ORIGINAL RESEARCH

GJB4 promotes gastric cancer cell proliferation and migration via Wnt/CTNNB1 pathway

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Background: Gap junction beta-4 protein (GJB4), or connexin 30.3, a member of integral membrane proteins, has been shown to involve and may function as a tumor promoter in tumorigenesis. However, the role of GJB4 in gastric cancer (GC) is still unclear.

Materials and methods: We used Progression-free survival Kaplan-Meier analysis and Western blot analysis to detect the expression of GJB4 in GC tissues and cells. In addition, both in vitro and in vivo assays were used to determine the effect of GJB4 on malignant behavior in GC cells.

Results: We found that GJB4 was overexpressed in gastric cancer tissues and cells compared with normal tissues and cells. The high GJB4 expression was significantly associated with poor overall survival of GC patients. Knocking down GJB4 in GC cells significantly suppressed cell proliferation and migration. We found that the effects of GJB4-knockdown on GC cells were associated with downregulation of CTNNB1 and its downstream MYC, MMP7 and CCND1 expression. In addition, we found that the promotive effect of GJB4 overexpression on cell proliferation and migration was negated by XAV-939, which is the inhibitor of Wnt/CTNNB1 pathway. Therefore, we revealed a novel mechanism by which GJB4 could activate the Wnt/CTNNB1 pathway to promote GC cell's proliferation and migration.

Conclusion: This study offer insights into GJB4 function and indicate that GJB4 is a promising biomarker and therapeutic target for gastric cancer patients.

Keywords: GJB4, cell proliferation, migration, gastric cancer, CTNNB1

Introduction

Gastric cancer (GC) is the most gastrointestinal malignancy and the second most common cause of cancer-related death worldwide, with an estimated 700,000 mortalities annually worldwide.[1–3] Various factors contribute to the pathogenesis of GC, including the genetic background of patients and environment factors.[4] Despite great advances in the diagnosis and therapy of patients with GC, the prognosis of GC patients remains poor with a 6-month survival rate of <15%.[5] Therefore, it is of great urgent to explore the molecular mechanisms underlying gastric cancer progression and develop novel therapeutic targets for improving the treatment for GC patients.

Gap junctions are transmembrane channels, which mediate the transfer of small molecules between the cytoplasm of neighbouring cells.[6] Gap junctions play an important role in cell cycle, cell differention, migration and invasion by regulation of signal transduction.[7–10] They are formed by proteins named connexins, which are homologous four-transmembrane-domain proteins, belong to a family of 21

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isotypes in human cells.[11] Among these isotypes, the amino acid sequences of transmembrane domains are highly conserved, whereas the intracellular carboxyl-terminal regions are highly variable.[12,13] GJA1 (connexin 43) and GJB2 (connexin 26) are the two of the most studied gap junction protein, have been shown to be expressed at higher levels in tumors.[14,15] High levels of GJA1 and GJB2 are reported to be associated with cell migration, invasion, and poor prognosis. In addition, GJA1 could also confer the chemoresistance of glioblastoma cells to temozolomide via activating pro-survival pathway.[16,17]

Gap junction beta-4 protein (GJB4), or connexin 30.3, has been recently reported that could promote metastasis and chemoresistance via Src activation in lung cancer.[18] However, the expression profile and biological function of GJB4 in GC remains largely unidentified.

In this study, we demonstrated that GJB4 was commonly expressed in GC cells. We found that downregulation of GJB4 efficiently suppressed cell proliferation, migration and tumorgenesis by regulating the Wnt/CTNNB1 pathway in GC cells. These data offer insights into GJB4 function and indicate that GJB4 as a potential target for GC patients.

Materials and methods
Cell culture and transfection
Human normal gastric mucosa epithelial cells GES-1, gastric cancer cell lines (MKN-45, SGC-7901, BGC-823 and HGC-27) and a retroviral packaging cell line (293FT) were purchased from the American Type Culture Collection (ATCC) (USA) and cultured as previously described.[19] GJB4-specific short hairpin RNA (shGJB4) and GFP-specific short hairpin RNA (shGFP) were purchased from GenePharma Co., Ltd (Shanghai, China), and coloned into the pLKO.1 vector. Sequences of the shGJB4 are given in Table 1. Vector encoding of human GJB4 were constructed by PCR-based amplification and subsequently subcloned into the pCDH-CMV-MCS-EF1-copGFP vector. Sequences of the primers used are given in Table 2. Lentivirus was produced as previously described.[19]

Cell proliferation analysis
Cell viability was assessed using the MTT assay. Cells (1×10^3 wells) were seeded into 96-well plates, and then were detected at the point from day 0 to 6. All experiments were carried out indenpendently in triplicate.

BrdU incorporation assay
Cells (2×10^4 cells/well) were seeded on coverslips in 24-well plates and incubated overnight. After treatment with 10 μg/ml BrdU (Sigma) for 30 min, cells were incubated sequentially with primary antibody against BrdU overnight and appropriate secondary antibody for 2 hrs. DAPI was added for nuclear staining. BrdU positive cells were observed and calculated from microscopy fields (Nikon 80i, Nikon Corporation, Tokyo, Japan).

Flow cytometry
For cell cycle analysis, cells were harvested and then fixed in 75% ethanol at 4°C for 24 h, and then incubated with propidium iodide (PI) and RNaseA at 37°C for 30 min.

Cells were examined by flow cytometry (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo software (version FlowJo 7.6; FlowJo LLC, Ashland, OR, USA).

Immunofluorescent analysis
Cells (1×10^4 cells/well) were grown on coverslips. After incubation with 4% paraformaldehyde for 20 min and 0.25% Triton X-100 for 15 min, the cells were incubated sequentially with primary antibody against MKI67 (Abcam) or PCNA (Abcam) overnight and appropriate secondary antibody for 2 hrs. Cell nuclei were stained with DAPI for 30 min. Cells were imaged and calculated from randomly chosen microscopic fields at X20 magnification (Nikon 80i; Nikon Corp.).

Transwell assay
24-well Boyden chambers (8 μm pore size, Corning) were used in the Transwell assay. Medium with 10% FBS as a chemoattractant was added to the lower chamber, and cells with serum-free media were placed in the upper chamber.

Table 1 Sequence of GJB4-specific shRNA

shGJB4-1-F	CCGGTGTATATGGCAACAGTATATGCTCGAGCATATACTGTTGCCATATACATTTTTG
shGJB4-1-R	AATTCAAAAATGTATATGGCAACAGTATATGCTCGAGCATATACTGTTGCCATATACA
shGJB4-2-F	CCGGCCACACTGTGGACTGTTACATCTCGAGATGTAACAGTCCACAGTGTGGTTTTTG
shGJB4-2-R	AATTCAAAAACCACACTGTGGACTGTTACATCTCGAGATGTAACAGTCCACAGTGTGG

Abbreviations: shGJB4, short hairpin-Gap junction beta-4.

Table 2 Primer pairs for real-time PCR

Gene	Sequence (5′-3′)
GJB4-F	TCCCTGTACGACAACCTGAG
GJB4-R	CGGTGGAAGATATAGAGGAAGCC
c-Myc-F	GTCAAGAGGCGAACACACAAC
c-Myc-R	TTGGACGGACAGGATGTATGC
MMP7-F	GAGTGAGCTACAGTGGGAACA
MMP7-R	CTATGACGCGGGAGTTTAACAT
CyclinD1-F	GCTGCGAAGTGGAAACCATC
CyclinD1-R	CCTCCTTCTGCACACATTTGAA
GAPDH-F	GGAGCGAGATCCCTCCAAAAT
GAPDH-F	GGCTGTTGTCATACTTCTCATGG

Abbreviations: GJB4, gap junction beta-4; RT-PCR, real time-polymerase chain reaction; c-MYC, MYC proto-oncogene; CyclinD1, G1/S-specific cyclin-D1; MMP7, matrix metalloproteinase-7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Cells were fixed in 4% paraformaldehyde (PFA) and then stained with crystal violet. Cells were imaged and calculated from randomly chosen microscopy fields (Nikon 80i, Nikon Corporation, Tokyo, Japan).

Wound healing assay

For the wound healing assay, cells were seeded in 6-well plates and allowed to grow to full confluence. Wounds were made using 10- μl pipette tip, and the wound healing process was monitored under a microscope.

Western blot

Western blotting was performed as previously described.[19] The primary and conjugated secondary antibodies used are as follows: GJB4 (Abcam), CTNNB1 (Abcam), CCND1 (CST), MMP7 (CST), MYC (Abcam), GAPDH (Proteintech).

Quantitative real-time PCR

Total RNA was extracted from gastric cell lines using TRIzoL Reagent (Invitrogen) and then reverse transcribed into cDNA for each sample. The expression of mRNA was defined based on Cq, and the individual values were normalized to that of the GAPDH control. All primer pairs were shown in Table 3.

Colony formation assay

To evaluate colony-forming ability, human gastric cancer cell lines (HGC-27 and SGC-7901) cells (1×10^3 wells)

Table 3 Primers pairs for human full-length GJB4

GJB4-F	ATGAACTGGGCATTTCTGC
GJB4-R	TTATGGATACCCACCTGCAT

Abbreviation: GJB4, gap junction beta-4.

stably transfected with shGFP and shGJB4 respectively were plated six-well plates with RPMI-1640 medium and 10% FBS for growth analysis. After culturing for 10 days, cells were fixed, stained with 0.1% crystal violet, and imaged and calculated in each well.

Xenograft assay

4-week-old Female NOD/SCID mice were purchased and housed in SPF room that was maintained at a constant temperature and humidity. Human gastric cancer cell lines (SGC-7901) cells (1×10^6 cells) stably transfected with shGFP and shGJB4 respectively were injected subcutaneously into the right flanks of 4-week-old Female NOD/SCID mice (6 mice/group). The tumors volumes were calculated daily based on caliper measurements of the tumor length and width. After 21 days of tumor growth, the mice were sacrificed by CO_2 inhalation, and the tumors were excised and measured. All studies were approved by the Animal Care and Use Committee of Chongqing Three Gorges Medical College, and carried out in conformity to the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006).

Statistical analysis

All experiments were carried out at least three triplicates. Statistical analysis was performed by Graph Pad Prism 5 system and the quantitative data were expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and a value of $p < 0.05$ was considered statistically significant.

Results

GJB4 is markedly upregulated in GC and is a prognostic indicator of patients with GC

To explore whether GJB4 could be a prognostic marker for GC, We performed a data analysis of public data sets, which indicated that GJB4 expression in GC tissues was higher than that in normal stomach tissues (Figure 1A and B). Moreover, the clinical significance of GJB4 expression was further evaluated by a Progression-free survival Kaplan-Meier analysis, which demonstrated that high expression of GJB4 was significantly associated with poor overall survival in patients with GC (Figure 1C). In addition, the Lauren classification divides gastric adenocarcinoma into intestinal, diffuse and mixed types on the basis of histology. Our analysis demonstrated that high GJB4 expression was also a poor prognostic factor in intestinal, disuse and mixed Lauren types GC (Figure 1D, E and Figure S1). To further explore the expression of GJB4 in
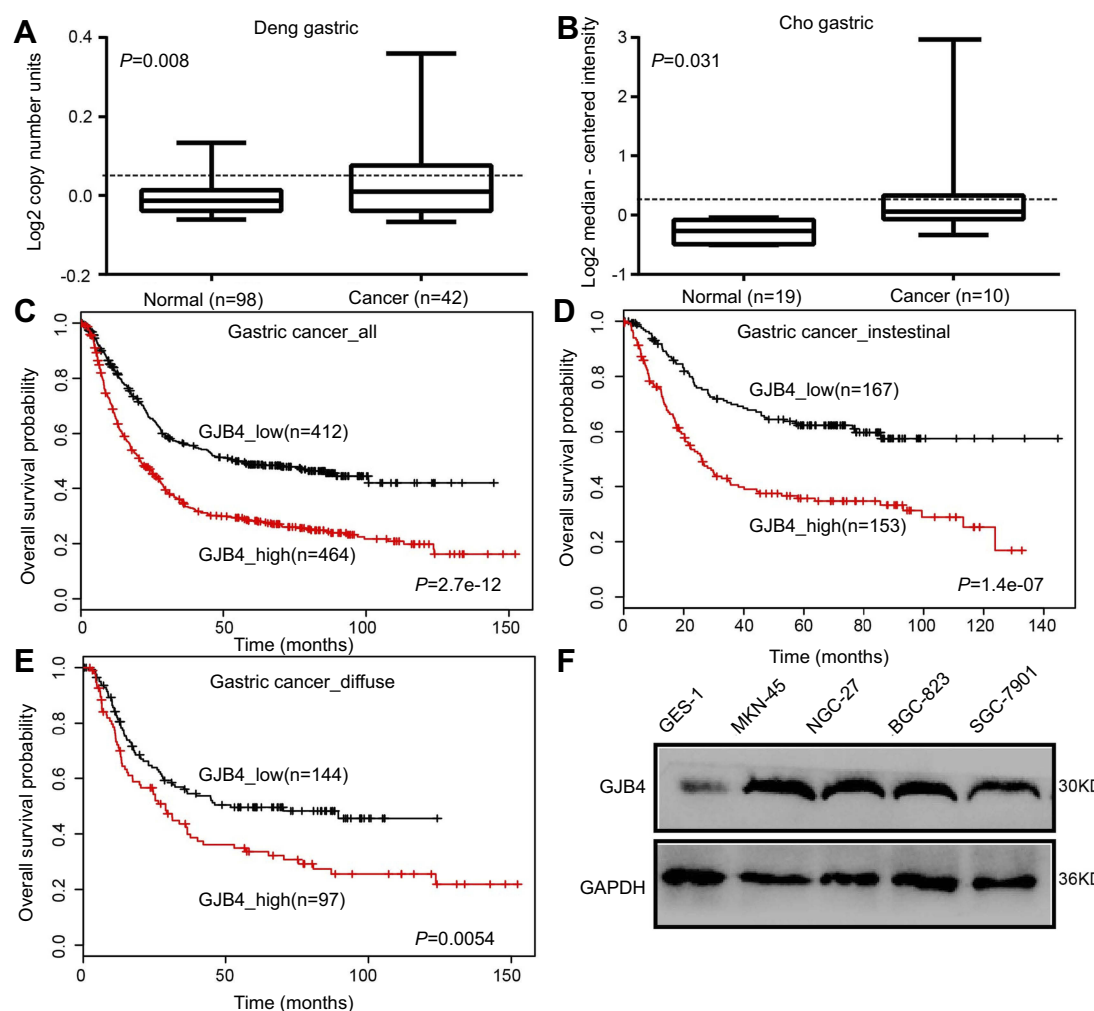
Figure 1 GJB4 is markedly upregulated in GC and is a prognostic indicator of patients with GC.

Notes: (**A, B**) Box plots of GJB4 expression levels in GC patients. GJB4 expression levels were downloaded from public data sets (Deng Gastric and Cho Gastric from Oncomine). (**C**) Kaplan-Meier overall survival for GJB4 expression in all Lauren types gastric cancer tumours (KM plotter gastric cancer dataset). (**D, E**) Kaplan-Meier overall survival for GJB4 expression in intestinal and disuse Lauren types gastric cancer tumours (KM plotter gastric cancer dataset). (**F**) Western blot analyses were performed to detect GJB4 expression in human gastric epithelial cell lines (GES-1) and four GC cell lines (HGC-27, SGC-7901, MKN-45, BGC-823). GAPDH served as endogenous and loading control.

Abbreviations: GC, gastric cancer; GJB4, gap junction beta-4.

GC, we detected GJB4 expression at the protein level in human gastric epithelial cell lines (GES-1) and four GC cell lines (HGC-27, SGC-7901, MKN-45, BGC-823). Compared with GES-1 cells, GJB4 expression was significantly increased in all four GC cell lines (Figure 1F). Taken together, we found that GJB4 was overexpressed in GC tissues and cell lines, and high GJB4 expression was associated with the poor prognosis of patients with GC.

GJB4 knockdown suppresses cell proliferation in GC cells

To investigate the function of GJB4 in GC cells, two shRNA sequences were used to knockdown GJB4 in HGC-27 and SGC-7901 cells, shGFP as a control (Figure 2A and B).

MTT assays showed that knockdown GJB4 significantly inhibited the growth of HGC-27 and SGC-7901 cells (Figure 2C and D). In addition, we performed BrdU, MKI67 and PCNA staining and found that the positive cells were significantly reduced after GJB4 knockdown (Figure 2E, F and Figure S2). Flow cytometry was performed to corroborate the data of proliferation, and the results showed that GJB4 knockdown led to cell cycle arrest at the G1 phase (Figure 2G and H).

GJB4 knockdown inhibits cell migration in GC cells

To determine whether GJB4 was related to the migration of GC cells, we performed Transwell assays. Then, the results
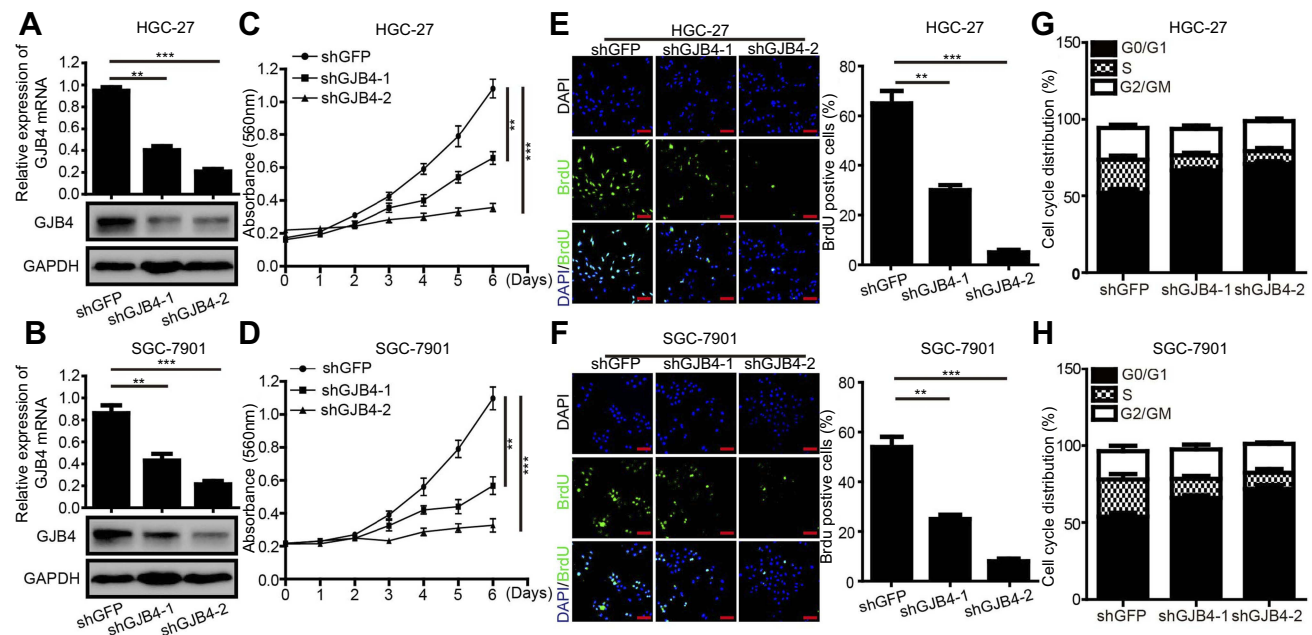
Figure 2 GJB4 knockdown suppresses cell proliferation in GC cells.
Notes: (**A, B**) The expression of GJB4 mRNA (upper) and protein (lower) in GJB4 knockdown HGC-27 and SGC-7901 cell lines and negative controls were detected by RT-PCR and Western blot analysis. GAPDH served as endogenous and loading control. (**C, D**) GC cell proliferation was measured by MTT assay. (**E, F**) BrdU incorporation assay was performed to examine the percentage of cells in S-phase. (**G, H**) Flow cytometric analysis of cell cycle distribution in HGC-27 and SGC-7901 cell lines. All data are shown as the means ± SD, *$P<0.05$, **$P<0.01$, ***$P<0.001$. All p-values are based on control versus treatment.
Abbreviations: GC, gastric cancer; GJB4, gap junction beta-4; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; BrdU, 5-Bromo-2-deoxyUridine.

showed that GJB4 knockdown significantly inhibited cell migration compared to controls (Figure 3A and B). In addition, wound healing assays demonstrated that the migratory ability was decreased in GJB4-knockdown cells (Figure 3C and D). These data indicated that downregulation of GJB4 significantly inhibited cell migration of GC cells.

GJB4 mediates cell proliferation and migration by regulating Wnt/CTNNB1 pathway

Wnt/CTNNB1 plays a key role in cell proliferation and migration in GC cells. To identify whether GJB4 influences the Wnt/CTNNB1 pathway, we performed the RT-PCR and Western blot analysis to examine the expression of CTNNB1 and its downstream molecule (MYC, MMP7 and CCND1) in GJB4-knockdown cells and control cells. We found that downregulation of GJB4 in the HGC-27 and SGC-7901 cells significantly reduced the mRNA and protein expression levels of CTNNB1, MYC, MMP7 and CCND1 (Figure 4A and B). XAV-939 is a potent Wnt/CTNNB1 inhibitor that inhibits proliferation and metastasis of cancer cells. To confirm whether the effects of GJB4 on GC cells were Wnt/CTNNB1 dependent, we treated GJB4 overexpression GC cells (HGC-27 and SGC-7901 cells) and their controls cells

with XAV-939. The accelerative effects of GJB4 on cell proliferation and migration were significantly blocked after XAV-939 treatment (Figure 4C, D and Figure S3A). In addition, Western blot analysis demonstrated that the protein levels of CTNNB1 and its downstream molecule (MYC, MMP7 and CCND1) were dramatically increased by GJB4 overexpression and were reduced by XAV-939 treatment (Figure 4E and F). Collectively, these results provided evidence that GJB4 mediates cell proliferation and migration by regulating Wnt/CTNNB1 pathway.

GJB4 knockdown inhibits self-renewal and tumor growth of GC cells

To explore the effects of GJB4 expression on colony formation of GC cells in vitro, colony formation assay was performed. As shown in Figure 5A and B, the colonies were smaller and fewer in GJB4-knockdown HGC-27 and SGC-7901 cells compared with the controls. In addition, the xenograft tumor growth assay was performed to elucidate the effect of GJB4 on GC growth in vivo. The results showed that GJB4-knockdown tumors were smaller than the controls in appearance, weight and volume (Figure 5C and D). Next, Western blot analysis showed that the protein level of GJB4, CTNNB1 and its
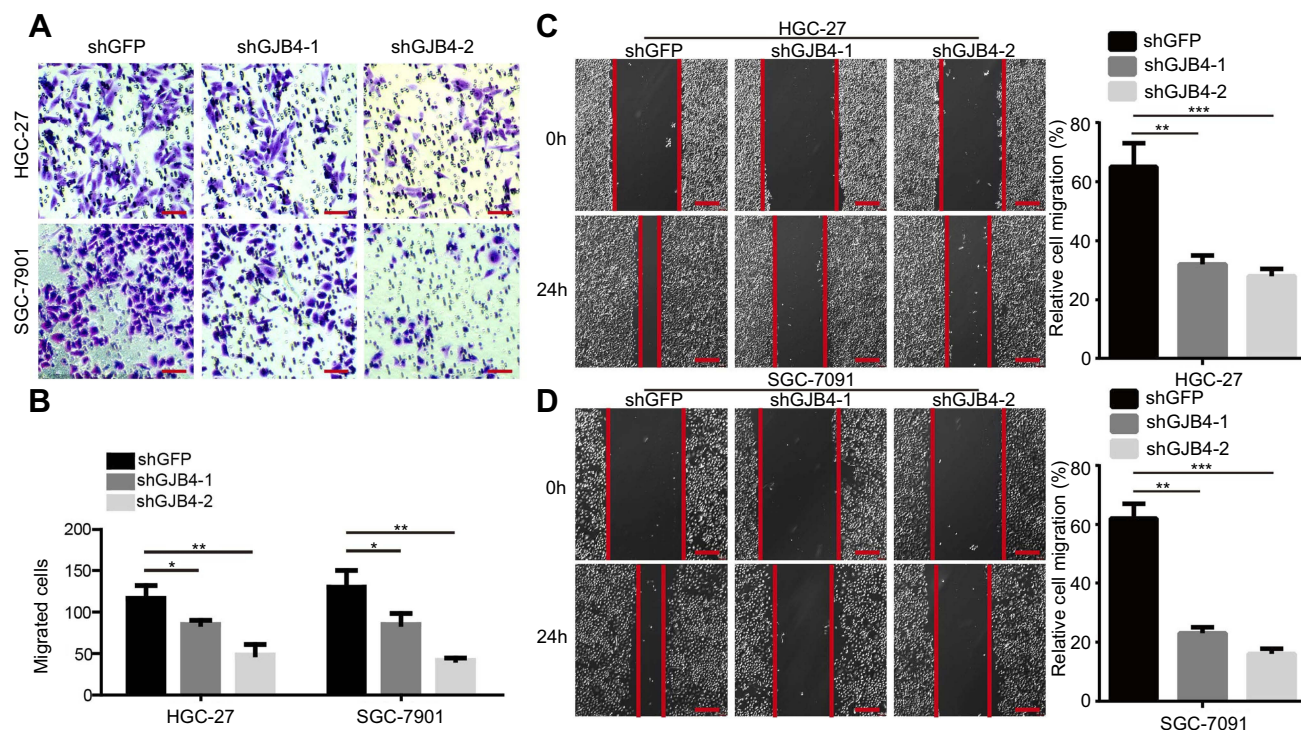
Figure 3 GJB4 knockdown inhibits cell migration in GC cells.
Notes: (**A, B**) Transwell assay was performed in GJB4-knockdown HGC-27 and SGC-7901 cell lines and negative controls. (**C, D**) Wound healing assay was performed in GJB4-knockdown cells. All data are shown as the means ± SD, *$P<0.05$, **$P<0.01$, ***$P<0.001$. All p-values are based on control versus treatment.
Abbreviations: GC, gastric cancer; GJB4, gap junction beta-4.

downstream gene were significantly changed in GJB4-knockdown tumors (Figure 5E). Taken together, these in vitro and in vivo results indicated that GJB4 potently promotes the tumor growth of GC cells.

Discussion

GC has long been great threats to human society, causing both patient sufferings and economic burdens. According to the latest global cancer statistics, in 2018, there will be 1.03 million new patients with GC worldwide.[20,21] Although advances in multimodal therapy involving surgical and medical management, the mortality rates of GC patients remain high.[22] Molecular-targeted therapy has become a promising treatment in cancer therapy, especially for GC.[23] Therefore, suitable molecular-targeted drugs are urgently needed for combating GC. Gap junction proteins are a family of transmembrane proteins that directly link the intercellular communication of neighboring cells by facilitating the transfer of ions and small molecules.[24,25] Abnormal expression of Gap junction proteins and loss of Gap junctional intercellular communication (GJIC) function were associated with the disease progression of numerous pathologies, including cancer.[26–29] The levels of different Gap junction proteins were significantly associated with degree of tumor

malignancy.[30] Recent studies have reported that the expression of GJB4, connexin30.3, was significantly upregulated in lung cancer cells. GJB4 promoted metastasis and enhanced chemoresistance of cancer cells by activating Src pathway. However, the biological function and molecular mechanism of GJB4 in GC have not been elucidated, especially in regards to the cell proliferation and migration of GC cells.

In the present study, we found that GJB4 was highly expressed in human GC and that GJB4 expression was positively associated with the survival probability of patients with GC. As to the biological study, we observed that downregulation of GJB4 significantly inhibited cell proliferation and migration in HGC-27 and SGC-7901 GC cells. Furthermore, GJB4 downregulation also inhibited self-renewal and tumor growth of GC cells. These results indicated that GJB4 was required for the growth and migration of GC cells.

GJB4 has been shown to modulate Src signaling in lung cancer cells. However, the molecular mechanisms of GJB4 in carcinogenesis and tumor progression remain largely unknown. Our study demonstrated that GJB4 silencing regulated the expression of various genes involved in cell proliferation and migration, such as MYC, MMP7 and CCND1, all of which are downstream molecules of the Wnt/CTNNB1 signaling. We found that GJB4 knockdown
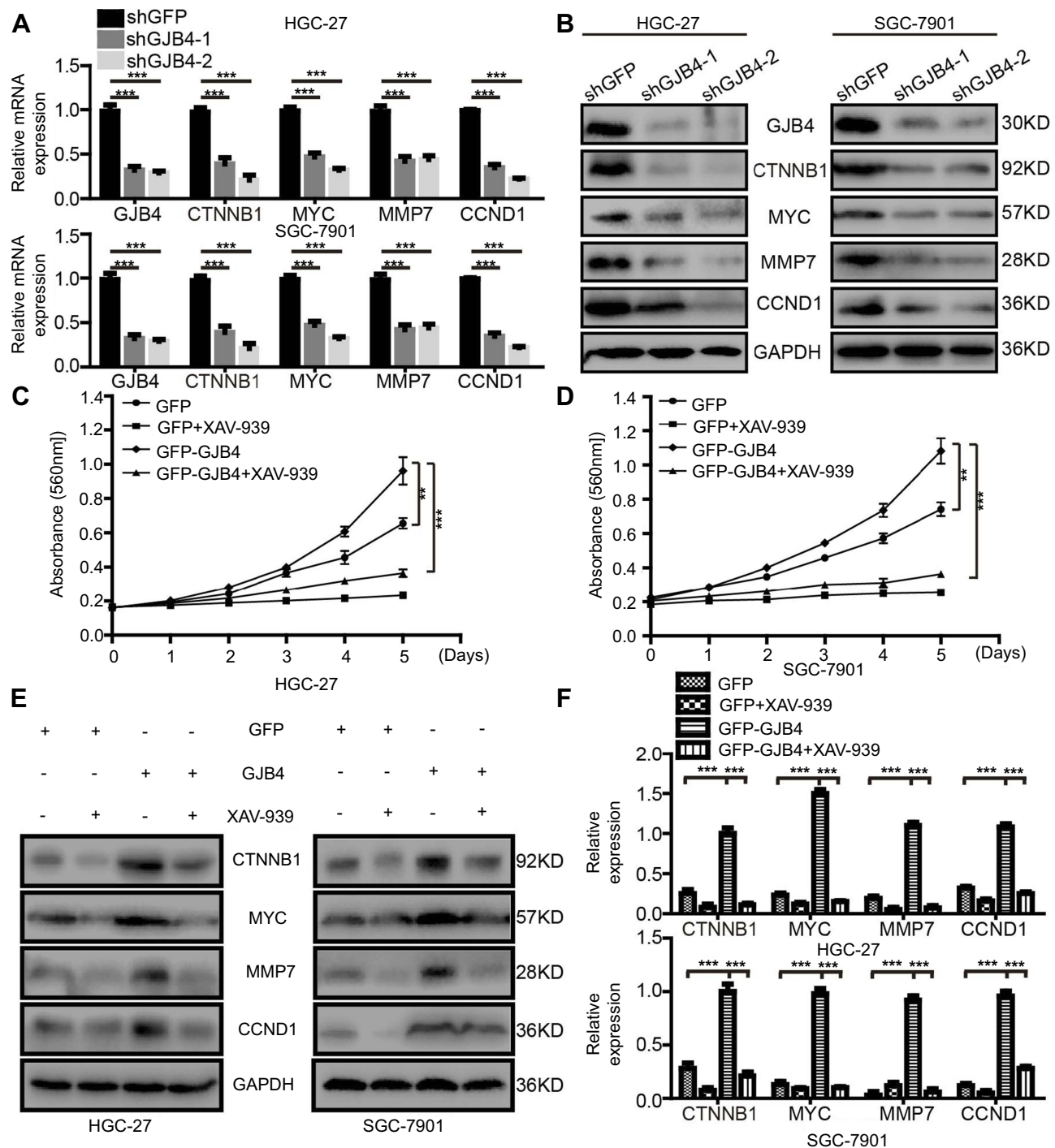
Figure 4 GJB4 mediates cell proliferation and migration by regulating Wnt/CTNNB1 pathway.
**Notes:** (**A, B**) Relative mRNA and protein expression of CTNNB1 and its downstream genes (MYC, MMP7 and CCND1) in GJB4 knockdown or control HGC-27 cells and SGC-7901 cells was detected by RT-PCR and Western blot analysis. (**C, D**) Cell proliferation of indicated stable cell lines was measured by MTT assay. (**E**) Cell migration of indicated stable cell lines was measured by Transwell assay. (**F, G**) Protein expression levels of GJB4, CTNNB1, MYC, MMP7 and CCND1 were analyzed by Western blot analysis. All data are shown as the means ± SD, *$P<0.05$, **$P<0.01$, ***$P<0.001$. All p-values are based on control versus treatment.
Abbreviations: GC, gastric cancer; GJB4, gap junction beta-4; RT-PCR, real time-polymerase chain reaction; MYC, MYC proto-oncogene; CCND1, G1/S-specific cyclin-D1; MMP7, matrix metalloproteinase-7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.

was associated with the suppression of CTNNB1 expression level in GC cells. Furthermore, the promotive effect of GJB4 overexpression on cell proliferation and

migration was blocked by treatment with Wnt/CTNNB1 inhibitor XAV-939. Therefore, we demonstrated that GJB4 could regulate the Wnt/CTNNB1 signaling.
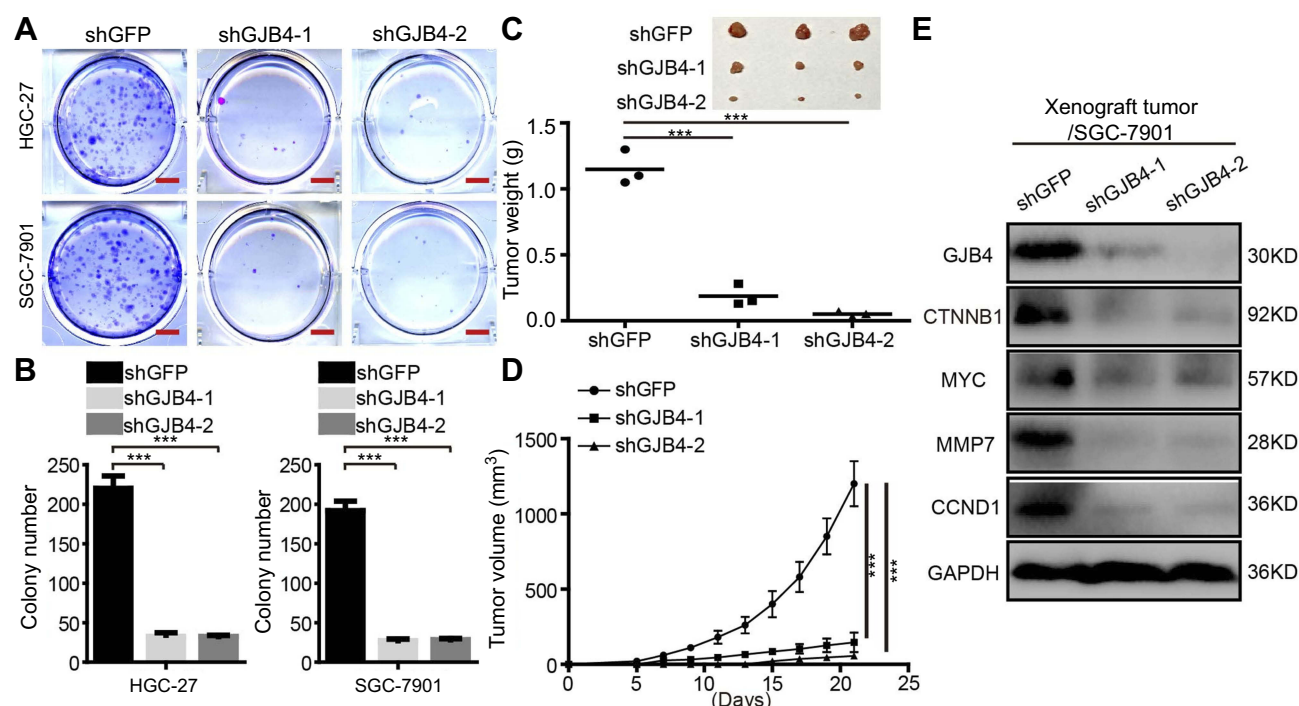
Figure 5 GJB4 knockdown inhibits self-renewal and tumor growth of GC cells.

Notes: (**A, B**) The effects of GJB4 on the colony formation in GJB4-knockdown HGC-27 and SGC-7901 cells. (**C**) Xenograft assays were performed after GJB4 knockdown in SGC-7901 cells. The size and weight of xenograft tumor were analyzed with 2-tailed Student t and P-value is indicated. (**D**) The growth curve of xenograft tumor was analyzed with 2-tailed Student t and P-value is indicated. (**E**) Immunoblotting of GJB4, CTNNB1, MYC, MMP7 and CCND1 in tumour samples. The GAPDH was used as a loading control. All data are shown as the means ± SD, *$P<0.05$, **$P<0.01$, ***$P<0.001$. All p-values are based on control versus treatment.

Abbreviations: GC, gastric cancer; GJB4, gap junction beta-4; MYC, MYC proto-oncogene; CCND1, G1/S-specific cyclin-D1; MMP7, matrix metalloproteinase-7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Conclusion

In summary, our study indicates for the first time that GJB4 promotes cell proliferation and migration through the Wnt/CTNNB1 signaling in human GC cells. Our findings provide new insights into the function of GJB4 and indicate that GJB4 is a promising biomarker and therapeutic target for gastric cancer.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials
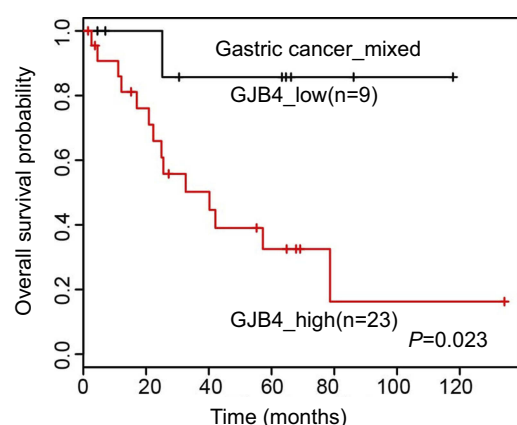
Figure S1 Kaplan-Meier overall survival for GJB4 expression in mixed types gastric cancer tumours (KM plotter gastric cancer dataset), and *P*-value is indicated.
Abbreviations: GC, gastric cancer; GJB4, gap junction beta-4.
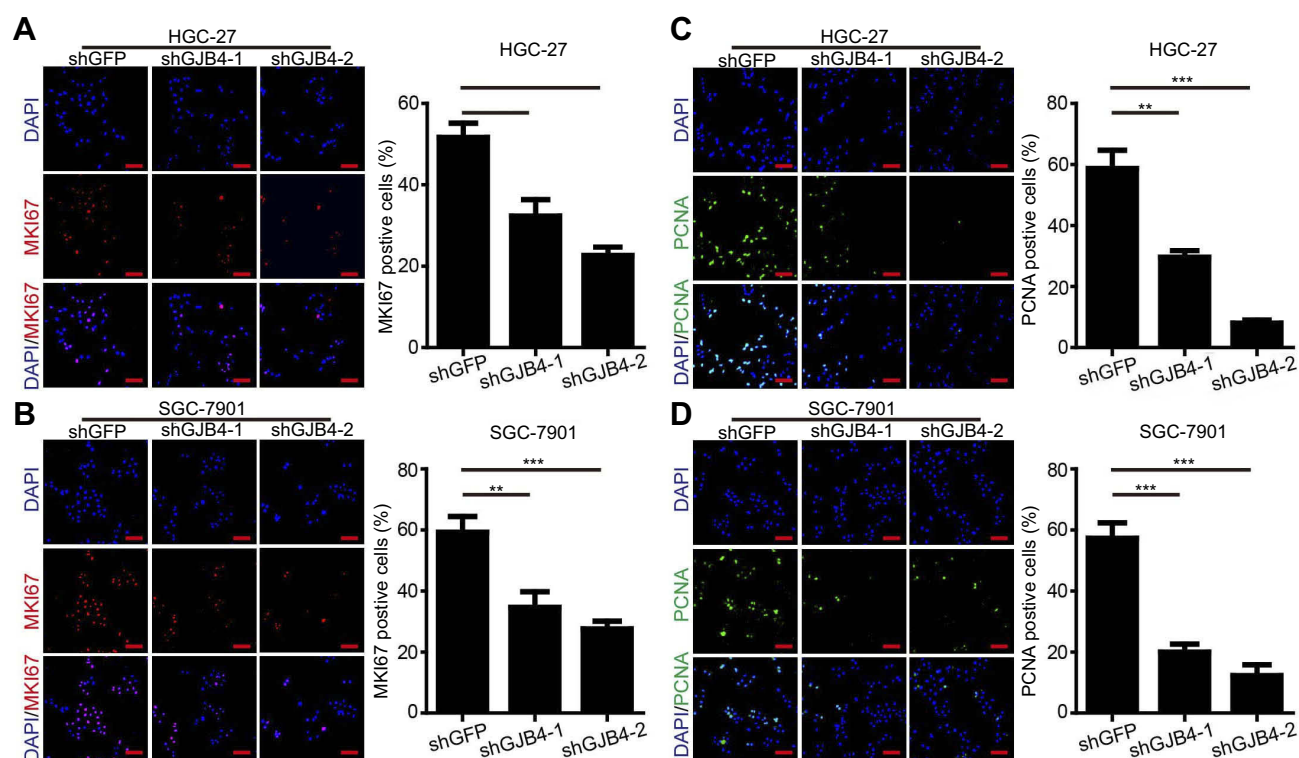
Figure S2 (**A, B**) Representative immunofluorescent images of MKI67 assay for HGC-27 and SGC-7901 cell lines. (**C, D**) Representative immunofluorescent images of PCNA assay for HGC-27 and SGC-7901 cell lines. All data are shown as the means ± SD, **p*<0.05, ***p*<0.01, ****p*<0.001. All *p*-values are based on control versus treatment.
Abbreviations: MKI67, marker of proliferation Ki-67; PCNA, proliferating cell nuclear antigen.
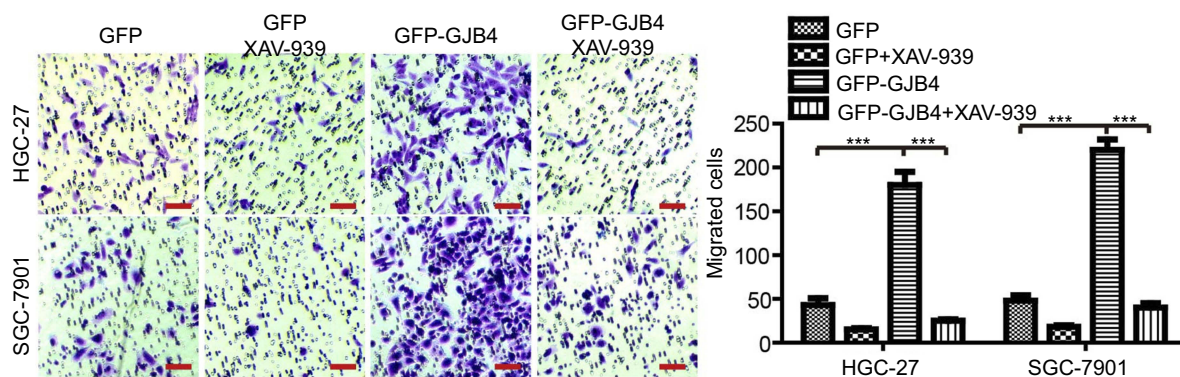
Figure S3 Cell migration of indicated stable cell lines was measured by Transwell assay. All data are shown as the means ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$. All p-values are based on control versus treatment.

Abbreviation: GJB4, Gap junction beta-4.