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CREBRF promotes the proliferation of human gastric cancer cells via the AKT signaling pathway

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Abstract: Gastric cancer (GC) is one of the most common malignant cancer around the world, however the mechanisms is still unclear. In the present study, we investigated the function of CREB3 regulatory factor (CREBRF) in human GC and explored its relevant molecular mechanism. We found that CREBRF was highly expressed in primary GC tissues and the expression level was associated with the clinicopathologic characteristics of GC. CREBRF silencing inhibited GC cell proliferation and induced G1/G0 to S phase cell cycle arrest through regulating Cyclin A, Cyclin D1 and CDK2 expressions. Furthermore, the results showed that knockdown of CREBRF suppressed the activation of AKT signaling pathway. We further discovered that activating of AKT rescued the effect of CREBRF silencing on cell growth and drove cell re-enter into the S phase of the cell cycle with SC79 (a AKT activator). Taken together, our study demonstrated that CREBRF might promote GC cell proliferation and induce G1-S phase transition through activating AKT signaling pathway. These findings suggest that CREBRF acts as a novel oncogene and may be a potential therapeutic target in therapy of GC.

Key words: Gastric cancer; CREBRF; Proliferation; Cell cycle; AKT signaling pathway.

Introduction

Gastric cancer (GC) is one of the most common malignant cancer and also the third cause of cancer-incurred mortality around the world (1). In China, GC was the second leading cause of cancer-related deaths after lung cancer until now (2). In the recent years, with rapid advances of diagnostic apparatus and therapeutic approaches, the morbidity and mortality rates of GC gradually decrease (3). However, the prognostic outcome of advanced stage disease still remains poor and 5-year overall survival rate is less than 30% (4,5). Existing therapeutic methods, such as surgery, radiotherapy, chemotherapy or multimodality therapy, are not satisfactory because of the advanced stage of the disease, and the fact that it is often accompanied by malignant growth, extensive invasion and lymphatic metastasis when diagnosed (6,7). Therefore, it is imperative to investigate the underlying molecular mechanism of GC in order to discover novel therapeutic approaches and to improve the patient survival.

CREBRF (CREB3 regulatory factor) is a novel cellular protein and a specific regulator of CREB3 that may recruit nuclear CREB3 to discrete foci in the nucleus and promote CREB3 protein degradation (8). CREB3 is the primary member of the CREB family proteins, including CREB1, CREB2 and CREB3. CREB family proteins can bind to the cAMP-responsive element recognition sequence of cAMP-sensitive genes to regulate transcription (9). CREB family members seem to play a role in the unfolded protein response, during which endoplasmic reticulum resident molecular chaperones and foldases are induced, attenuating translation to decrease the load (10). It is reported that CREB1 can upregulate autophagy genes (11). However, there is no evidence to date that CREBRF participates in tumorigenesis and progression of gastric carcinoma.

In the present study, we analyzed the expression of CREBRF in GC tissues using data from the Cancer Genome Atlas (TCGA). We performed quantitative real-time PCR in 65 pairs of GC tissues and normal tissues for evaluating CREBRF expression in GC. The role and molecular mechanism of CREBRF in GC were explored by a series of experiments. We found that CREBRF expression was remarkably up-regulated in GC and the expression level was correlated with the clinicopathologic characteristics, and CREBRF promoted GC cell proliferation through activating AKT signaling pathway. Our findings may provide novel insight into the underlying mechanisms of GC, and suggest that CREBRF may serve as a possible molecular target for GC therapy.

Materials and Methods

Preparation of human tissue samples

Human GC tissues (65) were collected from patients

who were diagnosed at the Department of Oncology Surgery, the First Affiliated Hospital of Xi'an Jiaotong University, PR China. Clinicopathological data were obtained through reviewing their pathology records. We obtained informed consent from each patient before specimen collection. All samples were stored at -80 °C. The study was approved by the Ethical Committee of Xi'an Jiaotong University Health Science Center.

Cell culture

Human GC cell lines (MKN-45, BGC-823 and SGC-7901) and normal human gastric epithelial cell line (GES-1) were purchased from the Cell Bank (Shanghai Genechem Co., Ltd., Shanghai, China). These cells were cultivated in RPMI-l640 medium (Gibco BRL, NY, USA) supplemented with 10 % (v/v) fetal bovine serum (Gibco) and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the cells and human tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was reverse transcribed to cDNA by using PrimeScript™ RT reagent Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). qRT-PCR were conducted using the iCycler iQ Multicolor qRT-PCR Detection System (Bio-Rad, Hercules, CA, USA). According to the manufacturer's instructions, the results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The primer sequences were as follows: CREBRF forward: 5'-TGAACAGCCAACTAAATGCAGTCC-3'; CREBRF reverse: 5'-TCATGCTCACTGCCGAATCC-3'; GAPDH forward: 5′-GAAGGTGAAGGTCGGAGTCA-3′; GAPDH reverse: 5′-TTGAGGTCAATGAAGGGGTC-3′. All reactions were performed in triplicate.

siRNA synthesis and transfection

Small interfering RNA (siRNA) was pre-designed for human CREBRF gene silencing. Human CREBRF siRNA (sense 5'-CUCCAACUUUAGCUCAACUUU-3', antisense 5'-AGUUGAGCUAAAGUUGGAGUU-3'), and negative control siRNA (NC-siRNA, sense 5'-UUCUCCGAACGUGUCACGUUU-3', antisense 5'-ACGUGACACGUUCGGAGAAUU-3') were chemically synthesized by GenePharma Corporation (GenePharma, Shanghai, China). Lipofectamine™-2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect siRNA according to the manufacturer's instructions. The siRNAs were diluted to 70 nM in the plated cells in future experimental procedures.

Cell proliferation assay

Cell proliferation was assessed using MTT assay (Sigma, St Louis, MO, USA). Human GC SGC-7901 cells (5,000 cells/well in 200 μl medium) were seeded into 96-well plates and cultured for 24 hours. Next, the cells were treated with NC-siRNA (70 nM), CREBRF siRNA (70 nM) or CREBRF siRNA (70 nM) + SC79 (AKT activator, 4 μg/ml) (MCE, USA) for 24, 48 and 72 hours, respectively. 20 μl of 5 mg/ml MTT solution was added per well. Cell viability was measured at 492 nm using multi-microplate test system (BMG Labtechnologies, Offenburg, Germany). Each experiment contained 5 replicates and was repeated at least 3 times.

Cell cycle analysis

The SGC-7901 cells were seed in 6-well plates and treated by NC-siRNA, CREBRF siRNA or CREBRF siRNA + SC79 for 48 hours. The cells were harvested and washed in PBS and fixed in 75 % ice-cold ethanol at 4 °C. The fixed cells were washed in PBS and stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 15 min. The cells were measured by fluorescence-activated cell sorting (BD Biosciences, San Diego, CA, USA). The cell-cycle populations were analyzed by ModFit software. Three parallel wells were made in each experiment, and the procedures were carried out in triplicate.

Cell apoptosis analysis

The SGC-7901 cells were seeded in 6-well plates for 24 hours and treated by NC-siRNA, CREBRF siRNA or CREBRF siRNA + SC79 for 48 hours. Next, the cells were harvested and performed with Annexin-V FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were measured through using a flow cytometer (BD Biosciences, San Jose, CA, USA). Quantification of apoptosis was analyzed by ModFit software. The procedures were repeated at least 3 times.

Western blot

Total protein was extracted via using a RIPA lysis buffer (Wolsen, Xi'an, China) from normal gastric tissues, GC tissues or GC cells. These proteins were separated in 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane (Roche, Basle, Switzerland). Next, the membrane was incubated with primary antibodies at 4 °C and then incubated with secondary antibody for 2 hours. The primary antibodies included mouse polyclonal antibody anti-CREBRF (1:1,000; Santa Cruz, CA, USA), rabbit monoclonal antibody anti-phospho-AKT (1:2,000; Santa Cruz, CA, USA), rabbit monoclonal antibody anti-Akt (1:1,000; Santa Cruz, CA, USA), mouse monoclonal antibody anti-Cyclin A (1:1,000; Santa Cruz, CA, USA), mouse monoclonal antibody anti-Cyclin D1 (1:1,000; Santa Cruz, CA, USA), rabbit monoclonal antibody anti-CDK2 (1:500; Santa Cruz, CA, USA), mouse monoclonal antibody anti-GAPDH (1:2,000; Cell Signaling Technology). The membranes were incubated with ECL (Pierce, Rockford, IL, USA) for chemiluminescence detection. The luminescent signal was scanned and the band density was examined with Syngene GBox (Syngene, UK).

Statistical analysis

The data were presented as mean ± SEM from at least 3 separate experiments. All statistical analysis was performed using SPSS 19.0 software (Abbott Laboratories, Chicago, IL). Pearson's chi-square test was employed to measure the relationships between CREBRF expression and clinicopathologic characteristics, and Student's t-test was used to analyze the difference between two groups. $P < 0.05$ were considered to indicate statistical significance.

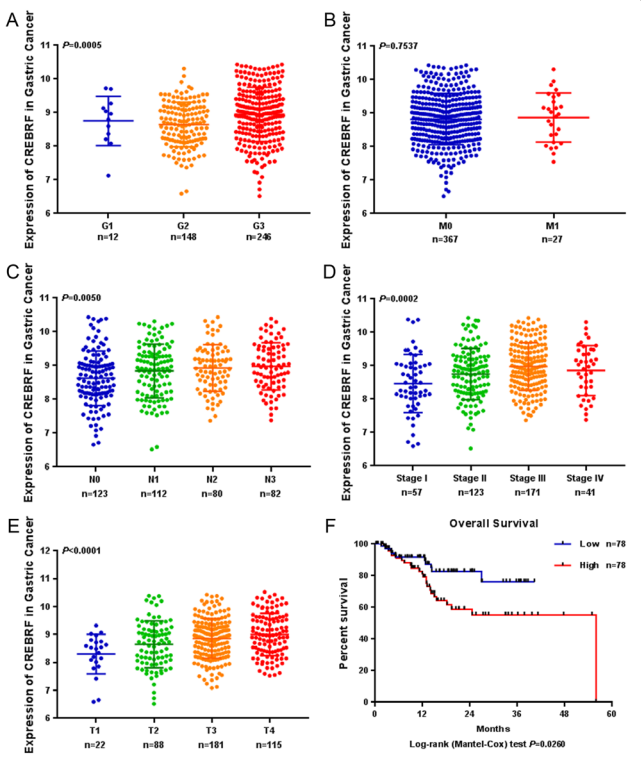

Figure 1. TCGA data show that the expression of CREBRF is correlated with the clinicopathologic characteristics of GC patients. (A) Correlation between CREBRF expression and poor tumor histology in GC patients using data from TCGA. (B) Correlation between CREBRF expression and M stage in GC patients using data from TCGA. (C) Correlation between CREBRF expression and N stage in GC patients using data from TCGA. (D) Correlation between CREBRF expression and TNM stage in GC patients using data from TCGA. (E) Correlation between CREBRF expression and T stage in GC patients using data from TCGA. (F) Panels represent patient survival estimation datasets of CREBRF high expression correlation group compared with low expression correlation group from TCGA.

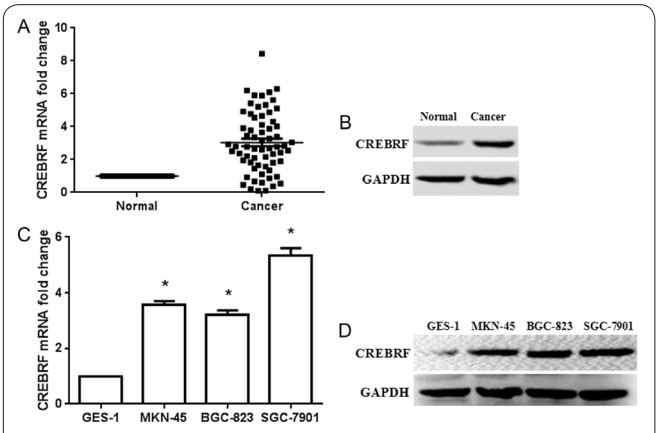

Figure 2. CREBRF is up-regulated in human GC tissues and cell lines. (A) CREBRF mRNA expression in GC tissues versus normal gastric tissues (n = 65; P < 0.01). (B) CREBRF protein expression in GC tissues and normal tissues (n = 12). (C) CREBRF mRNA expression in GC cell lines (MKN-45, BGC-823 and SGC-7901) and normal human gastric epithelial cell line (GES-1) (*P < 0.01). (D) CREBRF protein expression in GC cell MKN-45, BGC-823, SGC-7901 and GES-1, with GAPDH as an internal control.

Results

CREBRF is frequently up-regulated in GC tissues and cell lines

To explore the role of CREBRF in GC progression, we first analyzed the correlations between CREBRF expression and clinicopathologic characteristics. TCGA data showed that CREBRF expression was associated with G stage, N Stage, TNM Stage and T stage (P < 0.01) (Fig. 1A, C-E). No significant difference was observed in CREBRF expression between M0 and M1 cancers (Fig. 1B). In addition, the higher expression of CREBRF was positively correlated with decreased overall survival in these patients (P < 0.05) (Fig. 1F). To verify the change of CREBRF expression in GC tissues, we examined its expression levels in 65 GC tissues and matched adjacent non-tumor tissues by qRT-PCR. The results showed that CREBRF mRNA expression was significantly up-regulated in GC tissues compared with normal tissues (P < 0.01) (Fig. 2A). The expression of CREBRF protein was remarkably higher in GC tissues than in normal gastric tissues (Fig. 2B). The correlations between the CREBRF mRNA levels and clinicopathologic characteristics of GC patients are summarized in Table 1. High CREBRF expression was associated with poor tumor histology [well: 68.0% (17/25); moderate: 86.4% (19/22); poor: 100% (18/18)] (P < 0.05), T stage [T1/T2: 73.4% (28/38); T3/T4: 96.3% (26/27)] (P < 0.01) and TMN stage [I/ II: 76.9% (30/39); III/IV: 92.3% (24/26)] (P < 0.01). However, the expression was not associated with gender, age, tumor size, lymph node metastasis and lymphatic invasion. Next, this trend was further verified through detecting established GC cell lines, including MKN-45, BGC-823 and SGC-7901. The results showed that CREBRF mRNA expression in GC cells was significantly higher than that in normal human gastric epithelial cell line (GES-1) (P < 0.01), and CREBRF protein expression was up-regulated (Fig. 2C, D). These findings suggest that CREBRF may play an important role in the progression of GC.

CREBRF silencing suppresses GC cell proliferation and induces G1-S arrest

As CREBRF expression was up-regulated in GC tissues and cell lines, we hypothesised that CREBRF might act as oncogene and that CREBRF silencing could affect GC progression. We specifically silenced the expression of CREBRF in SGC-7901 cells by transfecting cells with CREBRF siRNA. After CREBRF siRNA had been transfected into SGC-7901 cells, we analyzed knockdown efficiency of CREBRF siRNA in mRNA and protein levels. Our results showed that CREBRF mRNA and protein expression reduced significantly in CREBRF siRNA group compared to the NC-siRNA group (P < 0.01) (Fig. 3A, B). Next, MTT assay was performed to detect cell viability. CREBRF siRNA and NC-siRNA were transfected into GC SGC-7901 cells. We found that CREBRF silencing inhibited remarkably GC cell growth at 24, 48 and 72 hours after transfection (P < 0.01) (Fig. 3C). The cell cycle is involved in the regulation of cell proliferation, so we detected the processes by using a flow cytometer 48 hours after transfection. We found that the cell cycles were remarkably arrested at G1/G0 phase in CREBRF siRNA group (P < 0.01), and the proportion of S phase decreased evidently (P < 0.01) (Fig. 3D). To measure the possible effects of CREBRF siRNA on cell death, we observed the detection of apoptosis by Annexin-V/PI staining. The results

Table 1. Correlation between CREBRF mRNA expression and clinicopathologic characteristics of gastric cancer patients.

Characteristics	Number of cases	CREBRF expression		*P* value
		High (n = 54)	Low (n = 11)	
Gender				0.726
Male	42	34	7	
Female	23	19	4	
Age				0.859
≥60 years	21	18	3	
<60 years	44	36	8	
Histology				0.017*
Well	25	17	8	
Moderate	22	19	3	
Poor	18	18	0	
Tumor size				0.534
<50 mm	26	22	4	
≥50 mm	39	32	7	
Lymph node metastasis				0.313
Yes	40	34	6	
No	25	20	5	
Lymphatic invasion				0.658
Yes	46	38	8	
No	19	16	3	
T stage				0.003*
T1/T2	38	28	10	
T3/T4	27	26	1	
TNM Stage				0.007*
I/ II	39	30	9	
III/IV	26	24	2	

* P < 0.05

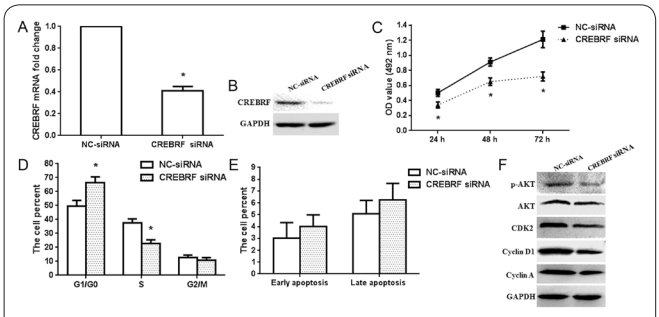

Figure 3. Silencing of CREBRF inhibits human GC SGC-7901 cell proliferation and induces G1-S arrest. (A) qRT-PCR was performed to measure CREBRF mRNA expression after transfection with CREBRF siRNA (*P < 0.01). (B) Western blot was performed to determine CREBRF protein expression after transfection. (C) MTT assay showed that CREBRF siRNA reduced the activity of SGC-7901 cells at 24, 48, and 72 hours (*P < 0.01). (D) Flow cytometry analysis showed the percentage of cells in the G1/G0, S and G2 phases. G1/G0 phase cells increased remarkably after CREBRF siRNA treatment (*P < 0.01). (E) Flow cytometry analysis showed the percentage of early and late apoptosis after transfection. (F) The expressions of AKT signaling pathway regulation proteins were analyzed in SGC-7901 cells at 24 hours after transfected with CREBRF siRNA by Western blot. GAPDH was used as a housekeeping control.

showed that there were no significant differences in apoptosis after transfection (Fig. 3E). Our data indicates that CREBRF could promote GC cell proliferation by regulating G1-S cell-cycle transition.

CREBRF silencing inhibits the AKT signaling pathway

To further study the potential molecular mechanism of CREBRF in the proliferation of GC SGC-7901 cells, the cells were transfected by CREBRF siRNA for 24 hours, and then the expressions of AKT signaling pathway were examined. No significant change was observed in the total protein expression of AKT, however, the expression of phosphorylated AKT decreased significantly in CREBRF siRNA group (Fig. 3F). Furthermore, we measured the expression of cell cycle regulators, including CDK2, Cyclin D1 and Cyclin A. The results revealed that CREBRF siRNA suppressed the expression of CDK2, Cyclin D1 and Cyclin A (Fig. 3F). The results suggest that CREBRF could activate the AKT signaling pathway in human GC SGC-7901 cells.

Activation of AKT signaling pathway eliminates the effects of CREBRF silencing on GC cells

To demonstrate that CREBRF exhibited oncogene function via AKT signaling pathway, we activated AKT signaling pathway by using p-AKT activator SC79 after CREBRF silencing. MTT assays showed that down-regulation of CREBRF expression led to suppression of GC cell growth, but activating of AKT eliminated the effect of CREBRF silencing on cell proliferation (P < 0.01) (Fig. 4A). Cell cycle assay also revealed that CREBRF silencing induced a remarkable increase of G1/G0 phase cells and a concomitantly significant decrease of S phase cells, CREBRF siRNA + SC79 was able to re-enter the S phase (P < 0.01) (Fig. 4B). The apoptotic

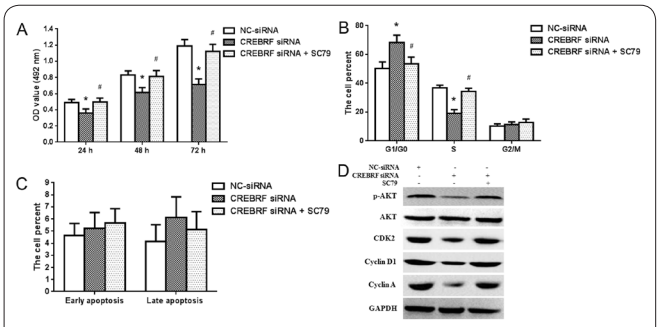

Figure 4. CREBRF promotes human GC SGC-7901 cell growth by activating AKT signaling pathway. (A) MTT assay showed that GC cell growth increased after treatment with CREBRF siRNA + SC79 in SGC-7901 cell (*P < 0.01, as compared with NC-siRNA group; #P < 0.01, as compared with CREBRF siRNA group). (B) Percentages of cells in the G1/G0, S and G2/M phases after treatment with CREBRF siRNA + SC79 (*P < 0.01, as compared with NC-siRNA group; #P < 0.01, as compared with CREBRF siRNA group). (C) Percentages of early-apoptotic and late-apoptotic cells after treatment with CREBRF siRNA + SC79. (D) The expressions of AKT signaling pathway regulation proteins were measured after treatment with CREBRF siRNA + SC79.

cells were no remarkable differences between CREBRF siRNA + SC79 group and CREBRF siRNA group (Fig. 4C). Our data also showed that the expression of p-AKT, CDK2, Cyclin D1 and Cyclin A up-regulated significantly in CREBRF siRNA + SC79 group compared to CREBRF siRNA group (Fig. 4D). These findings further demonstrate that CREBRF facilitates human GC cell proliferation by activating AKT signaling pathway.

Discussion

Carcinogenesis and progression of GC is a multifactorial and multistep process implicated in multiple epigenetic and genetic events, including the activation of oncogenes, inactivation of tumor suppressor genes and abnormal expression of cancer-related genes (12,13). Dysregulation of these genes has been shown to play a crucial role in regulating cell metabolism, survival, cycle, proliferation, differentiation, invasion, migration and apoptosis in cancer (14,15). Besides the cellular function of CREBRF, its role is largely unknown. Recently, it is reported that uteri of pregnant mice and embryos displayed enhanced CREBRF expression at all stages, and the expressed CREBRF was found to be localized specifically at implantation sites. CREBRF involved in the process of implantation in uteri and development of preimplantation embryos in mice (16). Furthermore, CREBRF regulated the malignant glioma progression through implicating hypoxia-induced glioblastoma cell autophagy (17). The main aim of this study was to explore the role of CREBRF and to elucidate the molecular mechanisms in human GC cells. In this experiment, for the first time, TCGA data and our results showed that CREBRF expression was up-regulated in GC tissues and associated with the clinicopathologic characteristics. Our experiments demonstrated that CREBRF silencing inhibited significantly GC cell proliferation, which suggests that CREBRF might be a novel oncogene.

G1 phase of cell cycle is a crucial stage when cells respond to environmental signals to regulate cell survival and proliferation. In the present study, we found that CREBRF silencing may induce G1/G0 to S phase cell cycle arrest. Cyclin A-CDK2 and Cyclin D-CDK4/6 protein kinase complexes, cell cycle regulators, possess the cellular progression by the G1/G0 to S phase of the cell cycle (18). The expressions of CDK4 and CDK6 are stable in a cell cycle-independent manner, whereas, the expression of Cyclins (D1, D2, and D3) fluctuates during the cell cycle, indicating that D-type Cyclins may play important roles in the regulation of cell cycle progression (19). D-Cyclins lead to release of the E2F transcription factors and drive cell entry into the S phase of cell cycle after the extracellular mitogenic stimulation. It is reported that Cyclin D1 is involved in human tumorigenesis (20). Cyclin A-CDK2 protein kinase complexes regulate cell growth and cell cycle in lung cancer and colon carcinoma (21). In this study, we demonstrate that CREBRF silencing inhibited the expression of Cyclin D1, Cyclin A and CDK2 in GC cells. These results indicate that CREBRF may drive cell entry into the S phase of cell cycle by regulating CDK2, Cyclin D1 and Cyclin A expressions.

PI3K/Akt signaling pathway is the primary mechanism for regulating cell survival, proliferation, metabolism, angiogenesis and malignant transformation in response to extracellular cues (22). The abnormity of AKT signaling pathway is involved in tumorigenesis and progression, including stomach, liver, breast, lung, prostate, colorectal and renal cancers (23). It is reported that the activation of AKT signaling pathway is associated with various clinicopathologic characteristics of cancer (24). AKT, a key downstream effector of the PI3K signaling pathway, regulates the function of generous substrates associated with cell cycle progression via directly phosphorylating the target proteins or indirectly regulating protein expression levels (23). AKT downstream regulating target genes Cyclin D1, Cyclin A and CDK2 are crucial transcriptional factors in the G0/G1 phase (25). We measured AKT and p-AKT expression, and found that CREBRF silencing inhibited AKT activation. After CREBRF silencing, activating of AKT rescued the effect of CREBRF silencing on cell proliferation by using SC79 (a AKT activator) (26), and drove cell re-enter into the S phase of the cell cycle. Our findings suggest that CREBRF may promote GC cell proliferation and induces G1-S phase transition via activating AKT signaling pathway.

In conclusion, our study demonstrated that CREBRF expression was up-regulated in GC tissues and associated with the clinicopathologic characteristics. CREBRF promoted GC cell proliferation through activating AKT signaling pathway. The findings provide evidence that CREBRF could be a useful marker in diagnosis of GC and a potential therapeutic target in therapy of GC.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

J.M. Han, L.Y. Zhao and C. Huang designed the study. L. Zhang and Q.Y. Jiang collected clinical data and sample. J.M. Han, L. Zhang, J. Zhang, D.D. Tong, L.Y. Zhao, X.F. Wang, and X. Gao performed the experiments. J.M. Han, L.Y. Zhao, performed the statistical analysis. J.M. Han, L. Zhang, L.Y. Zhao and C. Huang wrote and edited the manuscript. L.Y. Zhao, and C. Huang supervised the work.

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