Circular RNA circPFKP suppresses the proliferation and metastasis of gastric cancer cell via sponging miR-644 and regulating ADAMTSL5 expression

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ABSTRACT
The treatment of gastric cancer (GC) is extremely challenging; however, the specific pathogenesis of GC remains unclear. Circular RNAs (CircRNAs) are non-coding RNAs that can regulate gene expression both transcriptionally and post-transcriptionally. However, little is known about the circRNAs that are important in the progression of GC. This study identified significantly dysregulated circRNAs by analyzing gastric cancer patients and normal control tissues. The target gene was predicted using online bioinformatics tools and verified using RNA pull-down and luciferase reporter assays. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting were used to evaluate gene and protein expression. The malignant behavior of GC cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay, wound healing assay, Transwell invasion assay, and flow cytometry. CircPFKP is downregulated in GC tissues, and overexpression of circPFKP inhibits malignant behavior in GC cells. Bioinformatics predicted that circPFKP could bind to miR-644, and miR-644 could target disintegrin-like and metalloprotease domain-containing thrombospondin type 1 motif-like 5 (ADAMTSL5). Overexpression of circPFKP enhances the expression of ADAMTSL5 by decreasing the expression of miR-644 to suppress the growth of xenograft GC tumors in vivo and in vitro. In conclusion, the circPFKP/miR-644/ADAMTSL5 regulatory pathway inhibited the malignant progression of GC. These findings may extend our understanding of the effects of circRNAs on cancer development and provide novel targets for the diagnosis of GC.

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**Highlights**

- We first demonstrated that ADAMTSL5 is downregulated in gastric cancer.
- circPFKP is expressed at low levels in gastric tissue and plays an anticancer role.
- circPFKP/miR-644/ADAMTSL5 pathway inhibited the malignant gastric cancer progression.

**Introduction**

Gastric cancer (GC) is a malignant tumor with a high degree of malignancy and poor prognosis [1]. Although strategies for the diagnosis and treatment of GC have improved significantly, the 5-year overall survival rate for patients with advanced GC is only approximately 30% [2,3]. Surgery is the only radical treatment method for GC, and adjuvant or neoadjuvant therapy is often used in conjunction with surgery [4–9]. However, the molecular mechanisms underlying the occurrence, development, recurrence, and metastasis of GC are not yet fully understood. Therefore, identifying novel potential biomarkers for the early diagnosis and prediction of GC prognosis can provide new insights for the treatment of GC.

Circular RNAs, a type of non-coding RNA with a ring structure, have recently been reported to be dysregulated in many human cancers such as bladder cancer [10], colorectal cancer [11], and cervical cancer [12]. In addition, circRNAs such as hsa-circ-0008035, hsa-circ-0000670, and hsa-circ-0003159 have been found to be associated with GC tumorigenesis or metastasis [13–15]. MicroRNAs (miRNAs) are a group of non-coding RNAs with a length of 18–22 nucleotides [16,17]. It is well known that miRNAs can bind to target mRNAs, leading to their degradation or translational inhibition. Functionally, circRNAs can sponge miRNAs and block their interaction with target genes, thus changing the functions of cancer cells, such as proliferation, apoptosis, migration, and invasion [18,19]. Previous studies have indicated that circ-RanGAP1 sponges miR-877-3p to modulate vascular endothelial growth factor A (VEGFA) expression, thus facilitating gastric cancer development [18]. Circular RNA FAT1 (circFAT1), as a newly discovered circRNA, was found to inhibit the proliferation, migration, and invasion of gastric cancer cells by binding to miR-548 g [19]. These findings demonstrate that circRNAs and miRNAs play critical roles in the development of gastric cancer. However, the precise mechanisms underlying these effects remain unclear.

In this study, we screened dysregulated circRNAs using bioinformatics analysis based on the Gene Expression Omnibus (GEO) database. A novel circRNA, hsa_circ_0006608 (circular phosphofructokinase, platelet, circPFKP), was downregulated in gastric cancer tissues and cell lines. This study aimed to elucidate the regulatory function and molecular mechanism of circPFKP in the initiation and progression of gastric cancer and to provide potential therapeutic or diagnostic targets for human gastric cancer.

**Materials and methods**

**Microarray data analysis**

Circular RNA microarray data were obtained from the GEO database [20]. Differentially expressed circRNAs between tumor tissues and normal specimens were obtained by the differential analysis of the expression profiles of circRNAs using the online analysis tool GEO2R. The inclusion criteria were set as adjusted P-value < 0.05 and | fold change| > 2 for the identification of differentially expressed circRNAs.

**Tissue sample collection and cell culture**

A total of 25 tumor samples and paired non-cancerous tissues were acquired from GC patients diagnosed at the Shandong Provincial Hospital affiliated with the Shandong First Medical University. All the participants provided written informed consent. The tissues were immediately stored in liquid nitrogen and subsequently stored at –80°C for RNA extraction. This study was approved by the Ethics Review Board of the Shandong Provincial Hospital affiliated with the Shandong First Medical University (SWYX: NO. 2022–121). All the procedures complied with the guidelines of the Declaration of Helsinki.
**Cell culture**

Human gastric cancer cell lines, including HGC-27, NCI-N87, AGS, SGC-7901, and MKN45, and the normal gastric mucosal cell line, GES-1, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in Dulbecco’s Modified Eagle Medium (DME; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 mg/L streptomycin, and 100 mg/L penicillin at 37°C in an atmosphere containing 5% CO₂.

**Cell transfection**

Short hairpin RNAs (shRNAs) targeting circPFKP were procured from GenePharma (Shanghai, China). Nonspecific shRNAs were used as the negative controls. To upregulate circPFKP, circPFKP-overexpressing plasmid and negative control empty vector were designed and synthesized by GenePharma [21]. The mimic and inhibitor of miR-644 as well as their negative controls were acquired from RiboBio (Guangzhou, China). The disintegrin-like and metalloprotease domain-containing thrombospondin type 1 motif-like 5 (ADAMTSL5) overexpressing vector was obtained from RiboBio. Cell transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Reverse transcription was performed using extracted total RNA and SuperScript IV (Thermal, CA, USA). Quantitative real-time PCR was carried out in a 7500FAST Real-time PCR System (ABI, CA, USA) using a SYBR Green Mix kit (Takara, Dalian, China). The PCR thermocycling conditions were as follows: 94°C for 2 min, followed by 30 cycles at 94°C for 30s, 58°C for 30s, and 72°C for 30s [22]. β-Actin and U6 served as controls for mRNAs and miRNAs, respectively. The gene expression levels were calculated using the 2-ΔΔCt method. Each sample was analyzed at least three times. The sequences of the primers are as follows: circPFKP, F: 5’-CGGAGATGTGCGGGATGAA-3’ and R: 5’-ACCCCTTACAGCACCACCTTG-3’; miR-644a, 5’-GTGCTATCCAGTGCAGGGTCCAGGTATTCGACTGGC/CATTAA-3’; ADAMTSL5, F: 5’-CTCTGCTAGACTGTGGGTTTG-3’ and R: 5’-GCACACTGTAGGCTCTCGGAA-3’; β-actin, F: 5’-CGGAGTCAACGGATTTGGTCCTGACATG-3’ and R: 5’-AGCCCTTCCATGGTGCTGTA-3’; U6, F: 5’-CGCTTACGAATTTGCCTG-3’ and R: 5’-CTCGCTTCCGCGACACA-3’.

**3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay**

The MTT assay was conducted to evaluate cell proliferation. Following transfection, AGS and MKN45 cells were inoculated into 96-well plates at a density of 1,000 cells/well and cultivated at 37°C. Each well was supplemented with 10 μL of MTT solution (Dojindo, Kumamoto, Japan) at different time points (0, 24, 48, and 72 h) after incubation [23]. Then, the cells were cultured at 37°C for another 2 h, and the optical density (OD) value was examined at 490 nm with a microplate reader.

**Colony formation assay**

Transfected AGS and MKN45 cells were seeded in six-well plates (3 × 10⁵ cells/well) and cultured at 37°C for two weeks. The culture medium was replaced every 3 d. After washing with phosphate-buffered saline (PBS), colonies were immobilized and stained with 0.1% crystal violet [23]. Finally, the cells were observed under a microscope.

**Transwell assay**

The cell suspension (3 × 10⁵ cells/well) and 200 μL of serum-free medium were placed in the upper chambers, and the bottom of the chambers was complemented with 600 μL of complete medium containing 20% FBS. At 24 h post-incubation, migrated cells were fixed with 4% paraformaldehyde and treated with 0.5% crystal violet. Images of the migrated cells were obtained using a microscope in five randomly selected fields [23].
Western blot

Transfected AGS and MKN45 cells were lysed using a lysis buffer supplemented with protease inhibitors. Forty micrograms of protein were separated by 12% sodium-dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The proteins were then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking, the membranes were incubated with the primary antibody against ADAMTS5 (ab45047, 1: 2,500, Abcam, USA) at 4°C overnight [24], followed by incubation with hors eradish peroxidase (HRP)-labeled secondary antibody (ab6721, 1: 5,000, Abcam) for 2 h at room temperature. The blots were visualized using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control.

RNA pull-down assay

An RNA pull-down assay was performed using a Magnetic RNA-Protein Pull-Down kit (Pierce, Waltham, MA, USA). Briefly, transfected cells were harvested, trypsinized, sonicated, and incubated with a biotinylated control probe or miR-644 probe and streptavidin magnetic beads overnight at 4°C [25]. The expression of circPFKP and ADAMTSL5 in the eluted RNA was determined using qRT-PCR.

Luciferase reporter gene assay

The binding sites of circPFKP and ADAMTSL5 3’-UTR predicted to be targeted by miR-644 were inserted into the luciferase vector pmirGLO (Promega, USA) to construct wild-type luciferase reporter plasmids. Mutant vectors were generated by mutating the binding sites with miR-644. The AGS and MKN45 cells (1 × 10⁶ cells/well) were co-transfected with wild-type or mutant plasmids and miR-644 mimic or mimic control using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions [26]. After 48 h, luciferase activity was tested using the Dual-Luciferase Reporter Assay System (Promega).

Xenograft model

Gastric cancer cells in the logarithmic growth phase were digested, centrifuged, discarded from the old medium and resuspended in PBS. The cells were washed twice with PBS (removed the serum). The cell concentration was adjusted to 1 × 10⁶/mL, and 0.1 mL was injected subcutaneously into nude mice. After planting, obvious tumor nodules appeared at the inoculation site (generally 7–10 d), and tumor formation in nude mice was recorded. A tumor growth curve was then established. The tumor size was measured with a Vernier caliper, the longest diameter of the tumor (a) and the largest vertical transverse diameter (b) were measured, and the tumor volume was calculated according to the formula V (mm³) = ab²/2 [27].

Statistical analysis

The experiments were repeated at least three times. Data were analyzed using SPSS19.0 and are represented as mean ± standard deviation. Student’s t-test was used to evaluate the difference between the two groups. One-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test was used to evaluate differences among multiple groups. Statistical significance was set at P < 0.05.

Results

circPFKP is lowly expressed in gastric cancer

Increasing evidence has confirmed the crucial role of circRNAs in the progression of gastric cancer. Here, we screened the GEO database and selected three datasets, GSE93451, GSE89143, and GSE78092, for bioinformatic analysis and found 12 dysregulated circRNAs in gastric cancer (Figure 1a). Quantitative real-time PCR was performed to verify the expression of the circRNAs. The results showed that the expression levels of hsa_circ_0068610, hsa_circ_0075736, and hsa_circ_0007895 were upregulated, while those of hsa_circ_0006608 and hsa_circ_0005273 were significantly downregulated in gastric cancer (Figure 1b). We selected has_circ_0006608, termed circPFKP, for further study. We evaluated the expression of circPFKP in the gastric cancer...
tissues and cell lines. The results confirmed that circPFKP was weakly expressed in gastric cancer tissues and cells Figure 1(c,d).

**CircPFKP exists in gastric cancer cells**

Several experiments were performed to identify the circular characteristics of circPFKP. After RNase R treatment, linear and circular RNA expression was tested. The results showed that the expression of circular PFKP did not change, whereas the linear centrosome and spindle pole-associated protein 1 (CSPP1) level was significantly reduced Figure 2(a). After actinomycin treatment, linear and circular RNA half-life changes were observed. The results showed that circular PFKP has a higher stability and longer half-life as compared to that of the other RNAs Figure 2(b). The RNA in the cell cytoplasm and nucleus was separated to detect circPFKP expression by qPCR. The expression of circPFKP in the cytoplasm was much higher than that in the nucleus Figure 2(d). These results indicated the stability of circPFKP, which further verified its presence in gastric cancer cells.

**circPFKP inhibits gastric cancer progression**

To study the biological functions of circPFKP, cell proliferation, clone formation, and Transwell assays were performed. The qPCR results showed that shRNA plasmid transfection could effectively reduce the expression of circPFKP, while the overexpression vector of circPFKP notably promoted this effect Figure 3(a). The MTT and colony formation assays showed that circPFKP knockdown promoted the proliferation of gastric cancer cells, while overexpression of circPFKP inhibited this
**Figure 2.** The identification of circPFKP in the gastric cancer cells. 
(A) The expression of the linear and circular forms of phosphofructokinase, platelet (PFKP) was detected by quantitative polymerase chain reaction (qPCR). (B) The expression of the linear and circular forms of PFKP at different time points was detected by qPCR. (C) Divergent and convergent primers were designed, and PCR and gel electrophoresis were performed to detect the presence of circular PFKP (circPFKP). (D) RNA in the cytoplasm and nucleus was separated, and the expression of circPFKP was evaluated by qPCR. **P < 0.01.

effect Figure 3(b,c). The experimental results of the Transwell assay showed that circPFKP knockdown promoted invasion and migration, whereas circPFKP overexpression attenuated that of gastric cancer cells Figure 3(d,e).

**circPFKP sponges miR-644 in gastric cancer cells**

To elucidate the mechanism underlying the effects of circPFKP, we predicted potential miRNAs that may be targeted by circPFKP Figure 4(a). We then evaluated the expression of these miRNAs under circPFKP overexpression. Overexpression of circPFKP notably reduced the levels of miR-644, miR-591, and miR-941. We selected miR-644 for further analysis. Figure 3(b) shows the binding sequence of circPFKP and miR-644. Luciferase reporter assay indicated that circPFKP could bind directly to miR-644 Figure 4(b). The qPCR revealed that overexpression of circPFKP inhibited the expression of miR-644, while inhibition of circPFKP promoted this effect Figure 4(c). The RNA pull-down using the miR-644 probe demonstrated that the biotin-miR-644 probe could enrich circPFKP more than the control probe Figure 4(d). In the gastric cancer tissues, miR-644 was shown to be remarkably up-regulated Figure 4(e). Correlation analysis revealed that miR-644 expression was negatively correlated with circPFKP expression figure 4(f).

**miR-644 targets ADAMTSL5**

It is known that miRNAs bind to target genes to inhibit their expression, thus participating in the regulation of cell processes. To study the target genes of miR-644, we first predicted its binding target genes. Potential binding target genes were predicted using the TargetScan software. The binding sites are shown in Figure 5(a). The results of the luciferase assay showed that miR-644 could directly bind to ADAMTSL5 Figure 5(b). The RNA pull-down results revealed that biotin-miR-644 could hook ADAMTSL5 Figure 5(c). Furthermore, the qPCR assay showed that miR-644 could attenuate the expression of ADAMTSL5, while miR-644 knockdown could promote this Figure 5(d). The results of western blotting showed that miR-644 could reduce the protein level of ADAMTSL5, whereas the inhibition of miR-644 could promote this Figure 5(e). Moreover, bioinformatics analysis using qPCR revealed downregulated ADAMTSL5 expression in the gastric cancer tissues Figure 5(f).
miR-644 inhibitor and ADAMTSL5 overexpression both reversed the effect of circPFKP silencing in gastric cancer cells

First, we determined whether sh-circPFKP, miR-644 inhibitor, or ADAMTSL5 overexpression vector could change the expression level of ADAMTSL5. The qPCR results indicated that sh-circPFKP notably decreased the level of ADAMTSL5, while inhibition of miR-644 reversed this effect. In addition, ADAMTSL5 overexpression significantly increased ADAMTSL5 expression Figure 6(a). Cell proliferation and colony formation assays showed that circPFKP knockdown promoted the proliferation of gastric cancer cells, whereas miR-644 inhibition and ADAMTSL5 overexpression both reversed this effect Figure 6(b,c). Transwell experiments showed that circPFKP silencing promoted the invasion and migration of gastric cancer cells, while inhibition of miR-644 and ADAMTSL5 overexpression reversed this effect of circPFKP knockdown Figure 6(d,e).
Figure 4. circPFKP sponges miR-644 in gastric cancer cells.
(A) Candidate microRNAs (miRNAs) bound to circular phosphofructokinase, platelet (circPFKP) were screened by quantitative polymerase chain reaction (qPCR). (B) Bioinformatics analysis predicted the binding sites between circPFKP and miR-644. Luciferase assay was carried out to verify the binding between circPFKP and miR-644. (C) After circPFKP knockdown or overexpression, miR-644 expression level was detected by qPCR. (D) RNA pulled down using miR-644 probe was performed to detect the binding between circPFKP and miR-644. (E) Expression of miR-644 in gastric cancer tissues was detected. (F) Pearson analysis was performed to detect the correlation between circPFKP and miR-644. ** * P < 0.01.

Figure 5. miR-644 targets ADAMTSL5 in gastric cancer cells.
(A) Bioinformatic analysis predicted the binding sites between disintegrin-like and metalloprotease domain-containing thrombospondin type 1 motif-like 5 (ADAMTSL5) and miR-644. (B) Luciferase assay was performed to verify the binding between ADAMTSL5 and miR-644. (C) RNA pull-down using the miR-644 probe was performed to detect the binding between ADAMTSL5 and miR-644. (D, E) After miR-644 knockdown or overexpression, ADAMTSL5 expression levels were detected using quantitative polymerase chain reaction (qPCR) and western blotting, respectively. (F) ADAMTSL5 expression was detected in GC tissues. ** * P < 0.01.
circPFKP inhibits the growth of gastric cancer cells in vivo

To further confirm the biological effects of circPFKP on gastric cancer development, we investigated the carcinogenic effect of circPFKP at the animal level. The results showed that overexpression of circPFKP inhibited tumor growth and tumor weight, while knockdown of circPFKP promoted tumor growth Figure 7(a–c). We then evaluated the expression of circPFKP, miR-644, and ADAMTSL5 in the tumor tissues. As shown in Figure 7(d), circPFKP expression was elevated in the circPFKP overexpression group but...
Figure 7. circPFKP promotes tumor proliferation and metastasis. (A) The tumor volume was imaged. (B) The growth curve of the tumors in each group was established. (C) The weight of the tumors in each group. (D) Quantitative polymerase chain reaction (qPCR) was used to detect the expression level of circPFKP and miR-644. (E) Immunohistochemistry (IHC) was performed to assess the expression of disintegrin-like and metalloprotease domain-containing thrombospondin type 1 motif-like 5 (ADAMTSL5) in the tumor tissues, scale length: 200 μm. ** P < 0.01.

Discussion

Circular RNAs have been confirmed to play critical roles in regulating tumor development, including gastric cancer, by acting as sponges for related miRNAs [27–31]. Here, we screened dysregulated circRNAs in gastric cancer and found a novel circRNA (circPFKP) that was significantly downregulated in gastric cancer tissues and cell lines. Functional studies have indicated that circPFKP exerts an anti-cancer effect in gastric cancer both in vitro and in vivo.

MicroRNAs are involved in the regulation of signaling pathways associated with tumor growth. Studies have found that abnormal expression of multiple miRNAs in gastric cancer tissues is closely related to the occurrence and development of gastric cancer. Some circRNA molecules contain miRNA response elements (MREs), which can act as competitive endogenous RNAs and sponges to inhibit the function of miRNA [32–34]. To elucidate the mechanism of circPFKP, we predicted potential miRs that could be sponged by circPFKP. ADAMTSL5 was selected and verified to be sponged by circPFKP.

MicroRNAs are non-coding single-stranded RNA molecules encoded by endogenous genes and range in length from 19 to 25 nucleotides [35–37]. MicroRNAs play an important regulatory role in human pathophysiology and are involved in signal activation, cell proliferation, differentiation, and cell death by regulating signaling molecules [38–40]. MiR-644 has been rarely studied. It has been found to be upregulated in acute myeloid leukemia and bladder cancer. No studies have revealed the effect of miR-644 on the development of gastric cancer. In this study, first, we demonstrated the upregulation of...
miR-644 in gastric cancer. In addition, we found that miR-644 plays an oncogenic role in gastric cancer progression.

Disintegrin-like and metalloprotease domain-containing thrombospondin type 1 motif-like 5 is a newly discovered protein. It has a unique domain composition, comprising an N-terminal TSR, cysteine-rich module, spacer module, and C-terminal NTR module, which is connected to the spacer by a proline-rich segment. In addition, ADAMTSL5 can be secreted and interact with fibrillin-1 and fibrillin-2, thus modulating microfibril function [36]. In psoriasis, ADAMTSL5 is present in human leukocyte antigen (HLA)-restricted melanocytes that activate interleukin (IL) 17-producing T cells. However, the effect of ADAMTSL5 on cancer progression has not been reported. We first demonstrated that ADAMTSL5 is downregulated in gastric cancer and plays an anticancer role. However, the downstream signaling pathway that is modulated by ADAMTSL5 remains unclear. This should be explored in future studies. We will conduct an in vivo study to confirm the role of ADAMTSL5 in gastric development.

**Conclusion**

In summary, our data suggest that circPFKP is expressed at low levels in gastric tissue and plays an anticancer role. Mechanically, circPFKP targets miR-644 and promotes the expression of ADAMTSL5 as a ‘ceRNA.’ The circPFKP/miR-644/ADAMTSL5 signaling pathway may be critical in regulating the development and progression of gastric cancer and may be a therapeutic target for gastric cancer. Our findings provide new insights into the pathogenesis of gastric cancer.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Author contribution**

Shuai Kong and Shubu Tian performed the experiments Zhu Wang collected data and contributed to the statistical analysis Yulong Shi performed the informatics analysis Jizhun Zhang supported the experiments Hongqing Zhuo designed the study and wrote the manuscript

**Data availability statement**

All the data is available from the corresponding author due to reasonable request.

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