


Up-regulation of KLF17 expression increases the sensitivity of gastric cancer to 5-fluorouracil

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Zhao-jie An , Yong Li, Bi-bo Tan, Qun Zhao, Li-qiao Fan, Zhi-dong Zhang, Xue-feng Zhao and Shao-yi Li

Abstract

It has been reported that the expression of Krüppel-like factor 17 (KLF17) was associated with the occurrence, development, invasion, metastasis and chemotherapy resistance of various tumors. However, the detailed mechanisms by which KLF17 promotes chemotherapy resistance in gastric cancer (GC) have not been fully investigated. In the present study, we collected the GC tissues and non-tumor tissues (matched adjacent normal tissues with corresponding GC tissues) of 60 GC patients, used qRT-PCR, Western blot and immunohistochemistry assay to analyze the relationship between the expression of KLF17 and the clinical pathological data of the patients. The effect of KLF17 on the sensitivity of GC cell lines to 5-fluorouracil (5-FU), and the potential mechanism were detected by MTS assay, Flow cytometry assay, and Western blot. Compared with non-tumor tissues, the expression level of KLF17 in GC tissue was significantly down-regulated, and the expression level of KLF17 in GES-1 cell line and GC cell lines also had a similar trend. Down-regulated expression of KLF17 is related to tumor size, invasion, regional lymph node metastasis, and TNM staging. Furthermore, through upregulating the expression of KLF17, the sensitivity of BGC-823 and SGC-7901 cell lines to 5-FU was obviously increased. Mechanistically, upregulation the expression of KLF17 can inhibit the expressions of P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1), and B-Cell lymphoma-2 (BCL-2), which have been reported to be associated with drug resistance and cell proliferation. Collectively, these data implied that KLF17 has the biological effect of inhibiting chemotherapy resistance of GC, and it could be a potential strategy for the GC chemotherapy resistance.

Keywords

5-fluorouracil, chemotherapy resistance, gastric cancer, KLF17

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Introduction

Gastric cancer (GC) is one of the most common malignant tumors of the digestive tract with extremely high morbidity and mortality. According to statistics, there are approximately 951,000 new cases of GC and 723,000 deaths of GC worldwide each year.¹ In China, the morbidity and mortality of GC rank the second and third among all of malignant tumors respectively.² Currently, surgical removal of lesions is still the main treatment

for GC, however, given that many patients with GC have unresectable tumors or distant metastases,³ thus, chemotherapy plays a key role in the

Department of General Surgery, The Fourth Hospital of Hebei Medical University, Shijiazhuang, China

Corresponding author:

Yong Li, Department of General Surgery, The Fourth Hospital of Hebei Medical University, 12# Jiankang Road, Shijiazhuang, Hebei 050011, China.

Email: li_yong_hbth@126.com



treatment for GC. However, chemotherapy resistance is one of the most common reasons for tumor treatment failure. Among the classic pathways of GC chemotherapy resistance, multidrug resistance 1 (MDR1) is the first gene to raise concern, and the protein encoded by this gene is P-glycoprotein (P-gp),⁴ which can transport drugs from the cell to the outside of the cell through the energy-consuming way of active transport, leading to drug resistance. Multidrug resistance protein 1 (MRP1), similar with MDR1, is another important gene responsible for GC drug resistance.⁵ Glutathione-S-transferase (GST) can metabolize the drugs inside GC cells into non-toxic substances through detoxification, finally leading to drug resistance.⁶ The occurrence of tumor disease is a sign of the relaxation and regulation of the mechanism of apoptosis, which makes some abnormal or aging cells escape immune and enter the state of infinite proliferation. The change of apoptosis is not only related to the occurrence and development of tumor, but also related with the chemotherapy resistance.⁷ B-Cell lymphoma-2 (Bcl-2) family members are important regulators of mitochondrial apoptosis pathway.⁸ According to their functions, they can be divided into anti-apoptotic protein family and pro-apoptotic protein family, among which Bcl-2 is the main representative member of anti-apoptotic protein family. Bcl-2 associated X protein (Bax) is one of the main representative proteins of the pro-apoptotic protein family.⁹

Krüppel-like factor (KLF) belongs to the zinc finger transcription factor family, and its gene sequence structure is very conservative.¹⁰ It can participate in cell proliferation, apoptosis, differentiation, and migration through transcriptional regulation of target genes, playing an important role in the occurrence of multiple malignant tumors, cardiovascular diseases, and metabolic disorders.¹¹ KLF17 is a newly discovered member of the KLF family, which regulates the cell cycle and the function of epithelial-mesenchymal transition (EMT).¹² Previous studies have shown that KLF17 can inhibit the occurrence, development, invasion, and metastasis of esophageal squamous cell carcinoma, breast cancer, lung adenocarcinoma, hepatocellular carcinoma, and colorectal cancer.^{13–17} Zhang et al.¹⁸ have further shown that the member

of the KLF family-KLF8 is involved in hypoxia-induced MDR through inhibiting apoptosis and increasing the drug release rate by directly regulating MDR1 transcription. In the present study, we aimed to investigate the biological effects of KLF17 on GC, and its potential strategy for the GC chemotherapy resistance.

Materials and methods

Tissue specimens

A total of 60 patients with GC were collected at the Fourth Hospital of Hebei Medical University from March to August 2020. Participants included 40 males and 20 females, there were 10 patients in stage I, 9 in stage II, 38 in stage III, and 3 in stage IV. Each cancerous tissue and non-tumor tissues (matched adjacent normal tissues with corresponding GC tissue, at least 5 cm distant from the tumor site) was obtained from each participant. Each tissue specimen was approximately 1 cm × 1 cm × 0.5 cm in size. None of the cases received preoperative chemotherapy or radiotherapy. The consents for this study were obtained from all of the participants.

Cell lines and cell culture

The human GC cell lines NCI-N87, SGC-7901, BGC-823, HGC-27, and normal gastric mucosal cell line GES-1 were obtained from the Procell Life Science & Technology Co., Ltd and cultured in RPMI-1640 medium supplemented with 10% bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in a 5% CO₂ atmosphere.

Transfection of cell lines

The well-growing BGC-823 and SGC-7901 cell lines were seeded in a 6-well plate for 24 h prior to transfection with a density of 5×10^5 cells/well. When the cells grow to 70–80% confluence, pcDNA3.1-KLF17 and pcDNA3.1 (Vector) were respectively transfected into the GC cells in the 6-well plate according to the Lipofectamine™ 2000 (Invitrogen, USA) instructions, of which cells were cultured with RPMI 1640 of serum-free and antibiotics-free. Untransfected cells were used

as the blank control group (Ctr). After 6 h of transfection, the medium was replaced with a complex medium. The transfection efficiency was detected 24 h later by qRT-PCR and Western blot. The pcDNA3.1-KLF17 and pcDNA3.1 plasmids were designed and synthesized by Ribo Bio (Anhui, China). The above experiments were replicated for three times.

RNA extraction, reverse transcription, and qRT-PCR

Total RNA was extracted from tissue samples or cell lines with RNA Express Total RNA Kit (NCM Biotech, China), 3 µg of total RNA was reverse-transcribed using A5001 GoScript reverse transcription system (Promega, USA). Quantitative RT-PCR (qRT-PCR) was conducted with A6001 qRT-PCR Master Mix (Promega, USA) on a ABI PRISM 7500 sequence detection system (Applied Biosystems). RNA expression was normalized to GAPDH. Relative expression levels of KLF17 mRNA in tissues were calculated by using the $2^{-\Delta CT}$ equation, while the ones in cell lines were calculated by using the $2^{-\Delta\Delta CT}$ equation. The primers for KLF17 and GAPDH were designed by Sangon Biotech (Shanghai). The primer sequences used for RT-PCR were as follows:

KLF17 F: 5'-AGCTGAGTCCCAGTCATTGC-3',
R: 5'-TCCTGAGGCCTGGAGTTCTT-3'.
GAPDH F: 5'-TGAACGGGAAGCTCACTGG-3',
R: 5'-GCTTCACCACCTTCTTGATGTC-3'.

Protein extraction and western blot

Total protein was extracted from patient tissues and cell lines using RIPA lysis buffer (Solarbio, China) containing protease inhibitor cocktail (Roche, Mannheim, Germany). The concentrations were determined using the BCA assay (Thermal, USA) according to the manufacturer's instructions. The proteins (50 µg) were separated using 10–12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), and the membranes

were incubated for 1 h at room temperature with 5% fat-free milk. Next, the membranes were incubated overnight with the primary antibody at 4°C. The next day, after the membranes were washed with Tris-buffered saline containing Tween (TBST) three times, the membranes were incubated with the secondary anti-rabbit IgG for 1 h at room temperature and darkness. Target bands were detected using the Odyssey system (LI-COR Biosciences, USA), and β-actin was used as an internal control. All experiments were repeated three times.

MTS assay

BGC-823 and SGC-7901 cell lines transfected with pcDNA3.1-KLF17 and the corresponding untransfected cell line (Ctr) were plated onto a 96-well plate at 3×10^3 cells/well and continue to culture for 4–6 h. After the cells adhere to the wall, upon treatment with 5-fluorouracil (5-FU) for 48 h, 12 µL of CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA) was added to each well, followed by a 2 h incubation in a dark environment, the optical densities (ODs) was measured at a wavelength of 490 nm with a microplate reader after the plate being shaken for 15 min at room temperature. Analyze the values and draw the cell growth inhibition rate curves of the Ctr group and the pcDNA3.1-KLF17 group, calculate the IC50 values and make comparisons. The above experiments were replicated for three times.

Flow cytometry assay

BGC-823 and SGC-7901 cell lines transfected with pcDNA3.1-KLF17 and corresponding untransfected cell lines (Ctr) were treated with 5-FU (4 µg/mL for BGC-823 cell line and 1 µg/mL for SGC-7901 cell line) for 24 h, then collect the cells and washed twice with phosphate-buffered saline (PBS), and resuspended with $1 \times$ Binding Buffer according to the instructions of PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™, USA). PE and 7-AAD were added to the solution, followed by incubation for 15 min at room temperature in the dark. After diluted with $1 \times$ Binding

Buffer, the apoptosis rate of the cell lines in each group were measured by flow cytometry.

Statistical analysis

SPSS21.0 was used to analyze the data. All data are expressed as the mean \pm standard deviation, and the statistical differences between the data in each group are analyzed by one-way analysis of variance and *t* test. The count data was analyzed for statistical differences by chi-square test. *P* value <0.05 was considered as statistical significance.

Result

The expression of KLF17 is down-regulated in GC tissues and GC cell lines

We detected the expression of KLF17 at mRNA and protein levels in 60 pairs of GC tissues and non-tumor tissues by qRT-PCR and Western Blot. The results showed that the expression of KLF17 was down-regulated obviously in GC tissues than in the non-tumor tissues (Figure 1a and c). Furthermore, we measured the expression of KLF17 in GC cell lines, including NCI-N87, SGC-7901, BGC-823, HGC-27, and normal gastric mucosal cell line GES-1 by qRT-PCR and Western Blot. The results showed that the expression level of KLF17 in GC cells SGC-7901, BGC-823, and HGC-27 was significantly decreased, and moderately low in NCI-N87 cell line, while it was expressed at a high level in the normal gastric mucosal cell line GES-1 (Figure 1b and d). Next, we chose SGC-7901 and BGC-823 cells for subsequent experiments because of the low expression of KLF17.

The relationship between the expression level of KLF17 and the clinicopathological characteristics of patients

We analyzed the relationship between the expression level of KLF17 and the clinicopathological characteristics of 60 patients by immunohistochemical staining. The results showed that KLF17 was reduced in GC tissues of 44 patients (Figure 1e and f). The reduced expression of

KLF17 is related to tumor size and invasion, regional lymph node metastasis and TNM staging (Table 1).

Up-regulation of KLF17 increases the sensitivity of GC cell lines to 5-FU

QRT-PCR and Western blot demonstrated that KLF17 remained the same level in BGC-823 and SGC-7901 cell lines after it was transfected with pcDNA3.1 while mRNA and protein of KLF17 were significantly increased after it was transfected with pcDNA3.1-KLF17 (Figure 2). Furthermore, MTS assay illustrated that the sensitivity to 5-FU was significantly increased in cells BGC-823 and SGC-7901 transfected with pcDNA3.1-KLF17 compared with non-transfected cells (Figure 3a), and the IC₅₀ value was significantly lower than the control group (Figure 3b).

Up-regulation of KLF17 promote the apoptosis of GC cell lines treated by 5-FU

We further examined whether KLF17 have relationship to induce apoptosis. BGC-823 and SGC-7901 cell lines transfected with pcDNA3.1-KLF17 were treated with 5-FU (4 μ g/mL for BGC-823 cell line and 1 μ g/mL for SGC-7901 cell line) for 24 h, following which apoptosis rate was detected by flow cytometry. As shown in Figure 4, the apoptosis rate of the cells that transfected with pcDNA3.1-KLF17 was significantly higher than that of the untransfected cell lines (Ctr).

KLF17 increases the sensitivity of GC cell lines to 5-FU by reducing the expressions of P-gp, MRP1, and Bcl-2

To further explore the Mechanisms of KLF17 on the sensitivity of GC cell lines to 5-FU, the drug resistance-related proteins P-gp, MRP1, GST- π , and apoptosis-related proteins Bcl-2, Bax were detected in BGC-823 and SGC-7901 cell lines. The results showed that the expression of P-gp, MRP1, and Bcl-2 remarkably reduced after pcDNA3.1-KLF17 was transfected into BGC-823 and SGC-7901 cell lines, whereas changes were

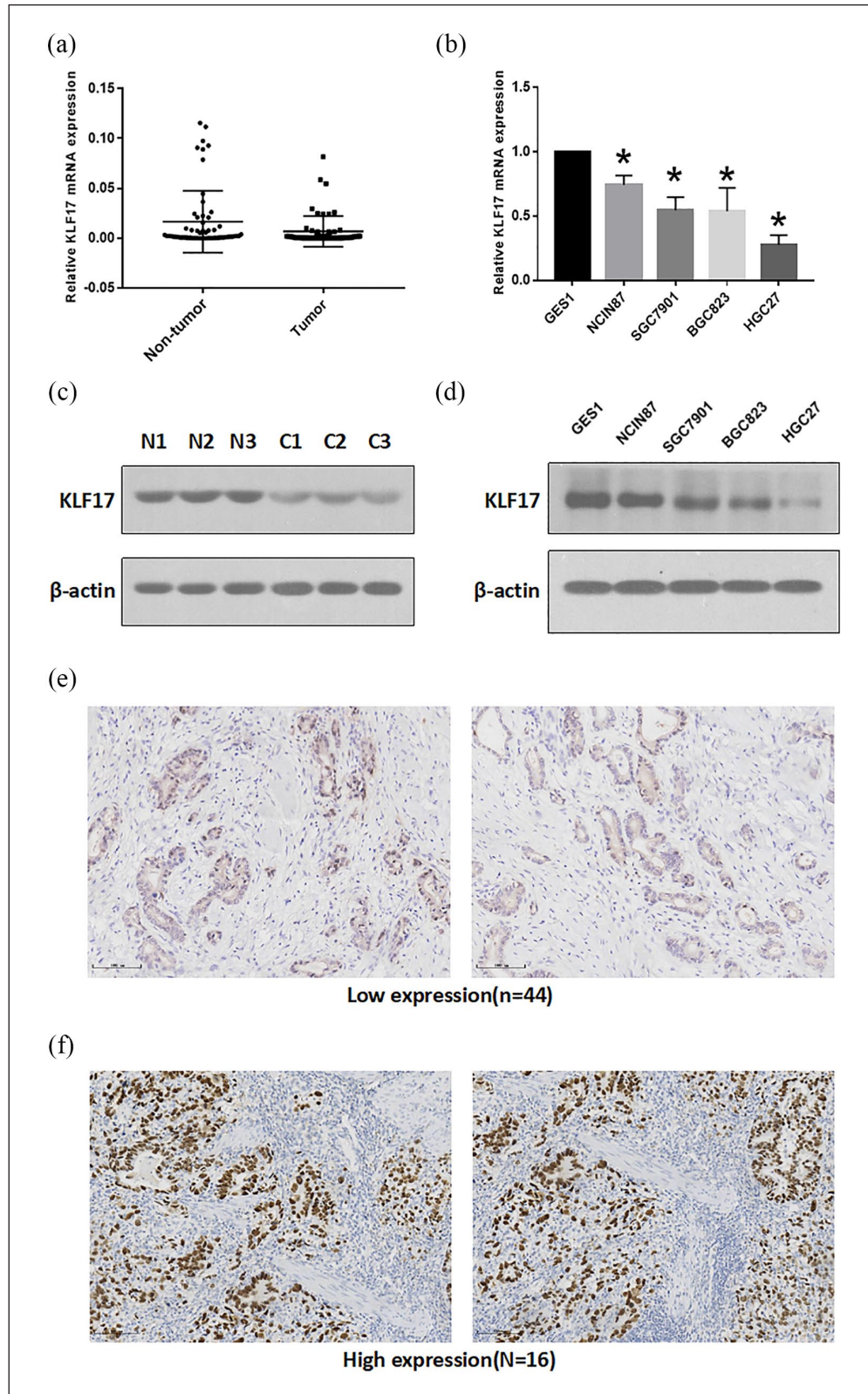


Figure 1. The expression of KLF17 is down-regulated in GC tissues and GC cell lines. (a) The mRNA expression levels of KLF17 in 60 pairs of GC tissues and non-tumor tissues were tested with qRT-PCR ($*P < 0.05$ vs non-tumor tissues). (b) The mRNA expression levels of KLF17 in various cell lines were tested with qRT-PCR. ($*P < 0.05$ vs GES-1 group). (c) The protein expression levels of KLF17 in 60 pairs of GC tissues (C) and non-tumor tissues (N) were tested with Western Blot. (d) The protein expression levels of KLF17 in various cell lines were tested with Western Blot. (e) KLF17 is low expressed in GC tissues according to immunohistochemical staining. (f) KLF17 is highly expressed in GC tissues according to immunohistochemical staining.

Table 1. The relationship between KLF17 expression and clinicopathological characters.

	KLF17 expression		χ^2	P-value
	High (16)	Low (44)		
Age (years)				
<55	3	13	0.256	0.613
≥55	13	31		
Gender				
Male	11	29	0.043	0.836
Female	5	15		
Tumor size(cm)				
<5	15	28	3.862	0.049*
≥5	1	16		
Tumor infiltration				
T1	5	3	5.784	0.044*
T2	2	5		
T3	0	0		
T4	9	36		
Regional lymph node metastasis				
N0	8	5	12.679	0.003*
N1	3	5		
N2	3	11		
N3	2	23		
Distant metastasis				
M0	16	41	0.161	0.688
M1	0	3		
TNM staging				
I	5	5	13.3461	0.001*
II	6	3		
III	5	33		
IV	0	3		

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

* $P < 0.05$.

not noticed in the expression of GST- π and Bax (Figure 5).

Discussion

The incidence rate of GC ranks first among digestive malignancies in China, and its mortality rate still ranks third among all malignant tumors.¹⁹ Chemoresistance of GC cell directly contributes to the failure of chemotherapy.²⁰ Previous studies have shown that the member of the KLF family can increase the drug release rate of GC by directly regulating MDR1 transcription. Therefore, we hypothesize that KLF17 may promote the sensitivity of GC cells to chemotherapy.

KLF17 (also known as ZNF393) is a newly discovered KLF transcription factor that can inhibit EMT of tumor cells. KLF17 can down-regulate the EMT process of tumor cells by regulating TGF- β /SMAD 3 or DJ-1/KLF 17/ID-1 signaling pathways. Thus, KLF17 plays an important role in inhibiting the invasion and metastasis of breast cancer, liver cancer, esophageal squamous cell carcinoma, and other tumors.¹³⁻¹⁷ There have been a few reports about the relationship between KLF17 expression and GC. Peng et al.²¹ confirmed that KLF17 down-regulation is an independent predictor of poor prognosis in patients with GC through multivariate Cox regression analysis, but there is no report on the biological function of KLF17 in GC.

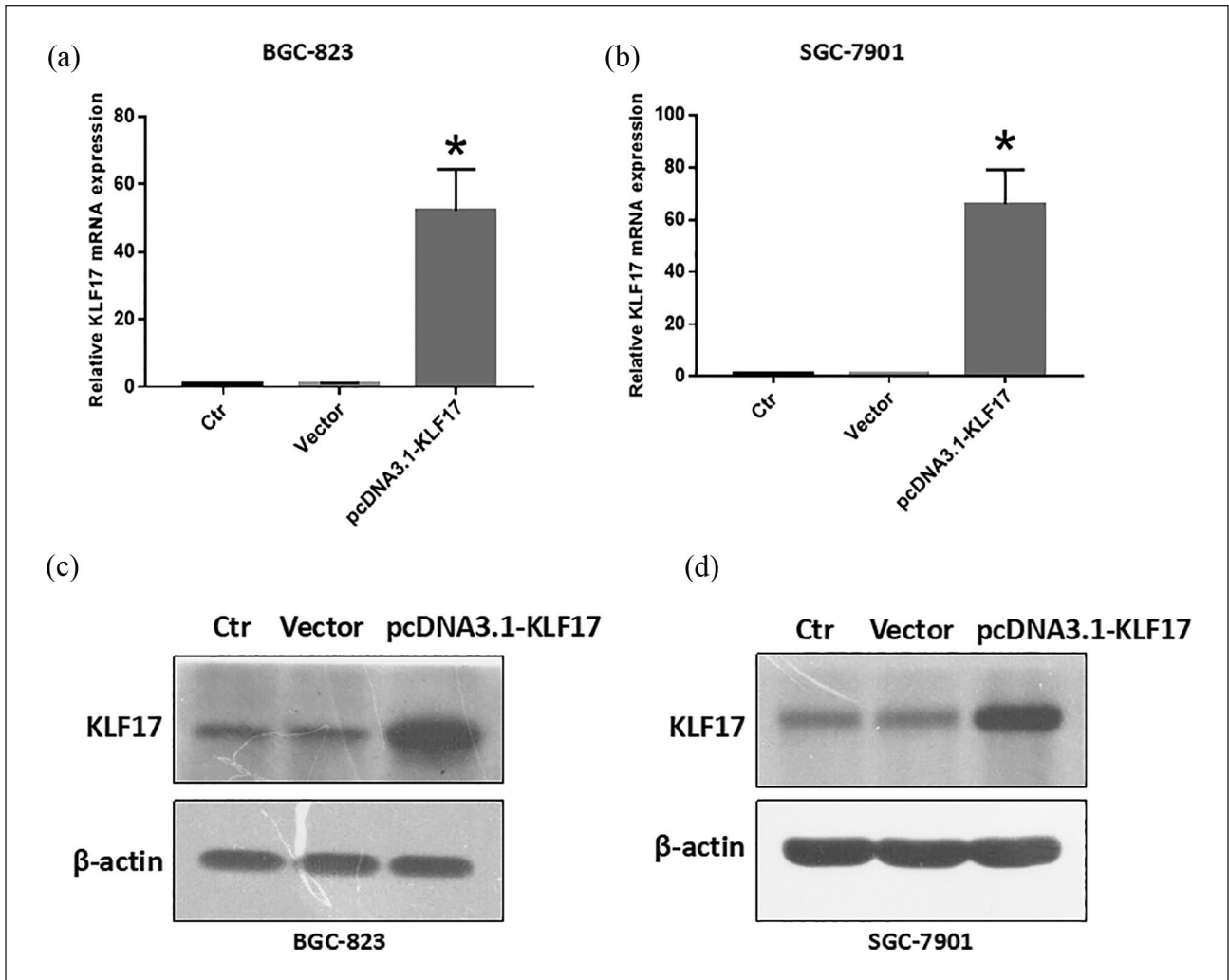


Figure 2. KLF17 overexpression in GC cell lines. (a) The mRNA expression levels of KLF17 in BGC-823 cells after they were transfected pcDNA3.1 and pcDNA3.1-KLF17 were tested with qRT-PCR ($*P < 0.05$ vs control group). (b) The mRNA expression levels of KLF17 in SGC-7901 cells after they were transfected pcDNA3.1 and pcDNA3.1-KLF17 were tested with qRT-PCR ($*P < 0.05$ vs control group). (c) The protein expression levels of KLF17 in BGC-823 cells after they were transfected pcDNA3.1 and pcDNA3.1-KLF17 were tested with Western Blot. (d) The protein expression levels of KLF17 in SGC-7901 cells after they were transfected pcDNA3.1 and pcDNA3.1-KLF17 were tested with Western Blot.

P-gp is a transmembrane glycoprotein (P170) with a molecular weight of 170 KD; it can not only bind to drugs, but also bind to ATP. ATP provides energy to pump intracellular drugs out of the cell, reducing the concentration of intracellular drugs and making the cancer cells resistant to the chemotherapy drugs.⁴ MRP1 is a glutathione transport pump (GS-X) which is widely expressed in human tissues. It is mainly distributed in the cytoplasm, with a small amount distributed in the cell membrane. It can promote the

excretion of glutathione-bound drugs from the cell and lead to multi-drug resistance.⁵ Bcl-2 (B-cell lymphoma-2) is one of the most important oncogenes in apoptosis research. It can enhance the resistance of cells to most DNA damage factors and inhibit the apoptosis of target cells caused by most chemotherapy drugs.²² After up-regulating the expression of KLF17 in GC cell lines by plasmid transfection in this study, the expression of P-gp, MRP1, and Bcl-2 decreased significantly. It indicates that KLF17

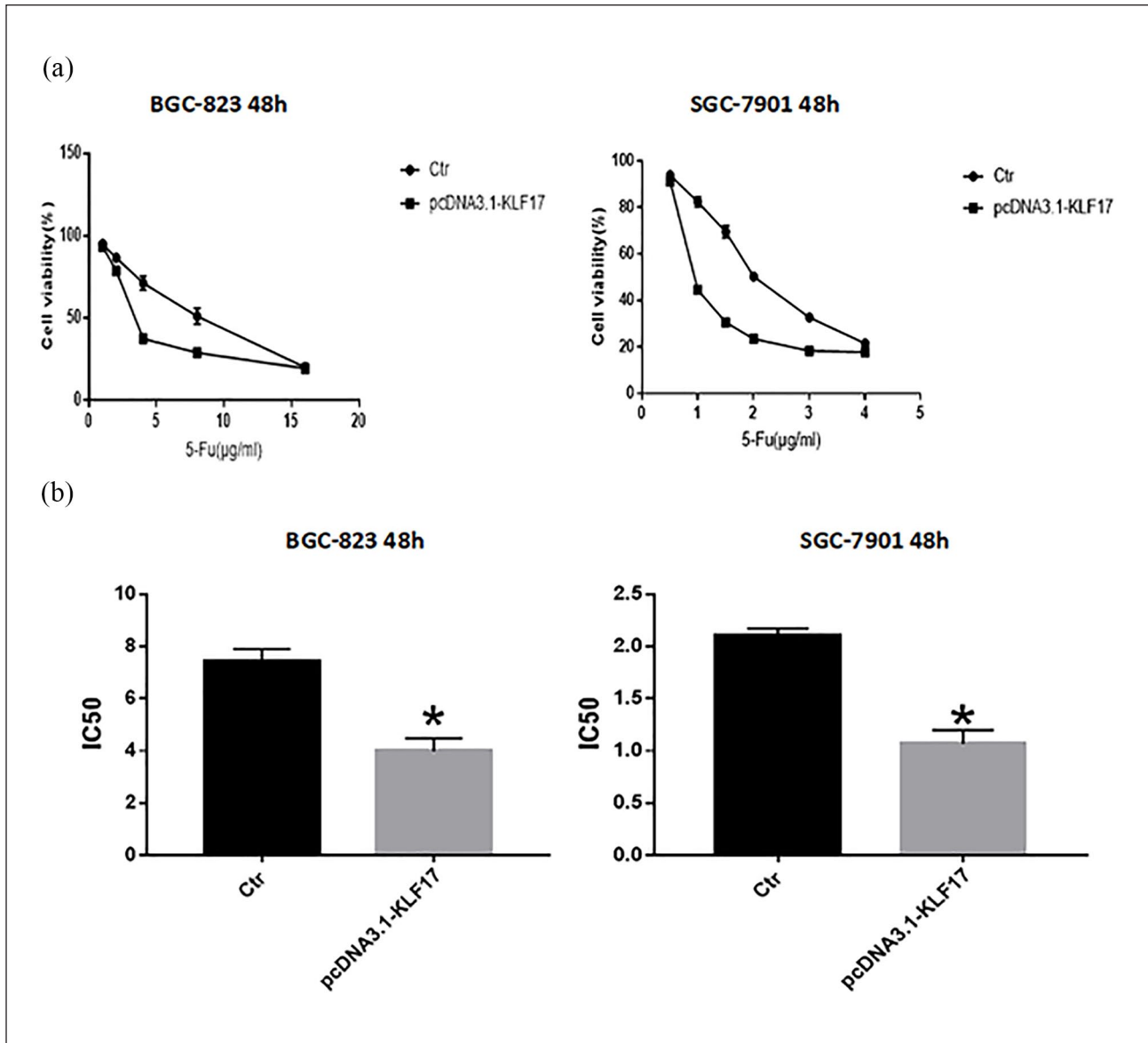


Figure 3. KLF17 expression and the sensitivity of GC cell lines to 5-FU. (a) The changes of sensitivity to 5-FU in cells BGC-823 and SGC-7901 after they were transfected pcDNA3.1-KLF17 were tested with MTS. (b) The IC50 value of BGC-823 and SGC-7901 cells after they were transfected pcDNA3.1 and pcDNA3.1-KLF17.

may affect the chemotherapy resistance of GC cells by down-regulating the expression of the above proteins through its transcriptional regulation function.

In this study, we verified that the down-regulation of KLF17 is related to the poor prognosis of GC patients, GC tissues and GC cell lines compared with non-tumor tissues and normal cell lines. Furthermore, cell function

experiments confirmed that up-regulation of KLF17 expression can increase the sensitivity of GC cells to 5-FU. This is the first study about the relationship between KLF17 expression and the chemotherapy resistance of GC. The results of the present study showed that KLF17 was abnormally expressed in GC tissues and cell lines, and we found that the over-expression of KLF17 markedly inhibited the

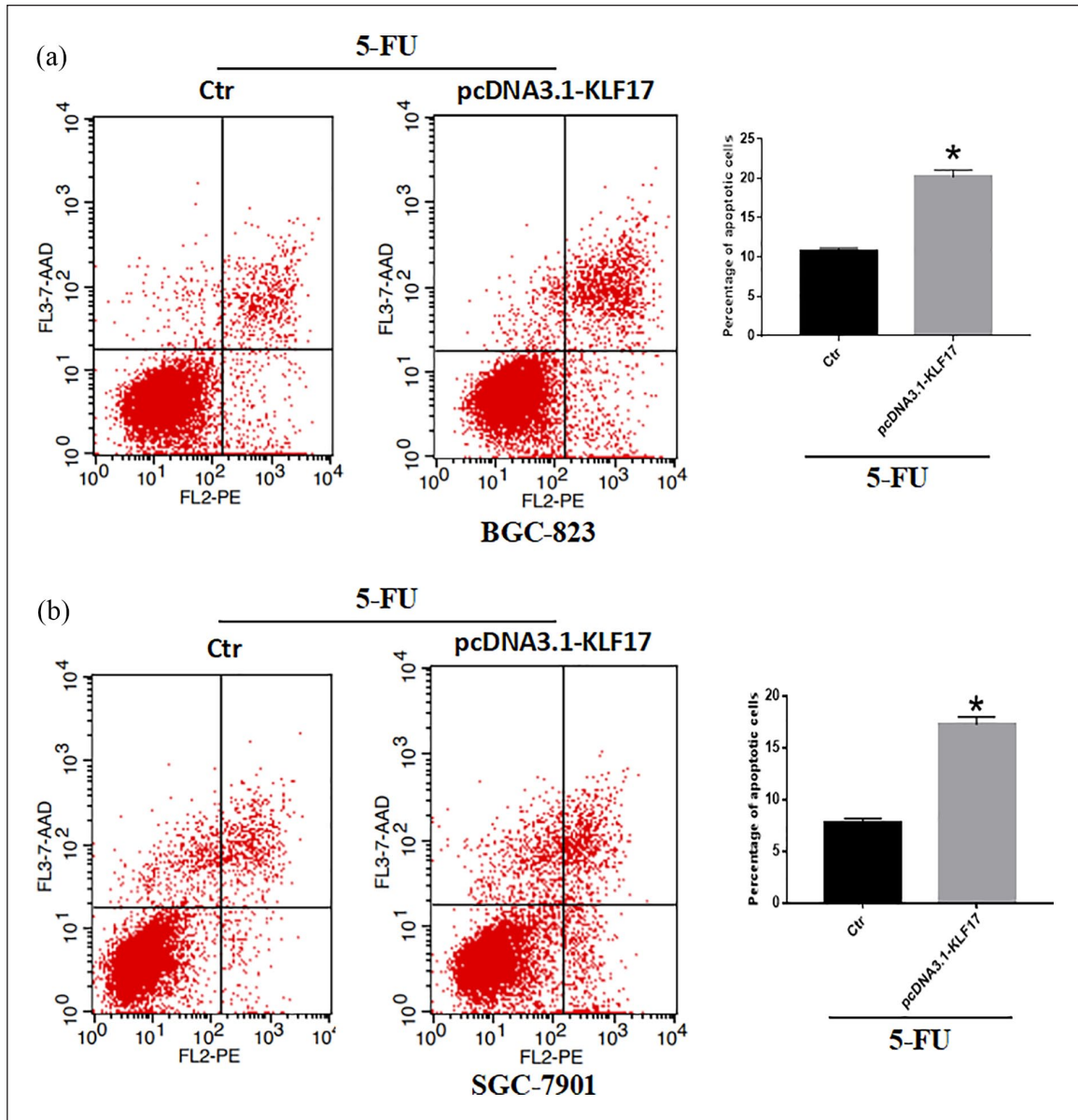


Figure 4. Up-regulation of KLF17 promote the apoptosis of GC cell lines treated by 5-FU. (a) The changes of cells apoptosis of BGC-823 cells after they were transfected pcDNA3.1-KLF17 (* $P < 0.05$ vs control group). (b) The changes of cells apoptosis of SGC-7901 cells after they were transfected pcDNA3.1-KLF17 (* $P < 0.05$ vs control group).

chemotherapy resistance of GC, which may be related to the down-regulation of P-gp, MRP1, and Bcl-2, but the specific mechanism by which KLF17 regulated the expression of these proteins is not clear and needs to be investigated in further study. Another limitation of this study is that due to the limited number of patients, our clinical sample size might be

relatively small, and we will make up for these deficiencies in subsequent further studies.

Conclusion

Collectively, our results suggest that KLF17 is crucial for inhibiting the chemotherapy resistance in human GC and it could be a new therapeutic

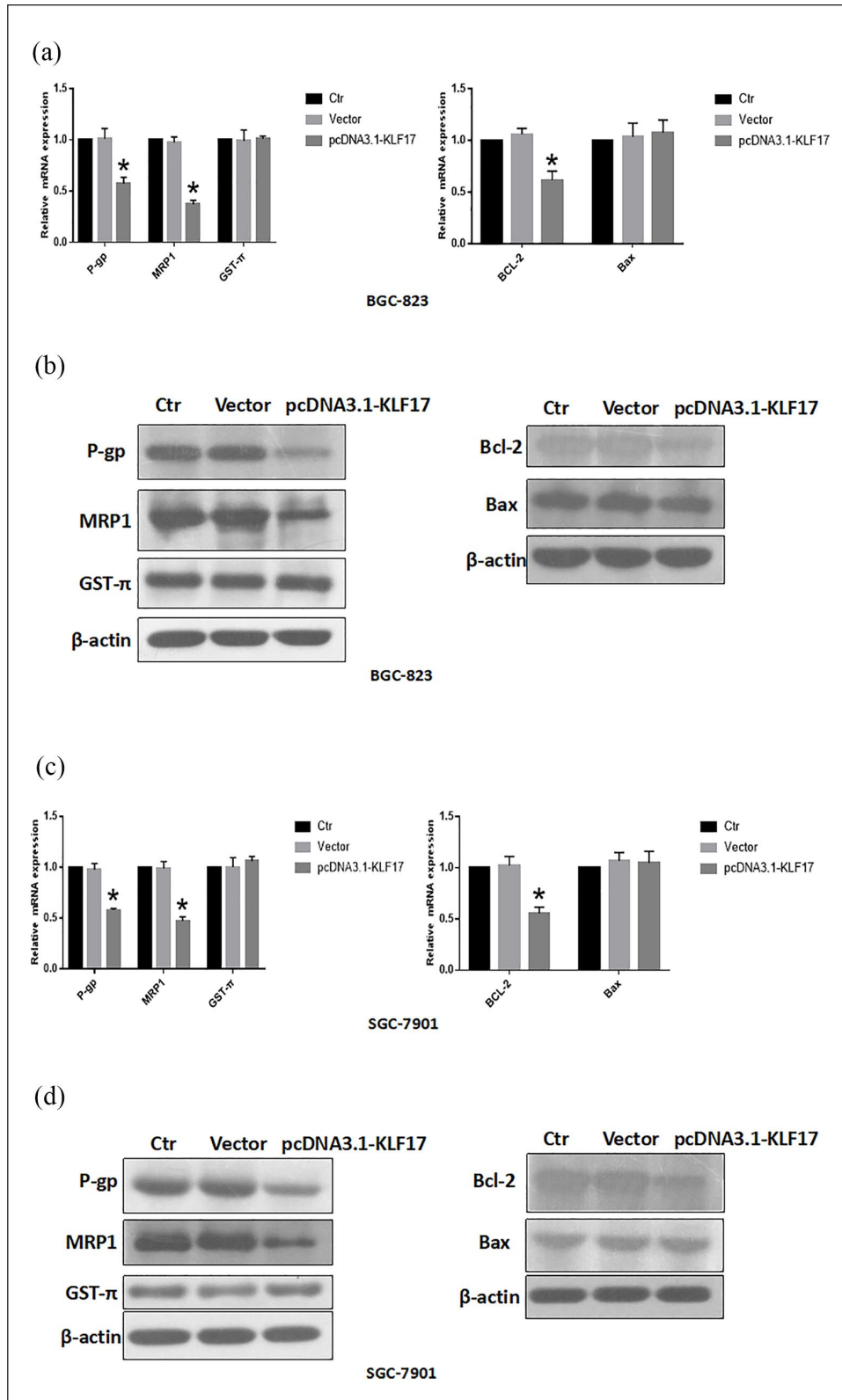


Figure 5. KLF17 increases the sensitivity of GC cell lines to 5-FU by reducing the expressions of P-gp, MRP1, and Bcl-2. (a) The changes of the drug resistance-related and apoptosis-related mRNA in BGC-823 cells after they were transfected pcDNA3.1-KLF17 ($*P < 0.05$ vs control group). (b) The changes of the drug resistance-related and apoptosis-related proteins in BGC-823 cells after they were transfected pcDNA3.1-KLF17 ($*P < 0.05$ vs control group). (c) The changes of the drug resistance-related and apoptosis-related mRNA in SGC-7901 cells after they were transfected pcDNA3.1-KLF17 ($*P < 0.05$ vs control group). (d) The changes of the drug resistance-related and apoptosis-related proteins in SGC-7901 cells after they were transfected pcDNA3.1-KLF17 ($*P < 0.05$ vs control group).

strategy for chemotherapy resistance of GC in the future.

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Declaration of conflicting interests

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Ethics approval

Ethical approval for this study was obtained from Ethics Committee of the Fourth Hospital of Hebei Medical University (2020KY106).

Informed consent

Written informed consent was obtained from all subjects before the study.

ORCID iD

Zhao-jie An  <https://orcid.org/0000-0001-6353-725X>

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