdown (Figure 4A, 4B).

Discussion

The occurrence of GC is a complex process involving multiple steps, pathogenic factors, and genes. Abnormal accumulation of the gene expression products and macromolecules, activation of proto-oncogenes, silence of tumor-suppressor

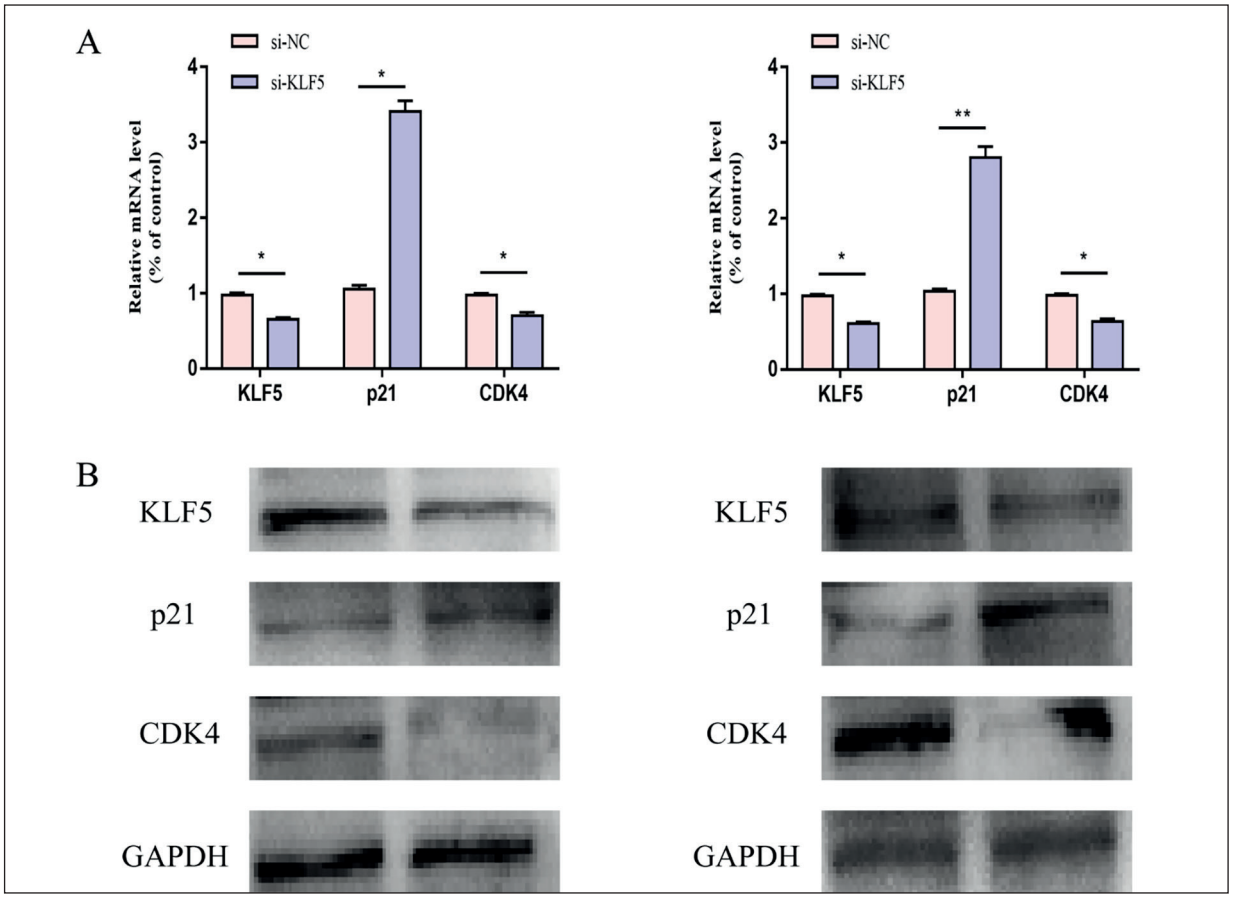

Figure 4. KLF5 accelerated GC to proliferate through regulating cell cycle-associated genes. **A, B,** The mRNA (**A**) and protein (**B**) levels of KLF5, p21 and CDK4 in SGC7901 and MGC803 cells transfected with si-NC or si-KLF5. **p*<0.05, ***p*<0.01.

genes, and inactivation of DNA repair genes, and cell adhesion factors are all key events in tumor molecular biology[15]. According to the different sources and mechanisms, these pathogenic factors are classified into endogenous and exogenous factors[16]. The former ones are from the external environment, including physical factors, chemical factors, tumorigenic viruses, mycotoxins, etc. The latter include genetic mutations, immune status, hormone levels, DNA repair ability, etc.[17]. Researches on tumor biology at genetic level have been well concerned.

The KLFs family is a class of zinc finger protein transcription factors binding to sequences with high GC content, serving as an oncogenic or anti-tumor effect. KLFs exert extensive and critical roles in tumor proliferation and differentiation by targeting the downstream genes[18,19]. Some studies have shown the involvement of KLFs in tumor progression. KLF13 inhibits the growth of prostate cancer cells by inactivating AKT[20]. KLF8 promotes Temozolomide resistance in glioma by activating β-catenin[21]. In colorectal cancer, KLF8 accelerates tumor growth and metastasis by activating FHL2 transcription[22]. MiRNA-9 is able to enhance metastatic ability in liver cancer by targeting KLF17[23].

KLF5 is mainly expressed in intestinal epithelial cells, also known as intestinal-enriched Krüppel-like factor (IKLF), and basic transcription element binding protein 2 (BTEB2). It exerts a positive regulatory role in cell proliferation and tumor formation[24]. The KLF5 gene is located at 13q21, spans 18.5 kb and contains four exons. The cDNA of KLF5 has 3,350 bp long, which spans 324 bp in the 5' end, and 1652 bp in the 3' end[25]. The DNA binding domain of KLF5 gene is located at the carboxyl end and contains three C2H2 zinc finger structures composed of highly conserved amino acids. It binds to CACCC or GT

elements in the promoter region of KLF5 gene and then, regulates the transcription of the downstream genes[26].

In this paper, KLF5 was markedly upregulated in GC tissues. KLF5 level was positively correlated to TNM staging, tumor size, and metastasis of GC. Meanwhile, high level of KLF5 predicted poor prognosis in GC patients. *In vitro* experiments demonstrated that the silence of KLF5 in GC attenuated proliferative ability and arrested cell cycle in G0/G1 phase. Moreover, cell cycle genes p21 and CDK4 were regulated by KLF5 in GC cells. It is believed that KLF5 was able to promote proliferation in GC by arresting cell cycle progression.

Conclusions

Collectively, these data showed that KLF5 is upregulated in GC and closely linked to the pathological characteristics of GC patients. High level of KLF5 indicates poor prognosis of GC. It is believed that KLF5 may be a potential therapeutic target for GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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