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Original Research

FAM196B acts as oncogene and promotes proliferation of gastric cancer cells through AKT signaling pathway

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Abstract: Gastric cancer (GC) is the second leading cause of cancer-related deaths worldwide, but the mechanisms remain unknown. Here we report that family with sequence similarity 196 member B (FAM196B) is highly expressed in primary GC tissues and the expression level is correlated with the clinicopathologic characteristics of GC. In this experiment, knockdown of FAM196B suppressed GC cell proliferation and induced G1/G0 to S phase cell cycle arrest by regulating Cyclin D1, Cyclin A and CDK2 expressions. Furthermore, we investigated the molecular mechanism of FAM196B action in GC. The results showed that knockdown of FAM196B inhibited the activation of AKT signaling pathway. We further revealed that activating of AKT rescued the effect of FAM196B knockdown on cell proliferation and drove cell re-enter into the S phase of the cell cycle with SC79 (a AKT activator). Our findings demonstrated that FAM196B may promote GC cell proliferation by activating AKT signaling pathway. Taken together, this study provides a new evidence that FAM196B functions as a novel oncogene and could be a potential therapeutic target in therapy of GC.

Key words: FAM196B; Gastric cancer; Proliferation; Cell cycle; AKT signaling pathway.

Introduction

Gastric cancer (GC) is the fourth most common human malignant cancer and the second leading cause of cancer-related deaths in the world (1,2). There are approximately 723,000 GC-related deaths every year (3,4). Almost two thirds of cases occur in developing countries, particularly in China (5). Most patients are diagnosed in an advanced stage and the five-year overall survival rate is only 20%-30% (6,7). Thus, GC is a considerable health threat and burden to people. Currently, surgery, radiotherapy, chemotherapy, and multimodality therapy is used to treat patients with GC. Unfortunately, a satisfactory therapeutic effect has not been achieved because it is a highly complex disease. Gastric carcinogenesis and progression are involving many genetic and environmental factors, and are multistep processes (8-11). Although the study of GC has made great progress to date, the underlying molecular mechanisms are largely unclear yet. Therefore, it is of vital significance to explore the molecular mechanisms of GC for diagnosis and treatment.

Family with sequence similarity 196 member B (FAM196B) locates at 5q35.1 of human chromosome. It includes transcript variant 1 (NM_001129891, 5981 bp) and transcript variant 2 (NM_001346304, 5962 bp). It was reported that FAM196B expressed at higher levels in the embryonic ovary as compared to the testis during mouse gonad development (12). More recently, Feng and and colleagues reported that FAM196B was up-regulated in clear cell renal cell cancer cell lines by expression microarray (13). Emerging evidence suggests FAM196B may participate in cancer development. However, the role of FAM196B in malignancy has not been researched to date.

In this study, we analyzed the expression of FAM196B in GC tissues using data from the Cancer Genome Atlas (TCGA). We performed quantitative real-time PCR in 42 pairs of GC tissues and their normal tissues to evaluate FAM196B expression in GC. The role and molecular mechanism of FAM196B in GC were explored by a series of experiments. We found that FAM196B expression was significantly up-regulated in GC and the expression level was correlated with the clinicopathologic features, and FAM196B promoted GC cell proliferation by activating AKT signaling pathway. These findings may provide novel insight into the underlying mechanisms of GC, and suggest that FAM196B may serve as a promising molecular target for GC treatments.

Materials and Methods

Human tissue samples

Tissues were obtained from 42 GC patients treated at the Department of Oncology Surgery, the First Affiliated Hospital of Medical College in Xi'an Jiaotong University, PR China. The patients had not been pretreated with chemotherapy or radiotherapy prior to surgery. Clinicopathological data were obtained by reviewing their pathology records. Informed consent was obtained from each patients. The study was approved by the Ethical Committee of Xi'an Jiaotong University Health Science Center.

Cell culture

Normal human gastric epithelial cell line (GES-1) and human gastric cancer cell lines (BGC-823, MKN-45 and SGC-7901) were obtained from the Cell Bank (Shanghai Genechem Co., Ltd., Shanghai, China). These Cells were cultured in RPMI-1640 medium (Gibco BRL, NY, USA) supplemented with 10% FBS (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2.

RNA extraction and quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the cells and tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized according to the manufacturer's protocol (Takara, Dalian, China). qRT-PCR was performed using the SYBR Green PCR kit (Takara, Dalian, China). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for mRNAs. The primer sequences were as follows: FAM196B forward: 5'-TCTGCAGGTAATTC-CACTGCTTC-3'; FAM196B reverse: 5'-ATGTGGG-CACGGTGAGCTA-3'; GAPDH forward: 5′-GAAG-GTGAAGGTCGGAGTCA-3′; GAPDH reverse: 5′-TTGAGGTCAATGAAGGGGTC-3′. All reactions were performed in triplicate using an IQ5 Multicolor qRT-PCR Detection System (Bio-Rad, USA).

Immunohistochemistry (IHC)

The tissue samples were made into paraffin sections and pretreated with microwave, blocked, and incubated with primary antibodies against FAM196B (Santa Cruz, CA, USA) at a dilution of 1:200. The sections were then incubated with secondary antibody. Examination was performed using 3, 3'-diaminobenzidine (DAB) and hematoxylin. Finally, digital images were taken with a Leica Photo Microscope (Leica).

siRNA synthesis and transfection

siRNA was pre-designed for FAM196B gene silencing. Human FAM196B siRNA (sense 5'- GUAACUC-CAUACACCCUUUUU-3', antisense 5'- AAAGG-GUGUAUGGAGUUACUU-3') and negative siRNA (NC-siRNA, sense 5'-UUCUCCGAACGUGUCAC-GUUU-3', antisense 5'-ACGUGACACGUUCGGA-GAAUU-3') were synthesized by Shanghai GenePharma Corporation (SGC, Shanghai, China). Lipofectamine™-2000 (Invitrogen, Carlsbad, CA, USA) was used to optimize siRNA transfection according to the manufacturer's instructions. The siRNA complexes would be added to the plated cells and diluted to 60 nM in future experimental procedures.

MTT assay

MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]-based assay was used to evaluate the effect of FAM196B siRNA on SGC-7901 cell viability. Cells (5,000 cells/well in 200 μl medium) were seeded into 96-well plates and incubated for 24 hours. These cells were treated with NC-siRNA (60 nM), FAM196B siRNA (60 nM) or FAM196B siRNA (60 nM) + SC79 (AKT activator, 4 μg/ml) (MCE, USA) for 24, 48 and 72 hours, respectively. Then, 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) solution was added per well and the cells were incubated for another 4 h. Cell viability was assessed at 492 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). Each experiment contained 4 replicates and was repeated at least three times. Data obtained were presented as mean ± SEM.

Cell cycle analysis

SGC-7901 cells were cultured in 6-well plates for 24 hours and treated by NC-siRNA, FAM196B siRNA or FAM196B siRNA + SC79 for 48 hours. The cells were harvested and fixed in 75 % icecold ethanol overnight at 4 °C. The fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 20 min at room temperature. Cell-cycle distributions were analyzed by fluorescence-activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA, USA). Three parallel wells were made in each independent experiment, and the procedures were carried out in triplicate. The data were summarized as mean ± SEM.

Cell apoptosis analysis

Cells were incubated in 6-well plates for 24 hours and treated by NC-siRNA, FAM196B siRNA or FAM196B siRNA + SC79 for 48 hours. Then, the cells were harvested and stained with Annexin-V FITC Apoptosis Detection Kit (Invitrogen) according to the manufacturer's instructions. Cells were examined by using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA), and the apoptosis populations were determined by ModFit software.

Western blot analysis

GC tissues, normal gastric tissues and SGC-7901 cells were lysed in RIPA lysis buffer. Total protein was extracted and subjected to electrophoresis using 10 % SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated with primary antibodies overnight at 4 °C and then incubated with secondary antibody for 2 hours at room temperature. The primary monoclonal antibodies included rabbit monoclonal antibody (mAb) anti-FAM196B (1:2,000; Santa Cruz, CA, USA), rabbit mAb anti-Akt (1:1,000; Santa Cruz, CA, USA), rabbit mAb anti-phospho-AKT (1:2,000; Santa Cruz, CA, USA), rabbit mAb anti-CDK2 (1:500; Santa Cruz, CA, USA), mouse mAb anti-Cyclin D1 (1:1,000; Santa Cruz, CA, USA), mouse mAb anti-Cyclin A (1:1,000; Santa Cruz, CA, USA), mouse mAb anti-GAPDH (1:2,000; Cell Signaling

Technology). The membranes were incubated with ECL (Amersham) for chemiluminescence detection. The luminescent signal was scanned, recorded and quantified with Syngene GBox (Syngene, UK).

Statistical analysis

Each experiment was performed at least 3 times independently. The data were presented as mean ± SEM. Statistical analysis was performed with SPSS software (Abbott Laboratories, Chicago, IL). Student's t test, two-way ANOVA, and Pearson's chi-square test were used according to the data characteristics. $P < 0.05$ were considered to indicate statistical significance.

Results

FAM196B is significantly up-regulated in GC tissues and cell lines

To explore the role of FAM196B in GC progression, we first analyzed the expression of FAM196B in GC tissues. TCGA data showed that FAM196B expression was significantly higher in GC tissues than in normal gastric tissues ($P < 0.01$) (Fig. 1A), and the higher expression of FAM196B was positively correlated with decreased overall survival in these patients ($P < 0.05$) (Fig. 1B). In addition, the bioinformatics data also showed that FAM196B expression was associated with poor tumor histology ($P < 0.05$) and T stage ($P < 0.01$) (Fig. 1C, D). To verify the change of FAM196B expression in GC tissues, we examined its expression levels in 42 GC tissues and matched adjacent non-tumor tissues (> 5 cm) by qRT-PCR. The results showed that FAM196B mRNA expression was significantly up-regulated in GC tissues compared with normal tissues ($P < 0.01$) (Fig. 2A). The correlations between the FAM196B mRNA levels and clinicopathologic characteristics of the involved GC patients are summarized in Table 1. High FAM196B expression was associated with poor tumor histology [well: 68.4% (13/19); moderate: 81.8% (9/11); poor: 91.7% (11/12)] ($P < 0.05$) and T stage [T1/

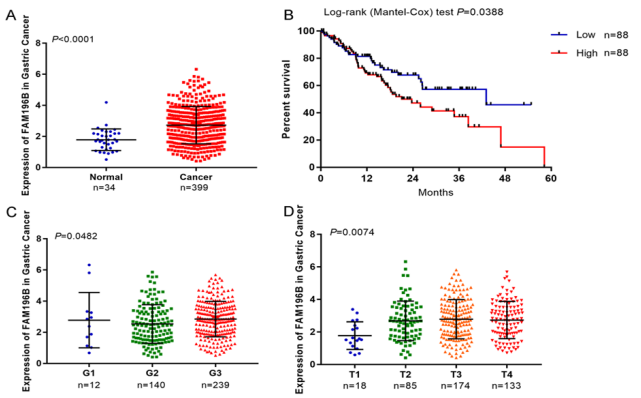

Figure 1. TCGA data show that FAM196B expression is up-regulated in GC tissues and is correlated with the clinicopathologic parameter of GC patients. (A) FAM196B expression in GC patients using data from TCGA. (B) Panels represent patient survival estimation datasets of FAM196B high expression correlation group compared with low expression correlation group from TCGA. (C) Correlation between FAM196B expression and poor tumor histology in GC patients using data from TCGA. (D) Correlation between FAM196B expression and T stage in GC patients using data from TCGA.

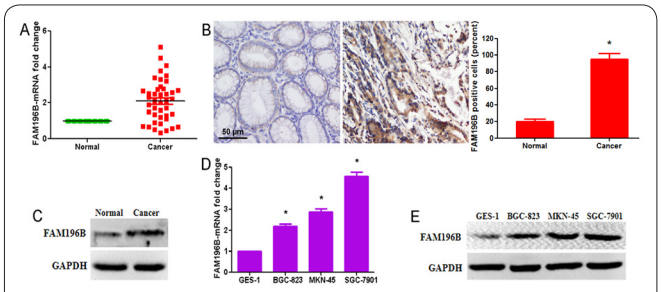

Figure 2. FAM196B is up-regulated in GC tissues and cell lines. (A) FAM196B mRNA expression in GC tissues versus normal gastric tissues (n = 42; $P < 0.01$). (B) IHC staining showed that FAM196B protein expression in GC tissues and normal tissues (n = 42). (C) Western bot showed that FAM196B protein expression in GC tissues and normal tissues (n = 12). (D) FAM196B mRNA expression in GC cell lines (BGC-823, MKN-45 and SGC-7901) and normal human gastric epithelial cell line (GES-1) (*$P < 0.01$). (E) FAM196B protein expression in GC cell BGC-823, MKN-45, SGC-7901 and GES-1, with GAPDH as an internal control.

Table 1. Correlation between FAM196B mRNA expression and clinicopathologic parameter of gastric cancer patients.

Characteristics	Number of cases	FAM196B expression		*P* value
		High (n =33)	Low (n= 9)	
Age				0.903
≥60 years	15	11	4	
<60 years	27	22	5	
Gender				0.521
Male	28	21	7	
Female	14	12	2	
Histology				0.018
Well	19	13	6	
Moderate	11	9	2	
poor	12	11	1	
Tumor size				0.127
<50 mm	19	13	6	
≥50 mm	23	20	3	
Lymph node metastasis				0.329
Yes	29	24	5	
No	13	9	4	
Lymphatic invasion				0.853
Yes	30	24	6	
No	12	9	3	
T stage				0.006
T1/T2	24	16	8	
T3/T4	18	17	1	
TNM Stage				0.236
I/ II	27	22	5	
III/IV	15	11	4	

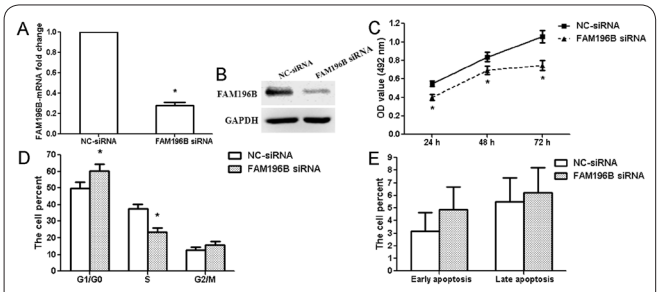

Figure 3. Silencing of FAM196B suppresses GC SGC-7901 cell proliferation and induces G1–S arrest. (A) qRT-PCR was performed to determine FAM196B mRNA expression after transfection with FAM196B siRNA (*P < 0.01). (B) Western blot was performed to examine FAM196B protein expression after transfection. (C) MTT assay showed that FAM196B siRNA decreased the activity of SGC-7901 cells at 24, 48, and 72 hours (*P < 0.01). (D) The results of flow cytometry analysis showed the percentage of cells in the G1/G0, S, and G2 phases. G1/G0 phase cells significantly increased after FAM196B siRNA treatment (*P < 0.01). (E) The data showed the percentage of early apoptosis and late apoptosis after transfection.

T2: 66.7% (16/24); T3/T4: 94.4% (17/18)] (P < 0.01). However, the expression was not associated with age, gender, tumor size, lymph node metastasis, lymphatic invasion, and TNM stage. The expression of FAM196B protein was significantly higher in GC tissues than in normal gastric tissues (Fig. 2B, C). This trend was further verified through detecting three established GC cell lines, including BGC-823, MKN-45 and SGC-7901. The results showed that FAM196B mRNA expression in GC cells was markedly higher than that in normal human gastric epithelial cell line (GES-1) (P < 0.01), and FAM196B protein expression was up-regulated (Fig. 2D, E). Taken together, FAM196B is up-regulated in GC tissues, suggesting that FAM196B may play an important role in the progression of GC.

FAM196B knockdown inhibits GC cell proliferation and induces cell cycle arrest

As FAM196B was expressed at high levels in GC tissues compared with normal gastric tissues, we hypothesised that FAM196B might act as oncogene and that knockdown of FAM196B expression could affect GC progression. We specifically silenced the expression of FAM196B in SGC-7901 cells by transduced cells with FAM196B siRNA. After FAM196B siRNA had been transfected into SGC-7901 cells, we analyzed knockdown efficiency of FAM196B siRNA in mRNA and protein levels. The results showed that FAM196B mRNA and protein expression decreased significantly in FAM196B siRNA groups compared to the NC-siRNA group (P < 0.01) (Fig. 3A, B). MTT assay was performed to measure cell viability. FAM196B siRNA and NC-siRNA were transfected into human GC SGC-7901 cells. We found that silencing of FAM196B significantly suppressed cell proliferation at 24, 48, and 72 hours after transfection (P < 0.01) (Fig. 3C). The cell cycle is involved in the regulation of cell growth, so we measured the processes using a flow cytometer 48 hours after transfection. The cell cycles were arrested significantly at G1/G0 phase in FAM196B siRNA group (P < 0.01) (Fig. 3D). To examine the possible effects of FAM196B siRNA on cell death, we observed the measurement of apoptosis by Annexin-V/PI staining. We found that there were no significant differences in apoptosis after transfection (Fig. 3E). These results indicate that FAM196B could promote GC cell proliferation by controlling cell cycle.

Silencing of FAM196B suppresses AKT signaling pathway

To further investigate the potential molecular mechanisms of FAM196B in the proliferation of SGC-7901 cells, the cells were treated by FAM196B siRNA for 24 hours, and then the expressions of AKT signaling pathway were measured. No significant change was observed in the total protein expression of AKT, but the expression of phosphorylation of AKT evidently decreased in FAM196B siRNA group (Fig. 4A). Furthermore, we examined the expression of cell cycle regulators, including Cyclin D1, Cyclin A and CDK2. We found that FAM196B siRNA inhibited the expression of Cyclin D1, Cyclin A and CDK2 (Fig. 4A). The data suggests that FAM196B activates AKT signaling pathway in GC SGC-7901 cells.

Activation of AKT eliminates the effects of FAM196B knockdown on GC cells

To demonstrate that FAM196B exhibited oncogene function through AKT signaling pathway, we activated AKT signaling pathway with p-AKT activator SC79 after FAM196B knockdown. The results showed that the p-AKT expression increased in FAM196B siRNA + SC79 group compared to FAM196B siRNA group (Fig. 4B). MTT assays revealed that down-regulation of FAM196B expression resulted in suppression of GC cell proliferation, and activating of AKT rescued the effect of FAM196B knockdown on cell proliferation (P < 0.01) (Fig. 4C). Cell cycle assay also showed that silencing FAM196B induced a significant increase of G1/G0

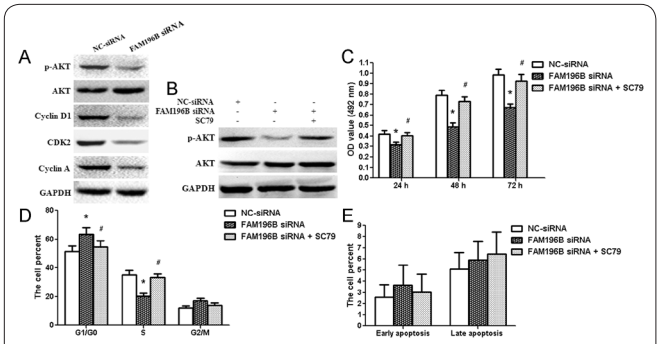

Figure 4. FAM196B promotes GC SGC-7901 cell proliferation by activating AKT signaling pathway. (A) Expression analysis for AKT signaling pathway regulation proteins in SGC-7901 cells at 24 hours after transfected with NC-siRNA or FAM196B siRNA by Western blot analysis. GAPDH was used as a housekeeping control. (B) The expression of p-AKT was examined after treatment with FAM196B siRNA + SC79. (C) MTT assay showed GC-cell proliferation after treatment with FAM196B siRNA + SC79 in SGC-7901 cell ($^{*}p < 0.01$, as compared with NC-siRNA group; $^{\#}p < 0.01$, as compared with FAM196B siRNA group). (D) Percentages of cells in the G1/G0, S, and G2/M phases after treatment with FAM196B siRNA + SC79 ($^{*}p < 0.01$, as compared with NC-siRNA group; $^{\#}p < 0.01$, as compared with FAM196B siRNA group). (E) Percentages of early-apoptotic and late-apoptotic cells after treatment with FAM196B siRNA + SC79.

phase cells and a concomitantly remarkable decrease of S phase cells, FAM196B siRNA + SC79 was able to re-enter the S phase ($P < 0.01$) (Fig. 4D). The apoptotic cells were no significant differences between FAM196B siRNA + SC79 group and FAM196B siRNA group (Fig. 4E). These results further demonstrate that FAM196B promotes GC cell proliferation by activating AKT signaling pathway.

Discussion

The main aim of this study was to explore the role of FAM196B and to elucidate the molecular mechanisms of action in human GC cells. Gastric carcinogenesis is a multifactorial process involved in multiple genetic and epigenetic events. As we know, dysregulation of oncogenes and anti-oncogenes has been shown to play an important role in control cell proliferation, survival, cell cycle, migration, invasion, and apoptosis in cancer (14-16). At present, it was only found that FAM196B was up-regulated in renal carcinoma (13). DNA methylation of FAM196B showed high differences in breast cancer (17) Our previous study found that the oncogene MeCP2 could bind to the methylated CpG islands in the promoter regions of FAM196B in GC (8), which implied that FAM196B might participate in GC development and progression. In the present study, for the first time, our results and TCGA data all showed that FAM196B expression was up-regulated in GC tissues and associated with poor tumor histology and T stage. Our studies demonstrated that FAM196B knockdown significantly suppressed GC cell proliferation, which indicates that FAM196B might be a novel oncogene.

The first gap (G1) phase of cell cycle is an important stage when cells respond to environmental signals to determine cell fate such as survival, proliferation and cellular senescence. In this experiment, we found that silencing FAM196B may induce G1/G0 to S phase cell cycle arrest. Important cell cycle regulators include Cyclin A-CDK2 and Cyclin D-CDK4/6 protein kinase complexes, which govern the cellular progression through the G1/G0 to S phase of the cell cycle (18). CDK4 and CDK6 are stably expressed in a cell cycle-independent manner, but the expression of Cyclins (D1, D2, and D3) fluctuates during the cell cycle, suggesting that the roles of different D-type Cyclins are key in the regulation of cell cycle variations (19). After the extracellular mitogenic stimulation, D-Cyclins lead to release of the E2F transcription factors and drive cell entry into the S phase of the cell cycle. It was found that Cyclin D1 is involved in the human tumorigenesis (20). Cyclin A-CDK2 protein kinase complexes regulate cell proliferation, survival, and cell cycle in lung carcinoma, renal carcinoma and colon carcinoma (21,22). Our results showed that silencing FAM196B suppressed the expression of Cyclin D1, Cyclin A and CDK2 in GC cells. These results suggest that FAM196B may drive cell entry into the S phase of cell cycle by regulating Cyclin D1, Cyclin A and CDK2 expressions.

PI3K/AKT signaling pathway is one of the most potent prosurvival pathways in cancer (23). The aberration of AKT signaling pathway has been associated with numerous cancers, including stomach, breast, lung, prostate, glioblastoma, and liver cancers (24). Some investigators have reported correlations between AKT activity and various clinicopathologic characteristics (25). In addition, AKT activation has been found to correlate with advanced disease and poor prognosis in some tumor types. AKT, a critical downstream effector of the P13K signaling pathway, regulates the function of generous substrates associated with cell cycle progression, either by direct phosphorylation of the target proteins or, indirectly, by controlling protein expression levels (24). AKT downstream target genes Cyclin D1, Cyclin A and CDK2 are key transcriptional factors in the G0/G1 phase (26). Therefore, we examined AKT and p-AKT expression, and found that FAM196B knockdown inhibited AKT activation. After FAM196B knockdown, activating of AKT rescued the effect of FAM196B knockdown on cell proliferation by using SC79 (a AKT activator) (27), and drove cell re-enter into the S phase of the cell cycle. These findings suggest that FAM196B may promote GC cell proliferation by activating AKT signaling pathway.

In summary, our study revealed, for the first time that FAM196B expression was up-regulated in cancer tissues and associated with poor tumor histology and T stage of GC. FAM196B promote GC cell proliferation through activating AKT signaling pathway. Our findings provide evidence that FAM196B could be a useful marker in prognosis of GC and a promising therapeutic target in therapy of GC.

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Conflict of interest

All the authors have no declaration of conflict of interest.

Author contributions

J. Zhang, L.Y. Zhao, and C. Huang designed the experiments. M. Xue, and Q.Y. Jiang collected clinical data and sample. J. Zhang, D.D. Tong, L.Y. Zhao, M. Xue, X.F. Wang, P.B. Yang, and L. Ni performed the experiments. J. Zhang, and D.D. Tong, performed the statistical analysis. J. 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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