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KLF11 promotes gastric cancer invasion and migration by increasing Twist1 expression

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Gastric cancer (GC) is a leading cause of global cancer-related death. The incidence and mortality rates of gastric cancer in China are second and third ranked in all forms of malignant tumors. Krüppel-like factor11 (KLF11) is a member of the KLF family, and previous studies have shown it significantly influences epithelial ovarian, pancreatic and liver cancer proliferation, differentiation and apoptosis. However, the expression and some biological functions of KLF11 in GC are still unclear. We therefore collected and analyzed the mRNA and protein expressions of KLF11 in 59 paired gastric cancer tissues and matched healthy gastric tissue samples. We then investigated the KLF 11 biological functions and potential mechanisms in BGC823 and HGC27 gastric cancer cell lines. Analysis of KLF11 in gastric cancer specimens confirmed up-regulation compared to adjacent healthy gastric tissues, and similar results were evident in the GC cell lines. Ectopic expression of KLF11 was significantly associated with GC cell invasion and migration. KLF11 functions were most effective in Twist1 expression and knockdown, and also in KLF11 up-regulation which was accompanied by corresponding change in Twist1 expression; but these effects were inhibited when KLF11 was silenced by the small interfering RNA (siRNA). The relative Twist1 promoter region activity increased gradually with increasing KLF11 plasma, and KLF11 therefore has a critical role in regulating gastric cancer migration and invasion by increasing Twist1 expression. Finally, the results of this study should improve understanding of the KLF11 and EMT regulating network and KLF11's use as a potential therapeutic target in gastric cancer.

Key words: KLF11, gastric cancer, cell invasion, cell migration, Twist1

Gastric cancer is a very common malignant tumor [1]. According to statistics, there are about 1 million new cases and 800,000 people in the world die from GC every year [2]. In China, the incidence and mortality rates of gastric cancer occupy the second and third position in all forms of malignant tumors in both sexes [3]. Furthermore, the incidence rate of GC is still on the rise. Despite improved GC therapeutic strategies, the prognosis of advanced GC patients remains unsatisfactory; with the 5-year survival rate less than 30% [4]. This poor diagnosis rate is due to the atypical clinical symptoms of the GC patients and the low sensitivity of the markers. Therefore, finding novel sensitive markers and therapeutic targets is essential.

Epithelial-to-mesenchymal transition (EMT) is a hallmark of cancer and it attracts great attention because of its pivotal role in facilitating cancer cell invasion and metastasis [5, 6]. Suppression of E-cadherin is the most important symbol of EMT, and this not only breaks cell-cell adhesions but also promotes cellular migration and invasion [6]. In addition to the above abilities, EMT also enables cancer cells to obtain the characteristics of stem cells, including self-renewal and resistance to apoptosis [7, 8]. Previous studies have shown that transcription factors have a most important role in EMT progression [9], and Snail and Twist are the two most commonly associated with EMT. Some studies have demonstrated that Snail and Twist restrain the expression of E-cadherin by combining with the CDH1 promoter and thus aid the EMT process [10–12]. However, EMT regulation is most complex because Twist1 also affects the EMT process by regulating Snail2 [13].

KLF is a highly conserved family of zinc finger transcription factors [14] associated with cellular proliferation, differentiation, migration, invasion, apoptosis, cardiovascular disease and metabolic disorders [15]. KLF11 is a member of this family and contains three C_2H_2-type zinc fingers, 2 acids and 2 regions rich in proline at the carboxyl and amino

terminals [16]. Previous studies have shown that KLF11 has significant influence on proliferation, differentiation and apoptosis in epithelial ovarian, pancreatic and liver cancer [17–20]. While KLF11 promotes local invasion and distant migration in the EMT process in hepatocellular carcinoma [21], its biological functions and relationship with EMT in gastric cancer are not clarified.

Patients and methods

Clinical samples. A total of 59 paired gastric cancer tissues and the matched healthy gastric tissue samples from at least 5cm from the tumor edge were frozen in liquid nitrogen. All samples were obtained from the Fourth Affiliated Hospital of Hebei Medical University. No patients had received chemotherapy or radiotherapy before operation.

Cell lines and cell culture. Human gastric cancer cell lines MKN28, MGC803, SGC7901, MKN45, BGC823, HGC27 and GES1 were obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum(FBS) (Excell, USA) and 1% streptomycin/penicillin in a humidified atmosphere containing 5% CO_2 at 37 °C (Thermal, USA). GES1 is one of the normal gastric epithelial cell lines.

RNA extraction, reverse transcription and qRT-PCR. Total RNA was extracted from tissue samples or cells with TRNzol universal reagent (TIANGEN, China). After measuring the concentration, 3 μg of the total RNA was reverse-transcribed using the A5001 GoScript Reverse Transcription System (Promega, USA). Real time PCR reactions were conducted by A6001 GoTaq(R) qRTPCR Master Mix (Promega, USA) on the ABI PRISM 7500 sequence detection system (Applied Biosystems). All reactions were set with three sub-holes. The GAPDH was used as the endogenous controls and the relative expression levels of KLF11were calculated using the $2^{-\Delta\Delta CT}$ equation. The primer of KLF11 was designed by and purchased from Sangon Biotech (Shanghai). The primer sequences were:
KLF11 F: 5'-GGGATGTCACCACCACTGT-3', R:5'-GGCTCTGAGGAGGAGTTATGC-3'.
GAPDH F: 5'-AATCCCATCACCATCTTCCAG-3', R: 5'-CCTTCTCCATGGTGGTGAAGAC-3'.

Protein extraction and western blot. Total proteins were extracted from clinical samples and all cell lines by RIPA lysis buffer (Solarbio, China) and 1× protease inhibitor cocktail (Roche, Mannheim, Germany). Bicinchoninic acid (BCA) protein assay (Thermal, USA) was used to measure the concentrations of proteins according to the manufacturer's instructions. The proteins were separated by 10–12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes for 2 h (Millipore, Billerica, MA). The membranes were blocked in 5% fat-free milk for 1h at room temperature and incubated with primary antibodies at 4 °C overnight. The next day, the membrane was incubated with the secondary antibody for 1h at room temperature and in the dark. Bands on the membrane were detected by Odyssey system (LI-COR Biosciences, USA) and β-actin was the endogenous control. all experiments were in triplicate.

Cell transfection, 5×10^5 BGC823 or HGC27cells were seeded into a 6-well plate per well for 24 h prior to transfection and the 6-well plate was cultured in humidified atmosphere with 2 ml RPMI-1640 overnight. When the cells grew to 70–80% confluence, the KLF11-siRNA or pcDNA-KLF11 was transfected into cells with Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions. The control group was set in each group. Then qRT-PCR and Western blot confirmed transfection efficiency. After 24h or 48h of transfection, cells were collected for following experiments. siRNAs and pcDNA plasmids were designed by and purchased from Ribo Bio (Guangzhou, China).

Wound healing assay. Cells were seeded in 6-well plates (5×10^5 cells/well). After transfection, when the cells confluence surpassed 80%, the cell layer was wounded gently by

Table 1. Relationships between KLF11 expression and clinical pathological data.

	KLF11 expression		χ^2	p-value
	High (46)	Low (13)		
Age (years)				
<55	19	5	0.034	0.854
≥55	27	8		
Gender				
Male	33	10	0.138	0.71
Female	13	3		
Tumor size(cm)				
<5	14	10	9.077	0.003*
≥5	32	3		
Tumor infiltration				
T1	3	2	10.135	0.017*
T2	5	4		
T3	5	4		
T4	33	3		
Regional lymph node metastasis				
N0	5	4	8.076	0.044*
N1	9	5		
N2	11	3		
N3	21	1		
Distant metastasis				
M0	44	13	0.585	0.444
M1	2	0		
TNM staging				
I	3	3	17.541	0.001*
II	7	8		
III	34	2		
IV	2	0		

T1 includes T1a and T1b, T4 includes T4a and T4b. N3 includes N3a and N3b. TNM staging I includes Ia and Ib, II includes IIa and IIb, III includes IIIa, IIIb and IIIc. *p<0.05

a 0.2 ml micropipette tip across the diameter of the well. Then, after washing twice with 1×PBS, cells were cultured with serum-free medium containing 1mM mitomycin which was used to inhibit cell division. Pictures were captured by inverted microscope at different time points after the scratching.

Transwell invasion assay. The invasion of GC cell lines was measured by 24-well Transwell chambers (Corning Costar, Cambridge, MA, USA). The upper chambers were pretreated with a thin layer of Matrigel (BD Biosciences, USA). 1×10^5 cells were re-suspended in serum-free medium and seeded in the upper chambers. Meanwhile, in the lower chamber, 600 µl RPMI-1640 with 10% FBS was supplemented and acted as the attractant. After incubation of 20 h at 37 °C, cells on the upper sides were wiped by a cotton swab, and the cells on the lower portion were stained with 2% crystal violet for 10 min. The numbers of stained cells in six random fields were counted under the inverted microscope and used for analysis.

Luciferase reporter assay. We established a luciferase reporter gene plasmid of Twist1 with the 2 kb sequence upstream of the promoter region. When the cells grew to 70% confluence, pGL3-Twist1-luc was co-transfected into cells along with different dose pcDNA-KLF11 (0.1, 0.2, 0.4, and 0.8 µg) and the pRL-TK was used as the reference. At 48 h after transfection, the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) measured the luciferase activity and this is presented as mean ± SD. The above experiments were in triplicate.

Statistical analysis. The statistical software SPSS21.0 processed experimental data. All quantitative data is presented as the mean ± SD. Comparisons between different groups was by one-way analysis of variance and t test. Chi-square analysis analyzed categorical variables and two tailed p-values <0.05 were statistically significant.

Results

KLF11 is up-regulated in GC tissues and various GC cell lines. The expressions of KLF11 were detected via qRT-PCR and WB in 59 paired gastric cancer tissues and the adjacent healthy gastric tissue samples. KLF11 was up-regulated in 46 GC tissues compared to matched healthy gastric tissues (Figures 1A, B). From HGC27, BGE823, MKN45, SGC7901, MGC803, MKN28 and GES1 groups, HGC27 had the highest KLF11expression, followed by GC cell lines BGC823,

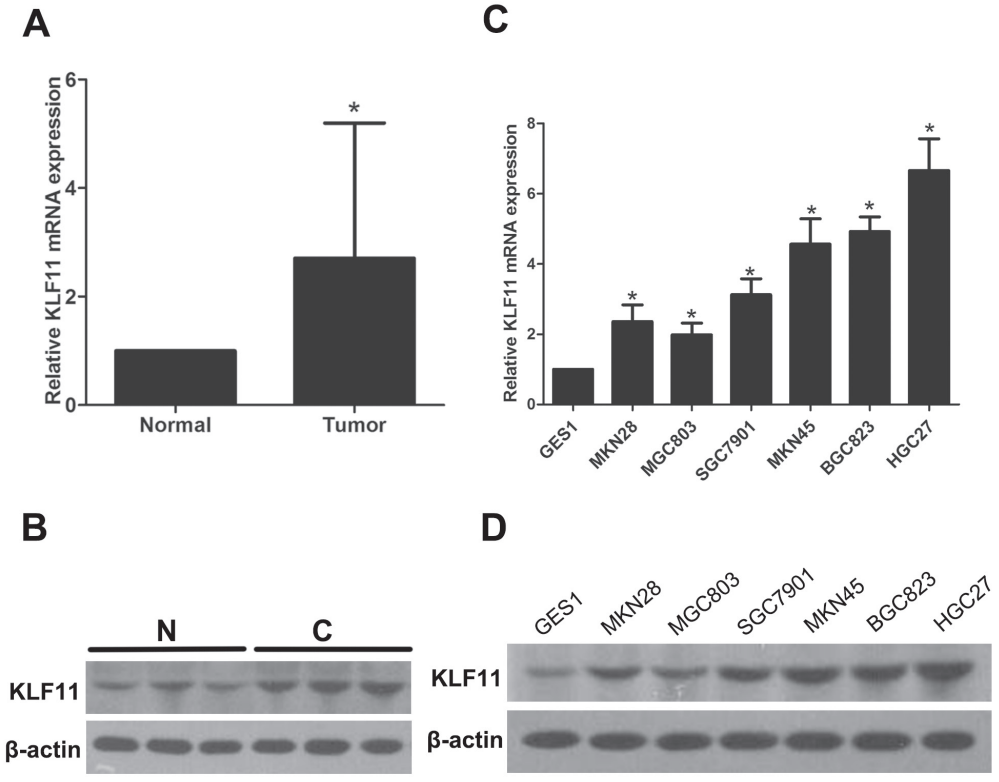

Figure 1. KLF11 expression in GC clinical tissues and various cell lines. A) The mRNA expression levels of KLF11 in 59 paired gastric cancer tissues were assessed by qRT-PCR. B) The protein expressions of KLF11 in GC tissues (C) and the paired para-carcinoma tissues (N) were shown by WB. C) The mRNA and protein expression levels of KLF11 in various cell lines were tested by qRT-PCR and WB. *p<0.05

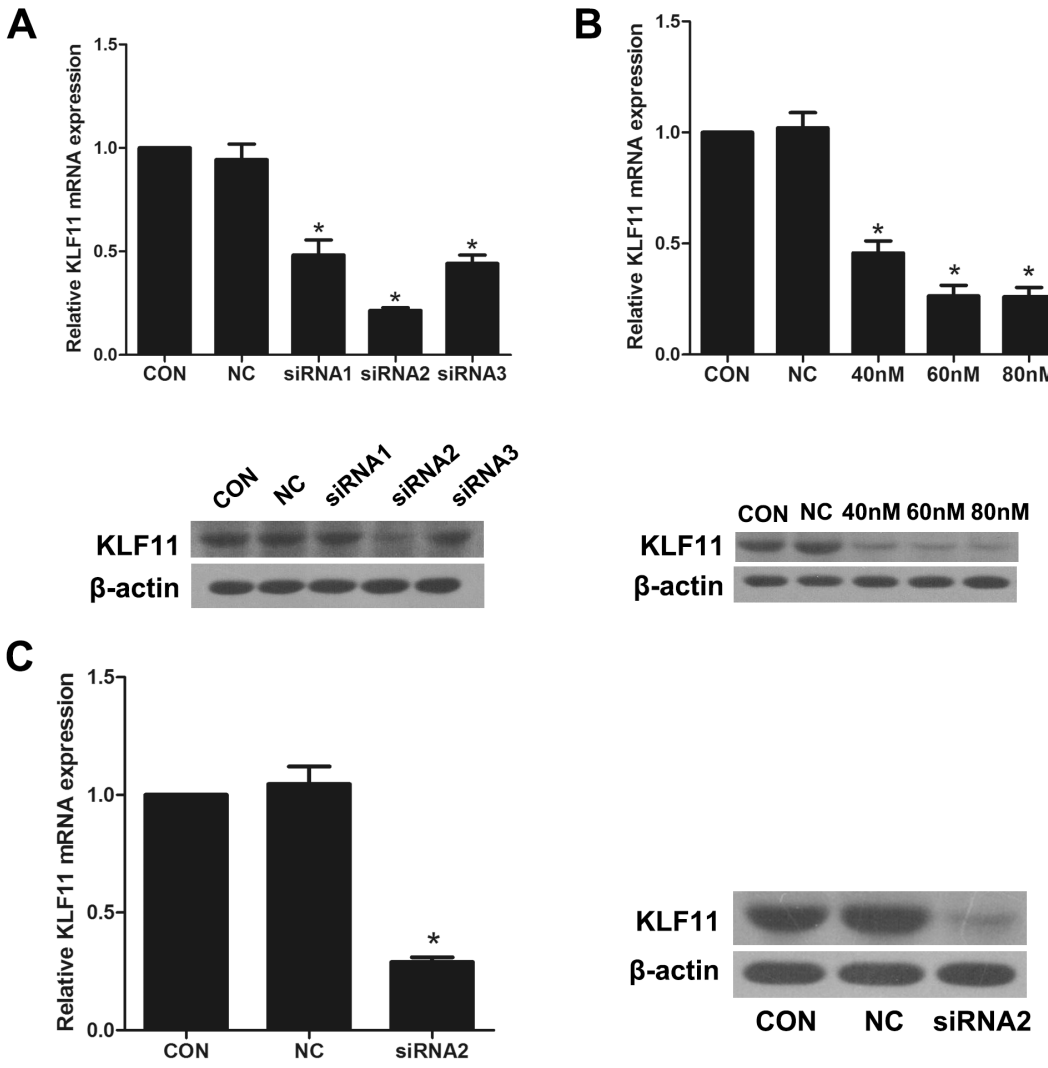

Figure 2. Knockdown of KLF11 in BGC823 cells. A) qRT-PCR and WB assessed the BGC823 cell mRNA and protein levels. These cells were transfected by KLF11-siRNA1, KLF11-siRNA2, KLF11-siRNA3 and NC-siRNA at the concentration of 60 nM. B) The BGC823 cell was transfected by different doses of KLF11-siRNA2 at 40 nM, 60 nM, and 80 nM, and the NC group with 60 nM concentration. C) The mRNA and protein levels of KLF11 were measured when KLF11-siRNA2 was transfected into the HGC27 cell. *p<0.05

MKN45, SGC7901, MGC803, MKN28; and the least was in the GES1 cell line (Figures 1C, D).

The relationship between KLF11 expression and clinical pathological characteristics. We found that KLF11 was up-regulated in gastric cancer tissues compared to matched adjacent gastric tissues. We analyzed the relationship between KLF11 expression and the clinical-pathological characteristics. The results showed that higher KLF11 expression was associated with tumor size and infiltration, regional lymph node metastasis and TNM staging (Table 1).

KLF11 is down-regulated in KLF11-siRNA cell line. The qRT-PCR and Western blot assays demonstrated that the mRNA and protein levels of KLF11 were stably reduced when the KLF11-siRNA2 was transfected in BGC823 cells. KLF11 remained at the same level in the NC-siRNA and Control groups (Figure 2A). We found dose-dependent effects of BGC823 with KLF11-siRNA2 transfected at concentrations ranging from 40 to 80 nM (Figure 2B). The mRNA and protein levels of KLF11 were also reduced when KLF11-siRNA2 was transfected into HGC27 cells (Figure 2C).

Down-regulation of KLF11 inhibits cell invasion and migration. To investigate the biological influences of KLF11, we conducted the wound-healing and Transwell invasion experiments. This assessed the migration and invasion of BGC823 and HGC27 cells which were transfected with NC-siRNA or KLF11-siRNA. As shown in Figure 3A, down-regulation of KLF11 notably inhibited the abilities of migration compared to the negative control

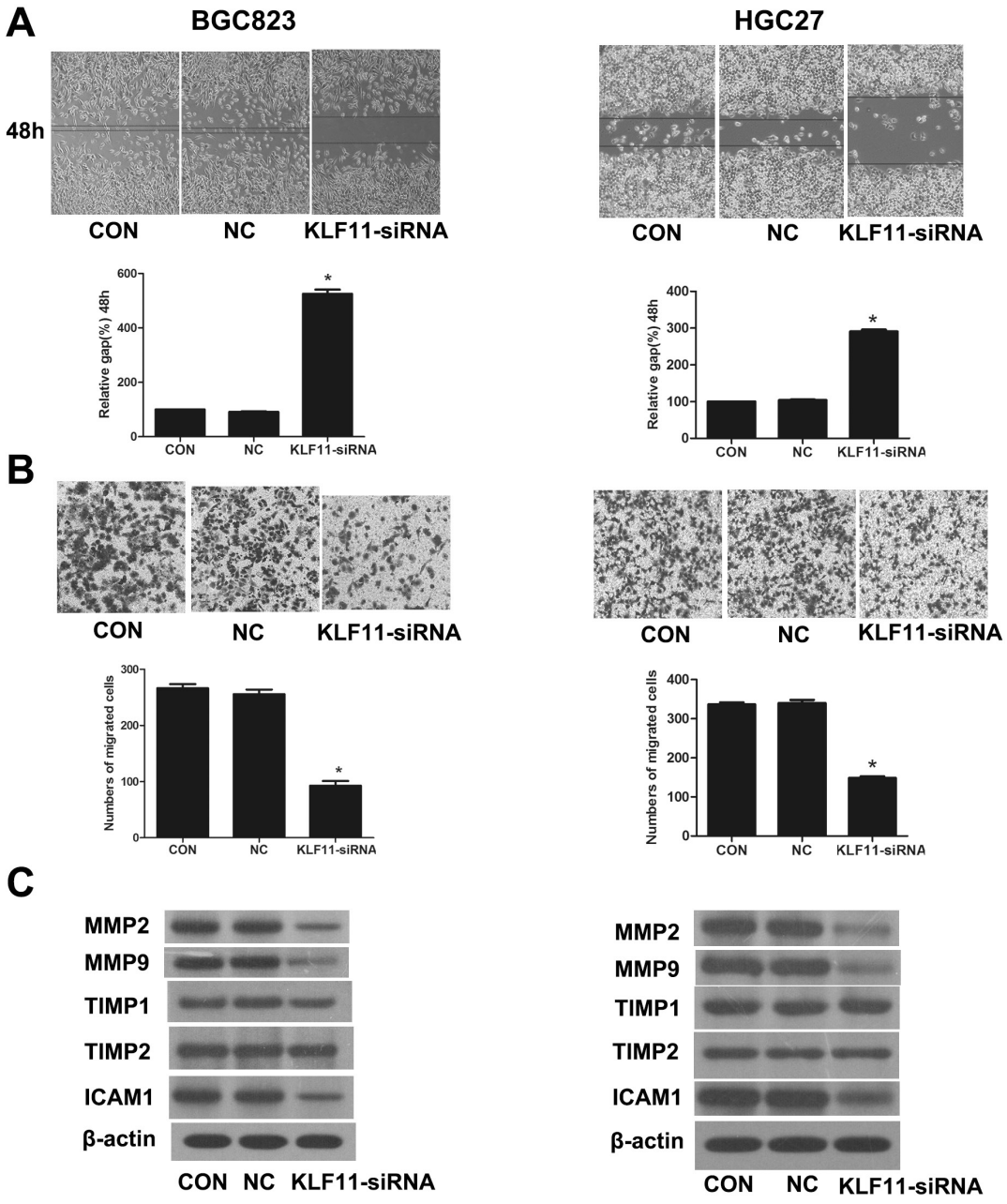

Figure 3. Down-regulation of KLF11 inhibits cell migration and invasion. A) Wound healing assay, B) Transwell invasion assay and C) The changes in MMP2, MMP9, TIMP1, TIMP2 and ICAM1 were tested in BGC823 and HGC27 cell lines by qRT-PCR and WB. *p<0.05

group. The numbers of migrated cells were observably reduced in the group of KLF11-siRNA (Figure 3B). We also detected the expressions of MMP2, MMP9, TIMP1, TIMP2 and ICAM1 proteins to explore potential mechanisms, and results revealed that the expression levels of protein MMP2, MMP9 and ICAM1 were obviously reduced in the group of KLF11-siRNA, while TIMP1 and TIMP2 remained at the same level (Figure 3C).

KLF11 may regulate Twist1 expression to induce EMT. We observed change in cell migration and invasion ability. Results indicated that KLF11 participates in the progress of EMT. We then detected the expressions of protein markers of EMT in BGC823 and HGC27 cell lines to confirm these observations. Figures 4A and B show that the E-cadherin epithelial cell marker protein was up-regulated and detected by qRT-PCR and WB assays in the KLF11-siRNA group and

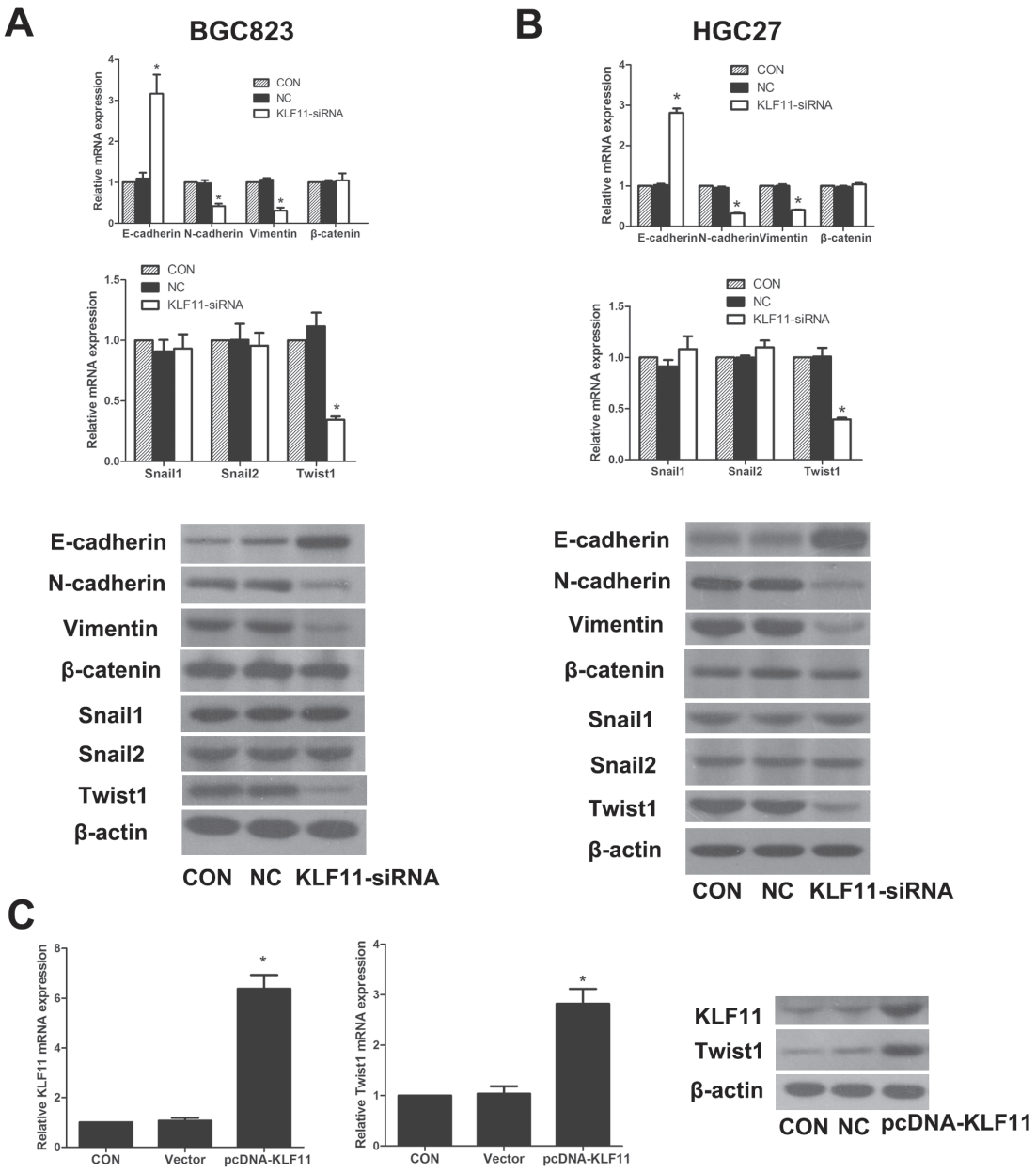

Figure 4. KLF11 may regulate Twist1 expression to induce EMT. The specific biomarkers of EMT including E-cadherin, N-cadherin, Vimentin and β-catenin were assessed by qRT-PCR and WB with BGC823 (A) and HGC27 (B) cell lines. Snail1, Snail2 and Twist1 were also checked by qRT-PCR and WB in the different groups. C) Following transfection with pcDNA-KLF11 or Vector, the BGC823 cells were assessed by qRT-PCR and WB assays to determine Twist1 expression. *p<0.05

the opposite expression of N-cadherin and Vimentin mesenchymal cell marker proteins were noted. There was no significant change in the expression of β-catenin. Snail and Twist were the two most significant transcription factors inducing EMT proceeding. To investigate the potential mechanism of KLF11-reduced EMT, we assessed the expressions of Snail1, Snail2 and Twist1 in BGC823 and HGC27 cell lines. The expression levels in mRNA and protein of Twist1 were reduced and Snail1 and Snail 2 remained at the same levels in the KLF11-siRNA group. In order to further confirm the KLF11-induced change of Twist1, we up-regulated the expression of KLF11 through transfecting pcDNA-KLF11; with corresponding improvement in Twist1 detected in the BGC823 cell line (Figure 4C).

KLF11 induces EMT through binding to the promoter region of Twist1. In order to further verify whether KLF11 induces EMT through binding to the promoter region of Twist1, we conducted Luciferase reporter assay in the

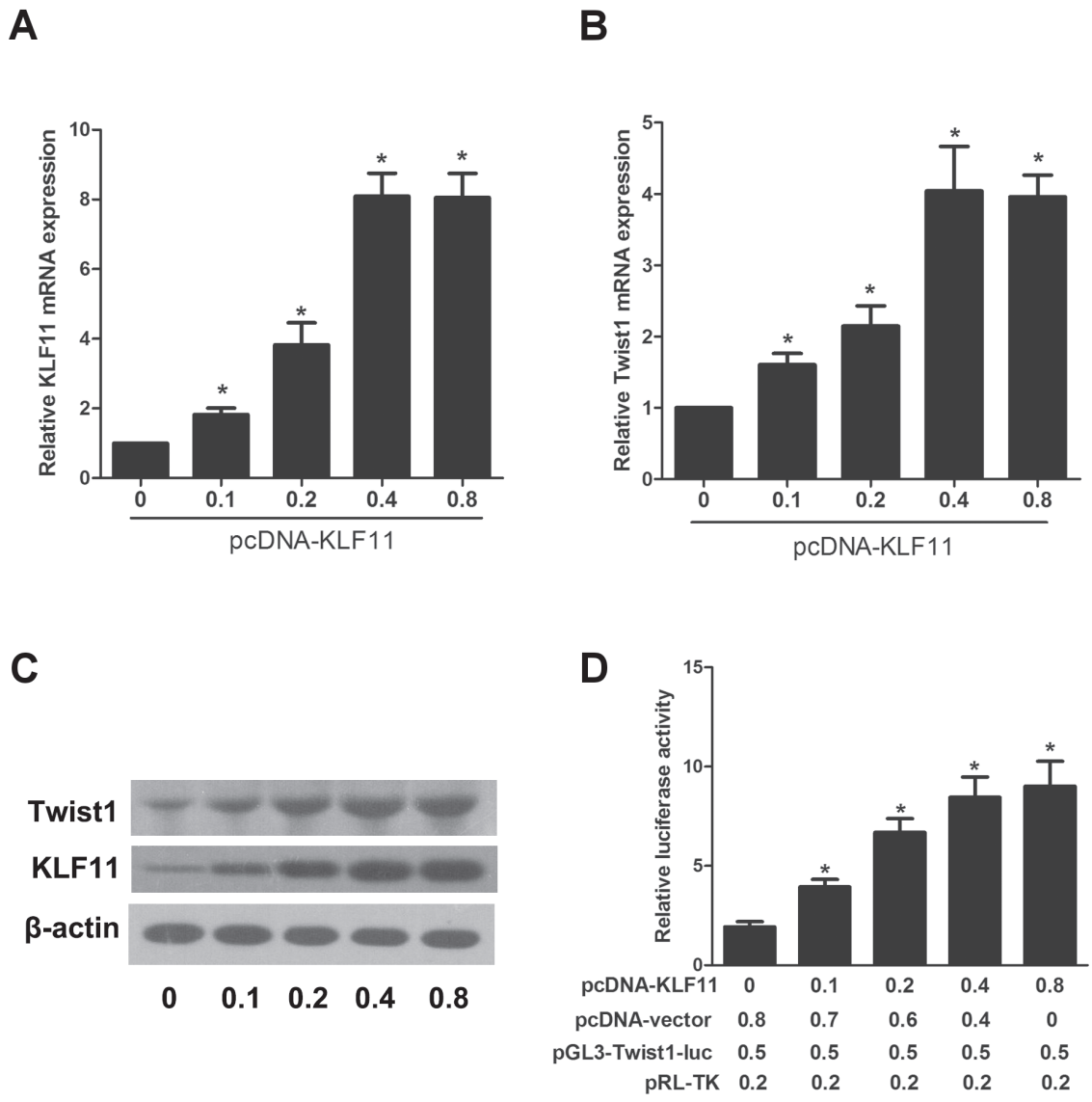

Figure 5. KLF11 induces EMT by binding to the Twist1 promoter region. (A, B, C) the corresponding changes in Twist1 mRNA and protein were detected by qRT-PCR and WB according to the increasing amounts of KLF11 plasmid (0, 0.1, 0.2, 0.4, 0.8 μg). D) The luciferase activities of the Twist1 promoter region were tested by Luciferase reporter assay with the different doses of pcDNA-KLF11 (0, 0.1, 0.2, 0.4, 0.8 μg). *p<0.05

BGC823 cell line. The results show that as the amounts of KLF11 plasmid increased (0, 0.1, 0.2, 0.4, 0.8 μg), activity of the Twist1 promoter region was gradually enhanced (Figure 5D). Corresponding changes in Twist1mRNA and protein were detected by qRT-PCR and WB (Figures 5A–C).

Discussion

Gastric cancer is one of the most common malignant tumors in the world. Although the mortality rate of GC appears to have decreased in the last decades, it still ranks third in all forms of malignant tumors [3]. Due to the lack of obvious symptoms and sensitive markers, many GC patients missed surgery until diagnosis. Previous studies showed that patients diagnosed in advanced stages had only 20–30% survival; and tumor invasion and metastasis were the main causes of cancer-related death [22]. It is therefore imperative to find new markers and therapeutic targets in gastric cancer.

KLF is a highly conserved family of zinc finger transcription factors associated with cellular proliferation, differentiation, migration, invasion, apoptosis, cardiovascular disease and metabolic disorders. KLF aids recruiting transcriptional regulatory proteins, including transcriptional co-activators

and co-repressors [14, 15]. In previous studies, KLF11 supported TGF-β signaling by terminating the smad7 loop and activating smad3; thus exerting anti-proliferative and pro-apoptotic functions in epithelial ovarian, pancreatic and liver cancer cells [17–20]. In the process of TGF-β-induced EMT, KLF11 significantly promoted hepatocellular carcinoma local invasion and distant metastasis by binding to the Smad7 promoter and suppressing transcription of Smad7 or directly up-regulating Smad2/3 expression [21, 23]. However, there are no reports on relationship between KLF11 and the EMT process in GC.

This is the first known study to establish increased expression levels of mRNA and protein of KLF11 in GC tissues and cell lines. Ectopic expression of KLF11 in gastric cancer cell notably promoted cell invasion and migration *in vitro*, and silencing KLF11 restrained these abilities.

The MMP-2 and MMP-9 members of the matrix metalloproteinases (MMP) family aid cancer cell spread by breaking down the extracellular matrix, and this is important in the EMT process [24, 25]. Our study of migratory and invasive proteins established that MMP-2, MMP-9 and ICAM1 [26] are reduced when KLF11 is silenced by siRNA, and results revealed that KLF11 partly mediates migration and invasion by regulating MMP-2, MMP-9 and ICAM1.

EMT is the process of transforming epithelial cells to mesenchymal cells under special physiological and pathological conditions. This is especially characterized by reduction in E-cadherin and over-expression of N-cadherin and Vimentin [27, 28]. Our results agreed with the above changes of EMT-related markers, and the combined results suggest that KLF11 has a crucial role in the EMT process.

It is known that the EMT process is affected by a complex regulating network, including various signaling pathways and transcription factors, and some non-coding RNAs and long non-coding RNAs also participate in EMT regulation [29]. Snail and Twist have been the most common transcription factors studied because they repress E-cadherin protein function [10, 30]. Recent research shows that MMPs promote transcription factors expression, including Snail, Twist and consequent EMT function in mammary epithelial cells. Snail is an established suppressor of E-cadherin and regulates MMP expression; a key factor in the EMT process [31, 32].

Other studies have shown that Twist1 is highly expressed in glioma and there is also up-regulated MMP2 expression in many tumor types. They also determined that Twist1 interacts directly with the MMP2 promoter to regulate MMP2 expression [33]. Twist1 has been proven to induce EMT in gastric cancer cells by reducing E-cadherin expression and increasing that of N-cadherin and Vimentin [34]. Herein, we reveal that Twist1 changed according to the expression of KLF11; not that of Snail 1 or Snail 2. Further research then confirmed that Twist1 is a down-stream target of KLF11, so that KLF11 then enhanced GC invasion and migration via binding to the TWIST 1 promoter region.

In conclusion, our study established that KLF11 is up-regulated in GC, and that it promotes the EMT process by increasing Twist1 expression. This is novel regulation of EMT, and our combined results may therefore improve understanding of EMT network regulation and KLF11 may be used as a therapeutic target in gastric cancer in the future.

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