Circular RNA circDDX17 suppression to gastric cancer progression via the sponging miR-1208/miR-1279/FKBP5 axis and encodes a novel circDDX17-63aa protein

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Abstract

Circular RNAs (circRNAs) have an important role in the development and progression of gastric cancer (GC). Hsa_circ_0063331 (circDDX17), a new circRNA shown to be considerably down-regulated in GC, was chosen for further study. The location of circDDX17 in GC cells was investigated using nuclear and cytoplasmic RNA fractionation and an RNA-FISH experiment. qRT-PCR was used to determine the expression levels of circDDX17, miR-1208, miR-1279, and FKBP5 in GC tissues. To investigate the functional mechanism of circDDX17 on the miR-1208/miR-1279/FKBP5 axis in GC cells, bioinformatics analysis, luciferase reporter, and IP were used. To investigate the role of circDDX17 and circDDX17-63aa in GC development, researchers used Transwell, wound healing, colony formation assays, CCK8, cell apoptosis tests, and Western blot. Finally, circDDX17 function in vivo was investigated using xenograft and metastatic mice models. We discovered that circDDX17 was down-regulated in GC tissues and cell lines in our investigation. CircDDX17 expression in GC patients was linked to tumor growth and lymph node metastasis. CircDDX17 decreased GC cell proliferation, migration, and epithelial-mesenchymal transition (EMT), while also promoting apoptosis in vitro and in vivo. CircDDX17 behaved as a sponge for miR-1208 and miR-1279, regulating FKBP5 expression in GC cells. FKBP5 interacts with ERK1/2 to control GC development through the ERK1/2 pathway. Furthermore, circDDX17 also encoded circDDX17-63aa, which inhibited GC cell proliferation, migration, and EMT. Finally, circDDX17 inhibits GC advancement via miR-1208/miR-1279/FKBP5 and encoded circDDX17-63aa to inhibit GC progression.

Introduction

Gastric cancer (GC) is one of the most frequent types of cancer worldwide. In 2020, there were 1.1 million instances of stomach cancer worldwide, with 66% being men and 34% being women. 60% of GC patients were found in East Asia, of which China accounted for 43.9%. Gastric carcinoma is a malignancy of a high aggressiveness, where the rate of median survival is less than 12 months for the advanced stage. In 2020, 770,000 individuals died from stomach cancer worldwide, with males accounting for 65% of fatalities and East Asia accounting for 56.6% of deaths worldwide, including 48.6% in China. The development of GC is influenced by a variety of environmental and genetic variables, including smoking, alcohol use, being overweight, eating a lot of salt, and Helicobacter pylori infection.

Noncoding RNAs (ncRNAs), which make up more than 98% of the human genome sequence, include genes. MicroRNAs (miRNAs), long ncRNAs (lncRNAs), and circular RNAs (circRNAs) are a few of the RNA species that make up ncRNAs. CircRNAs are extensively expressed in a variety of organs and cells. They are single-stranded, covalently closed ncRNAs lacking 5' end caps or 3' end poly (A) tails. It is produced by noncanonical splicing from its precursor mRNA and is extensively expressed in a variety of taxa, including viruses, plants, and mammals. Single-stranded non-coding RNAs called miRNAs, which are around 22 nt long, inhibit gene expression by interacting with the 3'-untranslated region (3'UTR). CircRNAs, which are miRNA's upstream molecules, have the ability to bind different miRNAs in order to block activity through miRNA sponging by miRNA response elements (MER). CircRNAs can competitively
bind miRNAs and operate as intracellular competitive endogenous RNA (ceRNA), which prevents miRNAs from inhibiting their target genes and controls miRNA activity and associated gene expression. Due to the absence of clear open reading frames (ORFs) in organisms, circRNAs were unable to be translated into proteins in the prior perspective. However, circRNAs may really encode proteins, according to a number of recent studies.

The immunophilin family, which also includes FKBP and the tetratricopeptide repeat (TPR) domain, includes FKBP5 (also known as FKBP51). Progesterone, androgen, and glucocorticoid receptors are peptidyl steroid hormone receptors, and FKBP5 is a well-known molecular chaperone of these receptors. The Oncomine database shows that FKBP5 was either up- or down-regulated in several malignancies. While FKBP5 is very weakly expressed in pancreatic cancer, colon cancer, testicular cancer, and gastric cancers. It is strongly expressed in brain cancer, prostate cancer, lymphoma, and melanoma. FKBP5 has been discovered to be connected to a number of signaling pathways, including the NF-κB pathway, the ERK pathway, and the AKT pathway.

CircRNAs are crucial regulators of the proliferation, apoptosis, migration, and invasion of cancer cells. In our study, circDDX17 had a low expression in GC tissues and cells, and it inhibited GC advancement through encoding circDDX17-63aa and circDDX17-miR-1208/miR-1279-FKBP5.

Methods

Patients, tissue samples, and ethics statement.

Between 2018 and 2020, 51 pairs of human GC and neighboring non-tumor tissues were gathered at Jiangsu University's First Affiliated Hospital. All patients gave their written, agreement for the use of their clinical records for investigational purposes. The Jiangsu University Ethical Committee gave its approval to all experimental methods.

Cells and Animals

Jiangsu University maintained the cell lines GES-1, HGC-27, MKN-45, MGC-803, and HEK-293T. All of the cells were grown in DMEM, which contains 10% fetal bovine serum (FBS; Becton, Dickinson and Company, USA). In a cell culture incubator with 5% CO₂, all cell lines were cultured at 37°C.

BALB/c nude mice that were 4 weeks old and weighed 15–20 g were acquired from Nanjing University's Model Animal Research Center. The "Guide for the Care and Use of Laboratory Animals" and the "Principles for the Utilization and Care of Vertebrate Animals" were strictly followed during animal care and experimentation. All animal testing was authorized by Jiangsu University's ethics committee.

Plasmid and Oligonucleotides transfection
Prof. Zhou (Nantong University) donated the pcicR-3.0 vector, which was utilized to overexpress circDDX17. Hanbio Biotech (Shanghai, China) provided the PcDNA-3.0 vector that was utilized to overexpress FKBP5. GenePharma (Shanghai, China) provided the si-circDDX17, si-FKBP5, miR-1208 mimics/inhibitors, and miR-1279 mimics/inhibitors. Sangon Biotech (Shanghai, China) supplied Lv-circDDX17, Lv-circDDX17-63aa, and Lv-circDDX17-mut, with Lv-vector serving as a negative control. Plasmid and oligonucleotide transfection was carried out using Lipofectamine 3000 (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's instructions.

**RNA extraction, gDNA extraction, quantitative Real-Time PCR, and RNase R Treatment**

Following the manufacturer's instructions, total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, USA). PrimeScript RT Reagent Kit from Takara, Japan was used for reverse transcription, and TB Green Premix Ex TaqII from Takara, Japan was used for quantitative Real-Time PCR. Sangon Biotech (Shanghai, China) created the primers. Table S3 contains a list of the PCR primer sequences. Following the manufacturer's instructions, PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, CA, USA) was used to extract genomic DNA (gDNA) from tissues. 2 mg of total RNA was treated with or without 3 U/mg RNase R for 30 min at 37°C as part of the RNase treatment.

**Fluorescence in situ hybridization (FISH)**

GC cells were found to have Cy3-labeled circDDX17 probe using a Fluorescent In Situ Hybridization Kit (GenePharma Biotech, China) in accordance with the manufacturer's instructions. In a 24-well plate that had been grown for 12 hours with 3×10^3 HGC-27 and MKN-45 cells, circDDX17-specific probes were hybridized overnight at 37°C. Then, for a further five minutes, the cell nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). Leica, Mannheim, Germany, fluorescence microscope was used to analyze the data and record the photographs.

**Transwell and wound healing assays**

Transwell assay (BD Falcon, Franklin Lakes, USA) was examined with 8 m pores. 3×10^4 HGC-27 cells, 4×10^4 MKN-45 cells, or 2×10^4 MGC-803 cells were planted into the cell culture inserts in 150 µL of serum-free DMEM before 10% FBS-containing culture media was added to the bottom chamber. The cells on the lower side of the filter were fixed, stained, and seen under a microscope after 24–48 hours of incubation. Cells were sown in a 6 or 12-well plate and cultured in DMEM containing 10% FBS for wound healing tests. The single cell layer was then scratched with a sterile 10 mL plastic pipette tip and refilled with serum-free DMEM. Images were taken with an Olympus light microscope at the specified periods (0 and 24 hours). All experiments were performed in duplicate and repeated three times.

**Colony formation and CCK8 assays**

A 12-well plate with 500 cells per well, culture media containing 10% FBS, and an incubator were used for the colony formation test. The cells were then stained with crystal violet after being fixed with methanol,
and colonies were photographed and counted. 1000 cells/well of culture media with 10% FBS were planted in 96-well plates for the CCK8 test. Following the addition of 10 µL of CCK8 reagent (Tongren, Shanghai, China) to each well and a 1-hour incubation period, the absorbance of the wells was spectrophotometrically determined at 450 nm at 24, 48, 72, 96, and 120 hours.

**Flow cytometry**

An Annexin V-FITC/PI kit from Vazyme, China, was used to conduct the cell apoptosis experiment. Following a 48-hour transfection, adherent cells were digested with trypsin without the addition of EDTA, and recovered cells were resuspended in pre-chilled PBS. By adding annexin V-fluorescein isothiocyanate solution (FITC, 5 µL) and PI (5 µL) in suspension, the BD FACS Calibur flow cytometer (BD Falcon, Franklin Lakes, USA) was able to detect apoptosis.

**Western blot assay**

Cells were collected, lysed with RIPA buffer (Invitrogen, Carlsbad, USA) containing 1% PMSF, and centrifuged at 4°C for 20 min at 12000 rpm. SDS-PAGE was used to load and separate proteins. The gels were transferred to PVDF membranes (Millipore, USA), then blocked overnight at 4°C with primary antibodies and 5% nonfat milk (BD Falcon, Franklin Lakes, USA). An enhanced chemiluminescence (ECL) system (Image Quant LAS 4000 mini, Pittsburgh, USA) was employed, and secondary HRP-conjugated antibodies were applied.

**Immunohistochemistry (IHC) assay**

The experiment was conducted in accordance with the IHC kit manufacturer’s instructions at Boster Bioengineering (Wuhan, China). On 4 µm slices that had been deparaffinized with xylene and rehydrated with ethanol, IHC staining was carried out. To decrease endogenous peroxidase activity, sections were treated to 3% hydrogen peroxide for 10 minutes. Following this, sections were blocked with 5% BSA and incubated with the primary antibody at 4°C for an overnight period. Sections were incubated using IgG-biotin and SABC, followed by counterstaining with 3,3-diaminobenzidine and hematoxylin and microscope viewing.

**Luciferase reporter assay**

At Jiangsu University, the plasmid PmirGLO vector was kept on hand. Sangon Biotech (Shanghai, China) created CircDDX17-wild-type (wt) and CircDDX17-mutant (mut). For 36 hours, the HEK-293T cells were co-transfected with the plasmids and miR-1208/miR-1279 mimics. Using a dual-luciferase reporter assay (Promega, WI, USA), the luciferase activities were found.

**Immunofluorescence assay**

GC cells cultured on slides were fixed for at least 30 minutes with 4% paraformaldehyde. 5% BSA is used for blocking and 0.5% Triton-X-100 (Sigma-Aldrich, USA) is used for permeabilization. primary antibodies were incubated overnight at 4°C. DAPI and fluorescent secondary antibodies (from Beyotime, China) were
then added. Leica, Mannheim, Germany, fluorescence microscope was used to analyze the data and record the photographs.

**Enzyme-linked immunosorbent assay (ELISA)**

According to the manufacturer's instructions, commercial ELISA kits from Meimian (Jiangsu, China) were used to evaluate the expression levels of the cytokines TGF-, TNF-, and IL-1. A microplate reader (Thermo Fisher Scientific, CA, USA) was used to measure the optical density (OD) values after the supernatants were collected, incubated with reaction solution, and then stopped with stop solution.

**Statistical analysis**

GraphPad Prism 9 was used to examine experimental findings using a t-test (two-tailed) or an ANOVA. Using the chi-square test or Fisher's exact test, the connection between the expression of circDDX17, miR-1208, miR-1279, and FKBP5 and clinicopathological traits was computed and examined. Statistical significance for the differences was determined by $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

**Results**

**Result 1: CircDDX17 characterization of the existence, subcellular distribution, and expression in GC cells and tissues.**

The GEO database (https://www.ncbi.nlm.nih.gov/gds/) predicted the differentially expressed circRNAs. CircRNA microarrays were used in the GSE181769, GSE121445, and GSE100170 paired GC tissues and neighboring non-tumor tissues, as well as the GSE202538 GC cell line and GES-1 cells. From the overlap of four circRNA microarrays, we were able to isolate hsa_circ_0063331 (circDDX17) (Figure 1A). Additionally, GC cells and tissue both express circDDX17 at low levels. The transcript ddx17, which is cyclized from exons 2–5, has a length of 451 bp and is found on chromosome 22 (Figure 1B). The circular transcripts are amplified using divergent primers, while the linear transcripts are analyzed using convergent primers. CircDDX17 resistance to RNase R treatment after treating the cDNA of HGC-27 cells (Figure 1C). And only HGC-27 cells' cDNA's divergent primers could amplify circDDX17 (Figure 1D). Figures 1E and 1F from nuclear mass separation assay and FISH analysis respectively showed that circDDX17 was present in the cytoplasm of HGC-27 and MKN-45 cells.

The expression of circDDX17 is low in gastric tumor tissues when compared to 51 pairs of neighboring non-tumor tissues and normal tissues (Figure 1G). The expression of circDDX17 in tumor tissues was negative and substantially correlated with the size of tumor tissues and lymph node metastases in patients, according to an analysis of the clinicopathological importance of circDDX17 (Table 1). Additionally, the circDDX17 gene expression in the GC cell line is lower than that in GES-1 cells (Figure 1H).

**Result 2: CircDDX17 promotes cell apoptosis, and inhibits cell proliferation, migration, and EMT GC cells.**
Utilizing MGC-803 cells to silence circDDX17 and HGC-27 cells to overexpress circDDX17, researchers were able to better understand the role of circDDX17 in GC. The overexpression or silencing of circDDX17 in cells was successfully accomplished, according to qRT-PCR data (Figure 2A), however the expression levels of ddx17 mRNA were unaffected (Figure 2B). Results of colony formation (Figures 2C and 2D), CCK8 (Figures 2E-2G), and the cell apoptosis test (Figure 2H) revealed that GC cells overexpressing circDDX17 decreased cell proliferation and promoted apoptosis, whereas silencing circDDX17 increased cell proliferation and blocked cell apoptosis. Data from a Western blot analysis of the proteins involved in proliferation and apoptosis (Figure 2I-2L) showed that HGC-27 and MKN-45 cells overexpressing circDDX17 could increase the expression of cleaved-PARP and BAX while suppressing the expression of BCL and PCNA. CircDDX17-induced cleaved-PARP and BAX expression were repressed by MGC-803 cells, and BCL and PCNA expression were decreased.

In order to investigate the role of circDDX17 in GC cell migration and EMT, wound-healing tests revealed that overexpression of circDDX17 significantly reduced HGC-27 and MKN-45 cell migration (Figure 2M) whereas si-circDDX17 significantly increased MGC-803 cell migration (Figure 2N). The overexpression of circDDX17 then significantly decreased the migration of GC cells, as seen by Transwell experiments (Figure 2O). As opposed to the NC group, the silencing of circDDX17 promoted the migration of GC cells (Figure 2P). According to the findings of a Western blot analysis of the proteins involved in migration and EMT (Figure 2Q–2T), overexpression of circDDX17 enhanced E-cadherin expression while suppressing the expression of N-cadherin, Snail, Vimentin, MMP2, and MMP9 in GC cells. GC cells silenced circDDX17 suppressed E-cadherin expression and enhanced N-cadherin, Snail, Vimentin, MMP2, and MMP9 expression.

By administering HGC-27 cells that had been stably transfected with either the circDDX17 vector or NC vector cells to the nude mice, the effect of circDDX17 on tumor development in vivo was investigated. The subcutaneously implanted tumors were still present after four weeks. According to xenograft tumor test results, circDDX17 overexpression led to smaller xenograft tumors than the NC vector (Figure 3A). According to an IHC experiment, overexpression of circDDX17 decreased the expression of N-cadherin, MMP2, MMP9, and PCNA while inducing the expression of E-cadherin (Figure 3B). According to the results of the Western blot, overexpression of circDDX17 increased the expression of the proteins cleaved-PARP, E-cadherin, and p-ERK1/2, while inhibiting the expression of the proteins PCNA, N-cadherin, Snail, Vimentin, MMP2 and MMP9 (Figure 3C). According to the results of qRT-PCR used to examine the mRNA expression of Xenograft tumor tissues (Figure 3D), overexpression of circDDX17 increased E-cadherin mRNA levels while decreasing N-cadherin, MMP2, MMP9, and PCNA mRNA levels. Figures 3E and 3F show an ELISA experiment used to examine the expression of TGF- and TNF- in serum from naked mice. According to the research, overexpression of circDDX17 decreased serum levels of TGF- and TNF-expression.

Results 3 CircDDX17 functioned as a miR-1208 and miR-1279 sponge.
Our work then investigated whether circDDX17 controlled the behavior of GC cells by sponging numerous miRNAs, one of the most crucial roles of circRNAs. First, we identified two miRNAs from the circBank (http://www.circbank.cn) using bioinformatics analysis. With circDDX17, miR-1208 and miR-1279 all potentially have binding sites. qRT-PCR results revealed that circDDX17 negatively influenced miR-1208 and miR-1279 expression in GC cells when investigating the connection between the two genes (Figures 4A and 4B). The full-length circDDX17-wild-type (wt) and circDDX17-mutant (mut) sequences were then cloned into the luciferase vector in the absence of miR-1208 or miR-1279 binding sites (Figure 4C). Assays using luciferase confirmed that miR-1208 and miR-1279 significantly decreased the luciferase activity of circDDX17-wt but not circDDX17-mut when compared to the miR-NC group (Figures 4D and 4E).

qRT-PCR was used to find the expression of miR-1208 and miR-1279 in GC cell lines, surrounding non-tumor tissues, and gastric tumor tissues in order to conduct additional study on the role of these molecules in GC. The findings showed that miR-1208 and miR-1279 exhibited high expression in the GC cell line and high expression in gastric tumor tissues as compared to surrounding non-tumor tissues (Figure 4F–4K). Table S1 shows a correlation between miR-1208 expression level and lymph node metastasis, and Table S2 shows a correlation between miR-1279 expression level and tumor size.

Results 4 miR-1208 and miR-1279 reversed the ability of circDDX17 on GC cell apoptosis, proliferation, migration, and EMT.

miRNAs are key players in the development of GC; to investigate their function in GC pathogenesis, GC cells must either overexpress or have miR-1208 or miR-1279 silencing. Results from the colony formation assay, CCK8 assay, Transwell assay, wound healing assay, and Western blot demonstrated that, in contrast to NC, miR-1208 and miR-1279 mimics enhanced GC cells' proliferation, migration, and EMT, while miR-1208 and miR-1279 inhibitors suppressed GC cells' proliferative behavior (Figure S1). The aforementioned results show that miR-1208 and miR-1279 have a tumor-promoting impact on GC.

As circDDX17 target miRNAs in this investigation, miR-1208 and miR-1279 were used. to find out if miR-1208 and miR-1279 interactions between circDDX17 and GC cells cause circDDX17 to decrease GC cell proliferation, migration, and EMT. We carried out rescue tests and co-transfected si-circDDX17 and an inhibitor of miR-1208/miR-1279 in MGC-803 cells, and circDDX17 vector and mimics of miR-1208/miR-1279 in HGC-27 cells.

Colony formation (Figure 5A and 5F), CCK8 assay (Figure 5B and 5G), Transwell, and wound healing assay (Figure 5D and 5I) results showed that circDDX17 suppressed the ability of proliferation and migration in HGC-27 cells. Further overexpression of miR-1208 or miR-1279 rescued proliferation and migration ability. And si-circDDX17 enhances the ability of proliferation and migration in MGC-803 cells, miR-1208-in or miR-1279-in reduced proliferation and migration ability (Figure S2). Western blot showed that, circDDX17 promoted cleaved-PRAP, BAX, and E-cadherin expression, and inhibited BCL, N-cadherin, Snail, and MMP9 expression, miR-1208 and miR-1279 reverse those trends (Figure 5C, 5E, 5H and 5J).
Results 5. CircDDX17 regulates FKBP5 by miR-1208 and miR-1279 in GC cells.

For research on the underlying mechanisms of miR-1208 and miR-1279 downstream factors, the bioinformatics tools (Targetscan and miRDB) were used to predict the potential targets of miR-1208 and miR-1279. As shown in Fig 6A, FKBP5 is miR-1208 and miR-1279 target gene. The expression of FKBP5 mRNA or protein was determined in miR-1208 and miR-1279 overexpression or knockdown cells, respectively in HGC-27 and MGC-803 cells. Results showed that the expression of FKBP5 mRNA (Figure 6B and 6C) and protein (Figure 6D-6F) was negatively regulated by miR-1208 and miR-1279. The expression of FKBP5 mRNA in human tissues was also identified. When compared to control tissues, the expression of FKBP5 was markedly downregulated in tumor tissues (Figure 6G and 6H). By analyzing the clinicopathological significance of FKBP5 in patients, the expression of FKBP5 in tumor tissues was negative and significantly associated with the size of tumor tissues (Table 2). IHC data also indicated that FKBP5 has low expression in gastric tumor tissues (Figure 6I). In addition, the expression of protein FKBP5 was lower expression in GC cells than in GES-1 cells (Figure 6J-6K). To sum up, the results above suggested that FKBP5 could be a target of miR-1208 and miR-1279.

For further research the effect of miR-1208/miR-1279-FKBP5 on the phenotype of GC cells, HGC-27 cells overexpression FKBP5, qRT-PCR to verify expression efficiency (Figure S3A). Overexpression of FKBP5 decreased colony numbers and suppressed cell viability (Figure S3B and S3C). Contrarily, transfected si-FKBP5 in MGC-803 cells, qRT-PCR to detect FKBP5 mRNA expression (Figure S3D), and knockdown FKBP5 promoted colony formation and cells viability (Figure S3E and S3F). Apoptosis and proliferation-related proteins were detected by Western blot, We found expression of FKBP5, cleaved-PARP, and BAX was markedly up-regulated in the FKBP5 overexpression group, and BCL and PCNA expression were down-regulated. Reverse, GC cells transfected si-FKBP5, the expression of FKBP5, cleaved-PARP, and BAX decreased, and BCL and PCNA expression were increased (Fig. S3G and S3H).

The goal of our investigation was to learn more about the function of FKBP5 in cell migration and EMT, wound healing assays, and Transwell assays. The findings showed that overexpressing FKBP5 reduced the ability of cells migration, while knocking down FKBP5 increased the ability of cells migration (Figure S3I-S3L). When HGC-27 cells overexpression FKBP5, migration and EMT-related proteins were detected by Western blot; N-cadherin, Snail, Vimentin, MMP2, and MMP9 expression were reduced, while E-cadherin increased. However, MGC-803 cells suppressed FKBP5, the expression of those proteins tended to go in the reverse direction (Figures S3M and S3N).

Our findings showed that FKBP5, which was down-regulated by miR-1208 and miR-1279, reduced proliferation, migration and EMT in GC cells. Thus, whether the effects of miR-1208 and miR-1279 were mediated by FKBP5 was studied. In HGC-27 cells, pcDNA-FKBP5 or pcDNA vector and mimics of miR-1208/miR-1279 or miR-NC were co-transfected; in MGC-803 cells, si-FKBP5 or si-NC and inhibitors of miR-1208/miR-1279 or inhibitor-NC were co-transfected. Results showed that overexpression of FKBP5 significantly lowered colony numbers and cell survival, whereas miR-1208/miR-1279 mimics may mitigate this impact (Figure 7A and 7B, Figure 7F and 7G). As Transwell and wound healing assay, the
results demonstrated, overexpression of FKBP5 could inhibit cell migration, however, this inhibitory effect was attenuated by down-regulation of miR-1208/miR-1279 mimics in HGC-27 cells (Figure 7D and 7I). Then, co-transfected si-FKBP5 and miR-1208/miR-1279 inhibitor showed opposite consequences (Figure S4A, S4B, S4D and S4E). Moreover, western blot data showed that the expression of cleaved-PARP, BAX, and E-cadherin was markedly up-regulated in the FKBP5 overexpression group, after transfecting miR-1208/miR-1279 mimics, the expression of these proteins was down-regulated. To the contrary, BCL, N-cadherin, Snail, and MMP9 were down-regulated in the FKBP5 overexpression group, after transfecting miR-1208/miR-1279 mimics, the expression of these proteins was up-regulated (Figure 7C, 7E, 7H and 7J).

**Results 6. FKBP5 regulated GC progression by the ERK1/2 pathway.**

According to the Oncomine database, FKBP5 was found to be either highly or lowly expressed in a variety of cancers. For example, FKBP5 was highly expressed in brain cancer, prostate cancer, lymphoma. But lowly expressed in pancreatic\(^{17}\), colon, testicular, and gastric cancers\(^{18}\). FKBP5 was first identified in HeLa cell cDNA library\(^{24}\). In this work, we discovered that FKBP5 had a low expression level in both GC cell lines and GC tissues, and that this expression was negative related with tumor size in GC patients. Overexpression FKBP5 decreased GC cell migration, EMT, and proliferation, while increasing apoptosis. Numerous tumor types grow as a result of abnormal ERK pathway activation, which also has an impact on cellular functions like metastasis, cell survival, and proliferation\(^{25}\).

To explore the underlying mechanism by which FKBP5 facilitates GC progression, bioinformatics tools (STRING, https://cn.string-db.org/cgi/network) were used to predict the interoperating protein, ERK1/2 as the target protein of FKBP5. Total proteins were IP using anti-FKBP5 antibody in order to further demonstrate the relationship between ERK1/2 and FKBP5. IP/Western blot was used to show that exogenous FKBP5 and ERK1/2 bind to HGC-27 and MKN-45 cells (Figure 8A). The expression and location of FKBP5 and ERK1/2 in HGC-27 cells were next examined using IF assay, and confocal imaging revealed that they co-localized in the cytoplasm (Figure 8B). When ERK1/2 was inactivated in HGC-27 cells with PD98059, Western blot (Figure 8C) and IF (Figure 8D-8F) data revealed that FKBP5, t-ERK1/2, and p-ERK1/2 expression levels were all considerably decreased. This finding suggests that PD98059 can concurrently limit the expression of FKBP5 and ERK1/2. Additionally, FKBP5 overexpression/knockdown in HGC-27/MGC-803 cells. Figure 8G shows that overexpression of FKBP5 decreased the expression of p-ERK1/2/t-ERK1/2 and p-STAT3/t-STAT3 in intracellular Western blot experiments. Figures 8H and 8I show that overexpression of FKBP5 inhibited the expression of p-ERK1/2 in cells, with the inhibitory effect being particularly noticeable in the nucleus. On the other hand, MGC-803 knockdown FKBP5 improved p-ERK1/2 activated in the nucleus and elevated p-ERK1/2/t-ERK1/2 and p-STAT3/t-STAT3 (Figure 8J-8L).

For a more thorough analysis of how circDDX17 affects the ERK1/2 and STAT3 pathway via altering the miR-1208/miR-1279/FKBP5 axis. First, we examined the effects of circDDX17 overexpression or knockdown in GC cells. As predicted, overexpression of circDDX17 decreased the effects on ERK1/2 and STAT3 phosphorylation in HGC-27 and MKN-45 cells (Figure S5A–S5D), whereas knockdown of
circDDX17 boosted ERK1/2 and STAT3 phosphorylation in MGC-803 cells (Figure S5E–S5F). When HGC-27 and control cells were overexpressed and injected into the nude mice, FKBP5 was upregulated in the tumor tissue by IHC and Western blot whereas ERK1/2 and STAT3 phosphorylation were downregulated (Figure S5G-S5I). Si-circDDX17 was transfected into MGC-803 cells, and IF results showed that circDDX17 knockdown boosted ERK1/2 and STAT3 activation by confocal microscopy in the nucleus (Figure S5J-S5M).

For study the circDDX17/miR-1208/miR-1279/FKBP5 axis function on ERK1/2 and STAT3 pathway, GC cells co-transfected circDDX17 vector and miR-1208/miR-1279 mimics, si-circDDX17 and miR-1208/miR-1279 inhibitor, pFKBP5 vector and miR-1208/miR-1279 mimics, si-FKBP4 and miR-1208/miR-1279 inhibitor, Western blot to analysis the influence on ERK1/2 and STAT3 expression and phosphorylation. The phosphorylation levels of ERK1/2 and STAT3 were decreased by the overexpression of circDDX17 and FKBP5, according to Western blot analysis. However, miR-1208/miR-1279 overexpression had the opposite effect (Figures S6A-S6D, S6I-S6L). On the other hand, GC cells that had their levels of circDDX17 and FKBP5 knockdown increased ERK1/2 and STAT3 phosphorylation. Then, the effects were reversed by the miR-1208/miR-1279 inhibitor (Figures S6E-S6H, S6M-S6P).

**Results 7. CircDDX17 encodes the 63aa tumor-suppressed protein**

Based on the results described above, we proved that circDDX17 suppressed the proliferation, migration, and EMT in GC cells. We further explored whether circDDX17 functioned by encoding a peptide, circDDX17 was mainly located in the cytoplasm, which has the potential molecular to encode a protein. The ability and sequence of the polypeptide encoded by circDDX17 were predicted by the public database ORF Finder (www.ncbi.nlm.nih.gov/orffinder), and the results showed that circDDX17 existed the ability to encode polypeptide, and 192nt (63aa) of which might have the possibility of encoding were selected for the study (Figure 9A).

According to the website prediction results, the peptides encoded by circDDX17 were expressed in eukaryotic plasmids according to different requirements, and a novel circDDX17 vector containing the GFP sequence was constructed to further assess the protein-coding ability of circDDX17. CircDDX17, the circularization created the tandem start codon ‘AUG’, which started the translation in combination with overlapping genetic codes. To dissociate the role of circDDX17-63aa from circDDX17, we constructed several GFP-tag vectors for circDDX17 (Figure 9B). Transfection with the LvcircDDX17 vector and LvcircDDX17-mut vector both successfully resulted in the overexpression of circDDX17, and the GFP-tagged proteins were detected by Western blot after extracting total cellular proteins from transfected HEK-293T cells after 48 h. (Figure 9C). LvcircDDX17 and LvcircDDX17-63aa could express GFP-tag, but Lv-vector and LvcircDDX17-mut unable expression GFP-tag. Vectors transfected in HGC-27 cells, GFP-tag expression on LvcircDDX17 and LvcircDDX17-63aa transfected cells, and GFP-tag almost located in the cytoplasm (Figure 9D). We further confirmed that circDDX17-63aa was translated from circDDX17 by SDS-PAGE and Western blot of the circDDX17-63aa was identified in the 10-kDa band, the specific protein fragments from circDDX17-63aa were successfully identified (Figure 9E and 9F).
To further explore the biological function of circDDX17-63aa, we transfection of those above-mentioned four vectors on HGC-27 cells. Overexpression of wild-type circDDX17-63aa resulted in decreased proliferation ability, evidenced by colony formation (Figure 9G and 9H) and CCK8 assay (Figure 9I). Moreover, circDDX17-63aa suppressed GC cell migration detected by Transwell (Figure 9J and 9L) and wound-healing assay (Figure 9K and 9M).

**Discussion**

CircRNAs have been shown to have an important role in the regulation of several cellular processes, including the development and spread of cancer. Numerous studies have revealed aberrant expression patterns of ncRNAs, including miRNAs, IncRNAs, and circRNAs, in a variety of cancer types. Multiple circRNAs are differentially expressed as a result of GC lead, and these differentially expressed circRNAs are crucial for GC diagnosis, therapy, and drug sensitivity research\(^{20,26,27}\). Studies that up-regulate CircFAM73A improve the stem cell-like characteristics of GC through the miR-490-3p/HMGA2 axis in a positive feedback loop. Studies that recruit HNRNPK to promote-catenin stabilization improve cell proliferation, migration, and resistance to cisplatin\(^ {28}\). Since CircORC5 is METTL14 downstream target, METTL14 knockdown boosted CircORC5 expression through reducing CircORC5 m6A level. Additionally, sponge miR-30c-2-3p inhibits the advancement of gastric cancer by reversing the METTL14-mediated up-regulation of miR-30c-2-3 and the down-regulation of AKT1S1 and EIF4B\(^ {29}\). We discovered circDDX17 in a circRNA microarray online database, which had low expression in GC tissue and GC cells. According to our findings, circDDX17 expression level inhibited GC cell proliferation, migration, EMT and induced apoptosis. It also showed a negative correlation between tumor size and lymph node metastasis. CircDDX17 expressed in both the cytoplasm and the nucleus, but mainly in the cytoplasm, according to the FISH and the nuclear and cytoplasmic isolation assay. These findings suggested that circDDX17 may act as a miRNA sponge to regulate mRNA expression. Then, we discovered that the circDDX17 target miRNAs miR-1208 and miR-1279 promote GC cell proliferation, migration, and EMT (Fig. 10).

According to earlier positions, eukaryotic mRNA is always translated through the conventional cap system, and eIF4E is a crucial component of the translation initiation process\(^ {30,31}\). The 5′ UTR of mRNAs contains sequences known as internal ribosome entry sites (IRESs), which can directly attract ribosomes to begin translation. When canonical translation is changed, IRES can direct begin mRNA translation\(^ {32,33}\). According to high throughput screening, IRES elements are present in around 10% of human mRNAs\(^ {34}\). CircRNAs may be the primary mechanisms for translation RNAs lack 3′ and 5′ ends\(^ {35}\). CircRNA has the potential to encode a protein since it has the start codon AUG and an ORF with a desirable length\(^ {36}\). The progression of sickness in mammals and the formation of circRNA-encoding proteins have now been linked in several studies. CircPPP1R12A encoded 73aa in malignancies to promote colon cancer cell growth, migration, and invasion\(^ {13}\). The 63aa protein that circDDX17 encoded in our study serves the same purposes as circDDX17. The cytoplasmic expression of circDDX17-63aa in GC cells promotes apoptosis while inhibiting cell proliferation and migration (Fig. 10).
Immunophilins have a distinctive role in the scaffold proteins by interacting with proteins to direct appropriate protein assembly. The first proteins of the immunosuppressive drug cyclosporin, that were found were cyclophilins and FK506 binding proteins (FKBPs). According to recent studies, immunophilins are crucial for the intrinsic characteristics of a tumor. The FKBP5 gene was the only immunophilin family member to be found to have a splicing variation. FKBP5 affects several essential elements of an organism's cell biology and physiology, including development, differentiation, metabolism, and immunological responses. It also possesses pleiotropic activities. FKBP5 had a low expression in GC cells and GC tissues in our study, which inhibited GC cell proliferation, migration, and EMT. FKBP5 participates in the NF-B, Akt, AMPK/mTOR, and TGF-/EMT signaling pathways, which are all signal transduction pathways. The ERK protein is a crucial component of the MAPK pathway, which plays a significant role in the development of gastric cancer by controlling the cell cycle, migration, apoptosis, and proliferation. Only ERK1 and ERK2 (also known as the p44 and p42 kinases, respectively) are involved in the RAS/MAPK signaling pathway out of the many isoforms of the ERK family, which range from ERK1 to ERK8. Active-ERK1/2 controls a wide range of cytoplasmic and nuclear targets via phosphorylation, which largely regulates cell proliferation, differentiation, survival, and death. Anomalous activation of the ERK-1/2 pathway has been associated with tumorigenesis. Hyperactivation of ERK-1/2 signaling has been reported in several malignancies including pancreatic cancer, breast cancer, and prostate cancer. In this study, FKBP5 interaction with ERK1/2, which they co-localized in GC cells, downregulated the expression of FKBP5. FKBP5 controlled GC development through the ERK1/2 pathway.

**Conclusion**

Through the circDDX17-miR-1208/miR-1279-FKBP5 axis and encoding circDDX17-63aa, circDDX17 affects biological activities such as proliferation, apoptosis, migration, and EMT of GC cells, and functions as an oncogenic factor in the development of GC.

**Abbreviations**

- GC: Gastric cancer
- circRNA: circular RNA
- miRNA: microRNA
- ORF: open reading frame
- RNase R: ribonuclease R
- FISH: fluorescence in situ hybridization
- IP: Immunoprecipitation
Declarations

Acknowledgements
Not applicable.

Authors' contributions
Shihe Shao and Feilun Cui provided ideas for the article. Tingjun Liu, Jiaxin Xue, Linqi Zhu, Wenjun Zhao, Jing Sun and Fan Wang researched data for the article. Tingjun Liu and Tieliang Ma edited the manuscript before submission. All authors approved the final version of the manuscript.

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Availability of data and materials
All the data in this article are included in the manuscript.

Ethics approval and consent to participate
The use of clinical samples was approved by the ethics committee of Jiangsu University and informed consent was obtained from all patients.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


Tables

Table 1

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Table 2 The clinicopathological features of GC patients with differential expressions of FKBKP5

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### Clinical Parameters

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**Figures**
Figure 1

Validation, Expression, and Characterization of circDDX17 in GC Tissues and Cell Lines

A Predicted the differentially expressed circRNAs by circRNA microarray of GC tissue and cell lines. B CircDDX17 location on the human chromosome. C Resistance RNase R role of circDDX17. D Validated circDDX17 circular transcripts by PCR. E The relative expression of circDDX17 in the nucleus and cytoplasm of GC cells was analyzed. U6 was used as nuclear control and 18s was used as cytoplasmic control. F FISH assay the location of circDDX17 in GC cells. G The expression of circDDX17 in GC and
matched non-tumor tissue samples (n=51) was analyzed by qRT-PCR. The expression level of circDDX17 in a series of cultured gastric mucosa cells (GES-1) and GC cell lines (HGC-27, MGC-803, MKN-45) was analyzed by qRT-PCR. *P<0.05, **P<0.001, ***P<0.0001.

Figure 2

Effect of circDDX17 on GC cell proliferation, apoptosis, migration, and EMT.
Used HGC-27 and MKN-45 cells to overexpression circDDX17, and MGC-803 cells to silence circDDX17. A qRT-PCR to analyze the expression level of circDDX17 after GC cells overexpression or knockdown of circDDX17. B qRT-PCR to analyze the expression level of ddx17 mRNA after GC cells overexpression or knockdown circDDX17. GC cells overexpression or knockdown circDDX17, analysis cells proliferation and apoptosis by the experiments as below. C and D Colony formation assay to analyze the ability of proliferation. E, F, and G CCK8 assay to analyze the ability of proliferation. H Flow cytometry assay cells apoptosis. I, J, K, and L Western blot to analyze the proteins of proliferation and apoptosis. M and N Wound healing assay to detect GC cell migration after cell overexpression or knockdown circDDX17. O and P Transwell assay to detect GC cell migration after cell overexpression or knockdown circDDX17. Q, R, S, and T Western blot to analyze the proteins of migration and EMT after GC cells overexpression or knockdown circDDX17. *P<0.05, **P<0.01, ***P<0.0001.

Figure 3

**In vivo**, CircDDX17 suppressed GC cell growth, migration, and EMT.

A CircDDX17 suppressed HGC-27 cells induced subcutaneous xenografts. B N-cadherin, E-cadherin, MMP2, MMP9, and PCNA expression were analyzed within the subcutaneous xenograft tumors through
IHC (scale bars = 100 μm). C Western blot to analyze the proteins expression of apoptosis, proliferation, migration, EMT, and ERK1/2 pathway of subcutaneous xenograft tumors. D qRT-PCR to detect the mRNA expression of N-cadherin, E-cadherin, MMP2, MMP9, and PCNA. E and F ELISA to detect the expression of TGF-β and TNF-α in nude mice serum. *P<0.05, **P<0.01, ***P<0.0001.
CircDDX17 functioned as a miR-1208 and miR-1279 sponge in GC cells.

**A and B** qRT-PCR to detect the expression of miR-1208 (A) and miR-1279 (B) after GC cells overexpression or silenced circDDX17. **C** Schematic representation of potential binding sites of miR-1208 and miR-1279 mimics with wt or mut circDDX17. **D and E** Dual-luciferase report assays to detect the interaction between miR-1208 (D) or miR-1279 (E) and circDDX17. **F and I** qRT-PCR to analyze the expression of miR-1208 (F) and miR-1279 (I) in the GC cell line. **G, H, J, and K** qRT-PCR to analyze the expression of miR-1208 (G and H) and miR-1279 (J and K) in adjacent non-tumor tissues and gastric tumor tissues. \*\(P<0.05\), **\(P<0.01\), ***\(P<0.0001\).

**Figure 5**

miR-1208 and miR-1279 mimic reserved circDDX17 function on GC cells apoptosis and proliferation.

Conduct the following experiments when HGC-27 cells co-transfected circDDX17 and miR-1208: The proliferative capacity was determined via **A** colon formation assay, **B** CCK8 assay; **C** The proteins of proliferation and apoptosis detected by Western blot; **D** The migration capacity were determined via Transwell and wound-healing assay; **E** The proteins of migration and EMT detected by Western blot. Conduct the following experiments when HGC-27 cells co-transfected circDDX17 and miR-1279: The proliferative capacity was determined via **F** colon formation assay, **G** CCK8 assay; **H** The proteins of proliferation and apoptosis detected by Western blot; **I** The migration capacity were determined via
Transwell and wound-healing assay; J The proteins of migration and EMT detected by Western blot; *$P<0.05$, **$P<0.01$, ***$P<0.0001$.

Figure 6

FKBP5 was a direct target of miR-1208 and miR-1279.
The potential target gene of miR-1208 and miR-1279 was predicted by Targetscan and miRDB database; B and C The mRNA level of FKBP5 in miR-1208 or miR-1279 overexpression (B) and knockdown (C) systems were determined by qRT-PCR; D, E, and F The protein level of FKBP5 in miR-1208 or miR-1279 overexpression and knockdown systems were determined by Western blot; G and H The expression of FKBP5 and corresponding normal tissues were detected by qRT-PCR; I The expression of FKBP5 and corresponding normal tissues were detected by IHC; J and K The protein level of FKBP5 in GC normal cells were measured by western blot; scale bars were 25 μm, *P<0.05, **P<0.01, ***P<0.0001.

**Figure 7**

**FKBP5 affects proliferation, apoptosis, migration, and EMT of GC cells.**

Perform the following experiment when HGC-27 cells co-transfected pFKBP5 vector and miR-1208 mimics: The proliferative capacity was determined via A colon formation assay, B CCK8 assay; C The proteins of proliferation and apoptosis detected by Western blot; D The migration capacity was determined via Transwell and wound-healing assay; E The proteins of migration and EMT detected by Western blot. Conduct the following experiments when HGC-27 cells co-transfected pFKBP5 and miR-1279 mimics: The proliferative capacity was determined via F colon formation assay, G CCK8 assay; H The proteins of proliferation and apoptosis detected by Western blot; I The migration capacity were determined via Transwell and wound-healing assay; J The proteins of migration and EMT detected by Western blot; *P<0.05, **P<0.01, ***P<0.0001.
Figure 8

Identify the interaction protein with FKBP5

A IP to analyze the interaction between FKBP5 and ERK1/2; B Confocal laser detects the colocalization between FKBP5 and ERK1/2; C and D Western blot to analyze the proteins when HGC-27 added PD98059; E, F, and G Confocal laser detect the expression level and location of FKBP5, t-ERK1/2, and p-ERK1/2 after HGC-27 added PD98059; G Western blot to analysis the proteins of ERK1/2 and STAT3 after HGC-27 cells overexpression FKBP5; H and I Confocal laser detect the expression and location of t-ERK1/2 and p-ERK1/2 after HGC-27 cells overexpression FKBP5; J Western blot to analyze the proteins of ERK1/2 and
STAT3 after MGC-803 cells overexpression FKBP5; K and L Confocal laser detect the expression and location of t-ERK1/2 and p-ERK1/2 after MGC-803 cells overexpression FKBP5; × 600, scale = 10 μm, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 9

CircDDX17 encodes a small unchartered protein circDDX17-63aa
The circDDX17 was potentially translated into a 63aa protein (circDDX17-63aa). B To establish a detectable circDDX17 expression vector. C The expression level of GFP-indicated circDDX17-63aa was detected by Western blot analysis. D GFP-tag expression and location on GC cells detected by confocal microscopy. E Protein bands of circDDX17-63aa were manually excised and submitted for identification by SDS-PAGE. F His-tag to analyze the circDDX17-63aa by Western blot. G and H Effect of circDDX17-63aa on the colony formation of HGC-27 cells; I CCK8 assay the role of circDDX17-63aa in HGC-27 cells; J and L Transwell assay after HGC-27 cells transfected with circDDX17-63aa vector; K and M Wound healing assay after HGC-27 cells transfected with circDDX17-63aa vector; IF × 600, scale =10 μm; Transwell and wound healing assay, × 200, scale=50 μm, *P<0.05, **P<0.01, ***P<0.001.

Figure 10

The function of circDDX17 in gastric cancer

CircDDX17 suppressed GC progression by circDDX17-miR-1208/miR-1279-FKBP5 axis and encoding circDDX17-63aa through ERK1/2 and STAT3 pathway.

Supplementary Files
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- Figures4.jpg
- FigureS5.jpg
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