CircHAS2 promotes the proliferation, migration, and invasion of gastric cancer cells by regulating PPM1E mediated by hsa-miR-944

Shuo Ma1,2,3, Xinliang Gu1,2,3, Lei Shen1,2,3, Yinhao Chen1,2,4, Chen Qian1, Xianjuan Shen4,5 and Shaoqing Ju1,6

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Gastric cancer (GC) is considered one of the most common gastrointestinal malignancies worldwide. Circular RNAs (circRNAs) are a new class of endogenous noncoding RNAs, which can be used as biomarkers and therapeutic targets for many tumors. However, the role and potential regulatory mechanisms of circRNAs in GC remain unclear. In this study, we demonstrated that a specific circRNA, circHAS2, was upregulated in GC tissues and cells and was positively correlated with tumor metastasis. In vitro experiments demonstrated that circHAS2 knockdown or the addition of hsa-miR-944 mimics inhibited the proliferation, migration, and invasion ability of GC cells and affected the epithelial-mesenchymal transition. In addition, hsa-miR-944 interacted with protein phosphatase, Mg2+/Mn2+-dependent 1E (PPM1E), and was found to be a target gene of circHAS2. The upregulation of PPM1E reversed the effects of circHAS2 knockout on GC cells. The circHAS2/hsa-miR-944/PPM1E axis may be involved in the progression of GC; thus, circHAS2 may be a potential biomarker and therapeutic target for GC.

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INTRODUCTION
Gastric cancer (GC) is considered to be one of the most common gastrointestinal malignancies, and its mortality rate ranks third worldwide [1, 2]. GC can be caused by many factors including Helicobacter pylori infection, precancerous lesions, diet, or genetic factors [3, 4]. It tends to spread through the lymph nodes to neighboring tissues or organs and produces more cancer cells in the bloodstream [5]. Although significant progress has been made in the diagnosis and treatment of GC in recent years, the overall survival (OS) of GC patients is still unsatisfactory, with a 5-year OS rate of less than 30% [6]. Therefore, it is urgent to identify effective biomarkers and understand their roles in the occurrence and development of GC.

Circular RNAs (circRNAs) are a special type of endogenous small RNAs, which are widely distributed and diverse [7]. They are formed by back-splicing to produce a covalently closed circular structure, usually by the cyclization of exons [8, 9]. Although Sanger et al. discovered the circRNA molecule as early as 1976, after more than 40 years of development, the biological functions of circRNAs are still largely unclear [10, 11]. CircRNAs are widely distributed in eukaryotic cells, have long half-lives, and are expressed at tissue- and stage-specific levels [12]. They can be used as a biomarker and therapeutic target for many tumors. For example, Ding et al. [13] found that circ-DONSON promoted the progression of GC by activating the expression of SRY-box transcription factor four via recruitment of the nucleosome remodeling factor complex. Fan et al. [14] found that circNR3C2 can act as a potential prognostic biomarker in breast cancer and negatively modulate breast cancer metastasis through the NR3C2/miR-513a-3p/Hrd1/vimentin axis. In recent years, studies have shown that circRNAs can encode regulatory proteins/peptides in unique ways [15–17]. However, owing to the small number of circRNAs that can encode, the encoding mechanism has not been effectively explained. The mainstream understanding of circRNAs as a molecular regulatory mechanism in diseases is that they function as a microRNA (miRNA) sponge to regulate tumor proliferation, metastasis, drug resistance, and other functions [18–20]. For example, circCUL2 regulates the expression of Rho-associated coiled-coil containing protein kinase two by regulating miR-142-3p, and regulates the tumor progression of GC by activating the autophagy pathway [21]. CircUBAP2 regulates the malignant behavior of osteosarcoma cells through the miR-204-3p/HMGA2 axis [22]. CircAGAP1 promotes the development of renal clear cell carcinoma by binding with miR-15-5p to promote EZF transcription factor 3 expression [23]. Recent studies have shown that circRNAs play a critical role in the genesis and development of GC [24, 25]. The mechanism of circRNA as a competing endogenous RNA (ceRNA) in GC has not been fully explored, and its physiological and pathological roles in GC are not completely understood.

In this study, we identified a novel circRNA, circHAS2. We confirmed that circHAS2 can competitively bind with hsa-miR-944 to affect the expression of PPM1E in GC and ultimately act as a
METHODS

Clinical samples
A total of 60 pairs of gastric and paracancerous tissues were collected from the Department of Pathology, Affiliated Hospital of Nantong University (Jiangsu, China). All of the specimens were immediately frozen in −80°C liquid nitrogen after resection, and total RNA was extracted. All of the specimens were pathologically diagnosed as GC. Tissues were collected from August 2016 to October 2021. The study was approved by the local ethics committee (ethical review report number: 2018-L055). Patients did not receive radiotherapy or chemotherapy before surgery. All of the GC patients and healthy control participants consented to the clinical trial and publication of the article.

RNA extraction and quantitative PCR
Total RNA from GC tissues and cells was extracted using TRIzol reagent (Invitrogen, Ontario, Canada). Total RNA was reverse transcribed into cDNA at 42°C for 10 min and at 72°C for 1 h using a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). The Roche LightCycler 480 (Roche, Switzerland) was used for quantitative PCR (qPCR) (20-μL total volume; denaturation 95°C, 10 s; annealing 60°C, 30 s; 45 cycles). GAPDH rRNA was used as an internal reference for hsa-miR-944. The primer sequences were forward-5′-GATGCAAAGGGCAACTGTTT-3′ and (reverse-5′-GC TGTGATTCCAAGGAG-3′); HAS2 (forward-5′-CCTCATCCTCAAAGGCTGT-3′ and (reverse-5′-GATGCAAAGGGCAACTGTTT-3′); PPM1E (forward-5′-GCTTACGGACAGGCAG-3′ and (reverse-5′-ACAGTCCCTTCTGTGCCATC-3′); GAPDH (forward-5′-TCCCCATCACCTATCTCCAGG-3′ and (reverse-5′-GATGACCTTTTGGCTTCCC-3′); U6 (forward-5′-AACGCTTCACGAATTTGCGT-3′ and (reverse-5′-CTC GCTTGGCAGACACA-3′).

The hsa-miR-944 primer sequence was obtained from RiboBio Co., Ltd. (Guangzhou, China). The 2−ΔΔCT method was used to calculate the relative expression levels of plasma circRNAs.

Cell culture
We obtained GC cells (AGS, MKN-1, HGC-27, MKN-45, SGC-7901, MGC-803, BGC-823) and gastric epithelial cells (GES-1) from the Chinese Academy of Sciences (Shanghai, China). All of the cells were cultured in RPMI-1640 medium (Corning, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 1% penicillin and streptomycin (Newcells Biotech Co., Ltd., Tyne UK). The culture condition was 37°C, 5% carbon dioxide. The media were changed every 3 days.

RNase R and actinomycin D assay
According to the amount of RNA, RNase R (Geneseed, Guangzhou, China) was used for treatment, and cells were incubated at 20°C for 20 min. After treatment, the expression of circHAS2 and HAS2 in the cells was detected by qPCR. The cells were cultured in medium containing actinomycin D (blocks RNA transcription) for 0, 2, 4, 8, 12, and 24 h, and then the RNA was detected by qPCR.

Plasmid construction and transfection
After the cells were grown to 60–70% confluency, they were transfected with short hairpin negative control (shNC), shRNA1, shRNA2, shHAS2-1, shHAS-2, mimics NC, hsa-miR-944 mimics, OE-NC, and OE-PPM1E using Lipofectamine 3000 (Thermo Fisher Scientific) and harvested after 24–48 h.

Cell counting Kit-8 assay
Cells transfected for 24–48 h were collected and counted. The cell suspension was diluted to 3 × 10^6/mL and plated in 96-well plates. Cells were incubated for 24, 48, 72, 96, or 120 h with four replicates for each timepoint. Then, 10 μL CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well at the corresponding timepoint. A microplate analyzer measured the absorbance of each well at 450 and 630 nm at 2 h.

Colony formation assay
Cells were collected at 24–48 h after transfection, and 1000 cells per well were seeded in 6-well plates. The culture medium was changed every 4 days, and after 2 weeks of culture, cells were fixed in paraformaldehyde for 12–24 h, stained with crystal violet, and photographed.

Transwell assay
Cells transfected for 24–48 h were collected, and the cell density was adjusted to 5 × 10^4/mL for the cell migration experiment and 8 × 10^5/mL for the cell invasion experiment. Then 500-μL RPMI-1640 medium was added to the 24-well plates. Then the transwell inserts were added to the plates. In the invasion experiment, Matrigel (Corning) and RPMI-1640 medium were mixed at a ratio of 1:6 and added to the upper chamber. The cell suspension was added to the chamber and removed after 48 h, fixed in paraformaldehyde, stained with crystal violet, and photographed under a microscope.

5-Ethynyl-2′-deoxyuridine assay
Cells were evenly seeded into 24-well plates at a density of 1 × 10^4 cells per well. The EdU Kit (RiboBio) was used to detect proliferating cells. After the cells adhered to the wall, the culture medium was changed to medium containing 50 μM EdU and incubated for 2 h. Then the cells were stained with Apollo and Hoechst 33342. The cells were observed by fluorescence microscopy. The EdU-positive cell rate (the ratio of EdU-positive cells to the total number of DAPI-stained cells) was calculated.

Fluorescence in situ hybridization
The cells were evenly inoculated in a 24-well plate. After the cells adhered to the wall, the cells were washed with phosphate-buffered saline and fixed in paraformaldehyde for 30 min. Then a permeability solution was added for permeability. A hybrid solution containing circHAS2 and HAS2 was added and the U6 probe (RiboBio) was added to each well overnight. Then cells were washed three times with liquid containing SSC (Sangon Biotech, Shanghai, China). Then nuclear staining was performed with Hoechst 33342 (RiboBio) or DAPI, followed by fluorescence microscopy.

Dual-luciferase reporter assay
CircHAS2 and PPM1E sequences containing wild-type (WT) and mutant (MUT) hsa-miR-944 binding sites were synthesized and inserted into a luciferase vector (RiboBio), which was co-transfected into GC cells with hsa-miR-944 mimics. After transfection for 48 h, the cells were lysed. A double-luciferase assay kit (Vazyme Biotech Co., Ltd., Nanjing, China) was used to detect luciferase activity.

Western blot analysis
After transfection for 48 h, the cells were lysed in RIPA buffer with PMSF (SolarBio Life Science, Beijing, China). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro- transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked in a rapid blocking solution (Shanghai EpiZyme Biotechnology, Shanghai, China) and then incubated with primary antibodies overnight at 4°C. Next, the membrane was washed 2–3 times with Tris-buffered saline with 0.1% Tween® 20 detergent (TBST), followed by incubation with horseradish-peroxidase (HRP)-labeled secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 2 h and then another 2–3 washes with TBST. Finally, proteins were visualized by enhanced chemiluminescence (Vazyme Biotech Co., Ltd., Nanjing, China).

RNA immunoprecipitation assay
The BersinBio™ RNA Immunoprecipitation (RIP) Kit (BersinBio, Guangzhou, China) was used to perform the RIP experiments. HGC-27 and MKN-45 cells overexpressing hsa-miR-944 were used. Cells were lysed in RNA lysis buffer and then incubated at 4°C overnight with RIP buffer containing coupled Argonaute-2 (AGO2) antibody (Proteintech, Rosemont, IL, USA) or negative control IgG. Then the magnetic beads were washed three times. After proteinase K treatment, the immunoprecipitated RNA was extracted with phenol-chloroform-isopropyl alcohol (25:24:1). Finally, qPCR was used to detect the expression of circHAS2 and hsa-miR-944.

Bioinformatics analyses
Cross-analysis of the prediction databases circAtlas (http://circatlas.biols.ac.cn/), Starbase (http://starbase.sysu.EdU.cn/), CircBank (http://www.circbank.cn/index.html), and Circinteractome (http://circinteractome.nia.nih.gov/)
was conducted. The target gene circHAS2 was obtained by the cross-analysis of four databases. Moreover, the MiRDB (http://mirdb.org/index.html), MiRWalk (https://mirwalk.umm.uni-heidelberg.de/), MiRPathDB (https://mpd.bioinf.uni-sb.de/overview.html), and Targetscan (http://www.targetscan.org/vert_72/) databases were used for the intersection and cross-analysis of target genes of hsa-miR-944.

Statistical analyses
SPSS16.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA) were used for statistical analyses. All of the experiments were conducted in triplicate, and all of the data are expressed as the mean ± standard deviation. Student’s t test or one-way analysis of variance evaluated the differences between two or more groups. The relationship between circHAS2 and linear HAS2 or hsa-miR-944 was evaluated using Pearson’s correlation coefficient. Survival was analyzed using the Kaplan–Meier survival curve. P < 0.05 was considered to be statistically significant.

RESULTS
CircHAS2 is upregulated in GC and is associated with GC progression
To investigate the biological function of circRNAs in GC, we analyzed the differentially expressed circRNAs in three pairs of GC tissues and corresponding paracancerous tissues from GSE121445 data. A total of 615 circRNAs were differentially expressed in the database, including 102 upregulated and 513 downregulated (Fig. 1A). We selected seven circRNAs (circAPBB2, circRHOBTB3, circRHOBTB3-1, circNAV3, circHAS2, circSOBP, and circZNF521) with the highest upregulated expression meeting the criteria of fold change >2 and P < 0.05. Their relevant information is shown in Supplementary Table 1. Subsequently, qPCR was used to verify the differences in expression levels of these seven upregulated circRNAs in 20 pairs of GC tissues and their corresponding paracancerous tissues. The results showed that only circAPBB2 and circHAS2 were significantly upregulated, and the upregulation of circHAS2 was the most significant (Fig. 1B). Then qPCR was used to further analyze the expression of circHAS2 in 60 pairs of GC tissues and corresponding paracancerous tissues, and the expression of circHAS2 in GC tissues was significantly higher than that in normal paracancerous tissues (Fig. 1C). According to different lymph node metastasis cases in GC tissues, the expression level of circHAS2 was found to increase with the increase in lymph node metastasis (Fig. 1D). In addition, the GC group was divided into a high expression group (expression level >3.0524) and low expression group (expression level ≤3.0524) based on the median expression level of GC tissue as the limit. After analyses of the clinical data, it was found that the expression of circHAS2 was correlated with T stage, tumor-node-metastasis (TNM) stage, lymph node metastasis, and nerve/vascular invasion but not with other characteristics (Table 1). After comparing survival curves, it was found that the survival status of the low expression group was significantly better than that of the high expression group (Fig. 1E). These results indicated that the expression of circHAS2 was upregulated in GC and correlated with the metastasis and prognosis of GC.

Characteristics and basic information of circHAS2
The circBase database (http://www.circbase.org/) and UCSC database (GRCh37/hg19) (http://genome.ucsc.edu/) showed that circHAS2 was located at chr8:122,640,953-122,641,580, with a mature transcript length of 627 base pairs (bp), which was formed by cyclization of exon 2 (Fig. 2A).
by 2.5% agarose gel electrophoresis, and the band length was 90 bp, consistent with the size of the primer products (Fig. 2B). In addition, Sanger sequencing further confirmed the reverse splicing site of circHAS2 (Fig. 2C). After agarose gel electrophoresis of qPCR products of genomic DNA and cDNA, it was found that the reverse amplification products of circHAS2 only appeared in cDNA, and the circular structure of circHAS2 was also verified by the above experiments (Fig. 2D). We treated AGS and MKN-45 cells

**Table 1. Clinical Bentley analysis of circHAS2.**

| Parameter                 | No. of patients | circHAS2(high) | circHAS2(low) | P value  |
|---------------------------|-----------------|----------------|---------------|----------|
| Sex                       |                 |                |               |          |
| male                      | 32              | 18             | 14            | 0.536    |
| female                    | 28              | 16             | 12            |          |
| Age (year)                |                 |                |               |          |
| <60                       | 15              | 9              | 9             | 0.319    |
| ≥60                       | 45              | 27             | 18            |          |
| Tumor size                |                 |                |               |          |
| <5                        | 43              | 28             | 15            | 0.074    |
| ≥5                        | 17              | 10             | 7             |          |
| Differentiation grade    |                 |                |               |          |
| Well-moderate             | 19              | 10             | 9             | 0.106    |
| Poor-undifferentiation    | 41              | 20             | 21            |          |
| T stage                   |                 |                |               |          |
| T1-T2                     | 35              | 21             | 14            | 0.0351*  |
| T3-T4                     | 25              | 14             | 11            |          |
| Lymph node status         |                 |                |               |          |
| Positive                  | 39              | 24             | 15            | 0.001*** |
| Negative                  | 21              | 16             | 5             |          |
| TNM stage                 |                 |                |               |          |
| I-II                      | 51              | 35             | 16            | <0.0001**** |
| III-IV                    | 9               | 7              | 2             |          |
| Nerve/vascular invasion   |                 |                |               |          |
| Positive                  | 33              | 19             | 14            | 0.0125** |
| Negative                  | 27              | 19             | 8             |          |

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

**Fig. 2** Basic information and characteristics of circHAS2. A Schematic diagram of chromosomal location and formation of circHAS2. B and C Agarose gel electrophoresis and Sanger sequencing verified the primer length and cyclization site of circHAS2. D Verify the circular structure of circHAS2. E and F RNase R and actinomycin D were used to verify the stability of circHAS2. G The expression levels of circHAS2 and HAS2 were detected by qPCR. **P < 0.01.
with RNase R and found that circHAS2 exhibited better antides-
tion ability compared to linear HAS2 (Fig. 2E). In addition, after
treatment with AGS and MKN-45 cells by actinomycin D, the
stability of circHAS2 was stronger than that of linear HAS2 (Fig. 2F),
further con
[86x178]fi
[193x178]rming the stability of the circular structure and
indicating that circHAS2 had good stability. By comparing the
expression levels of circHAS2 and its transcriptional gene HAS2 in
20 pairs of GC tissues and paired paracancerous tissues, it was
found that only circHAS2 showed signi
[188x138]fi
[193x138]cant differential expres-
sion in GC tissues (Fig. 2G). These results demonstrate that the
ring-like structure of circHAS2 may be a factor affecting the
progression of GC.

CircHAS2 can promote the proliferation and metastasis of GC
cells
To assess the effects of circHAS2 on cell proliferation, migration,
and invasion during the progression of GC, we conducted in vitro
functional experiments. The expression of circHAS2 in GC cells
was investigated by qPCR. As shown in Fig. 3A, the expression levels
of circHAS2 in GC cells (SGC-7901, HGC-27, MKN-45, MKN-1, BGC-823,
AGS) were all increased compared to GC epithelial cells. We
knocked down circHAS2 expression (shRNA1 and shRNA2) in HGC-
27 and MKN-45 cells (Fig. 3B). The CCK-8 assay and colony
formation assay demonstrated that cell proliferation was sig-
nificantly inhibited after circHAS2 knockdown compared to the
control group (Fig. 3C–E). Similarly, the EdU assay further
demonstrated that the number of proliferating cells was
significantly reduced after circHAS2 knockdown (Fig. 3F and G).
These experiments suggest that circHAS2 can promote the
proliferation of GC cells. Subsequently, we used the transwell
assay to investigate the migration and invasion ability of GC cells
after circHAS2 knockdown. The transwell assay demonstrated that
the migration and invasion abilities of HGC-27 and MKN-45 cells
after circHAS2 knockdown were significantly reduced (Fig. 3H–K).

**Fig. 3  circHAS2 can promote the proliferation, migration, and invasion of GC cells.** A The expression level of circHAS2 in GC cells. B Knocking efficiency of GC cells transfected with plasmids shNC,shRNA1, and shRNA2. C–G The effects of circHAS2 knockdown on the proliferation ability of HGC-27 and MKN-45 cells were evaluated by CCK-8, cell colony formation assay, and EdU assay. H–K The effects of circHAS2 knockdown on the migration and invasion ability of HGC-27 and MKN-45 cells were evaluated by Transwell migration and invasion assay. L After circHAS2 was knocked down, the expressions of N-cad and Vimentin, key proteins of EMT, were detected by Western blot.

***P < 0.001, ****P < 0.0001.
Western blot analysis showed that N-cadherin (N-cad) and vimentin expression in HGC-27 and MKN-45 cells was decreased after circHAS2 knockdown (Fig. 3L). To investigate the specific functional effects of circHAS2 on GC cells, we knocked down the expression of HAS2 in HGC-27 with MKN-45 cells and subsequently performed cellular assays. CCK-8, colony formation, and transwell assays demonstrated that knockdown of HAS2 had no effects on the proliferation, migration, and invasive abilities of GC cells (Supplementary Fig. 1). These results confirmed that circHAS2 promoted the proliferation, migration, and invasion of GC cells and was related to the epithelial-mesenchymal transition (EMT).

CircHAS2 regulates GC development by sponging hsa-miR-944

To evaluate the regulatory mechanism of circHAS2 in GC, nucleoplasmic separation and FISH assay were used to confirm that circHAS2 is mainly located in the cytoplasm (Fig. 4A and B). CircRNAs present in the cytoplasm mainly act as miRNA sponges to play a regulatory role in tumors [26, 27]. Therefore, we hypothesized that circHAS2 may be the ceRNA of GC. Thus, we predicted that the miRNAs hsa-miR-944 and hsa-miR-582-3p bound to circHAS2 through cross-analysis of four prediction databases: circAtlas, Starbase, CircBank, and CircInteractome (Fig. 4C). Subsequently, we used the luciferase gene reporter assay to demonstrate that hsa-miR-944 mimics inhibited the luciferase activity of WT circHAS2, while the luciferase activity of MUT type did not change (Fig. 4D). The hsa-miR-582-3p mimic did not affect the luciferase activity of WT and MUT circHAS2 (Fig. 4E). These experiments demonstrate that circHAS2 binds to hsa-miR-944 (Fig. 4F). The binding of circRNA to miRNA is dependent on AGO2, and the two interact through the action of AGO2 protein [28]. To further confirm the relationship between circHAS2 and hsa-miR-944 in GC cells, we performed anti-AGO2 RIP assays in MKN-45 cells. Compared with IgG, anti-AGO2 antibody effectively downregulated AGO2 in MKN-45 cells transfected with hsa-miR-944 mimics (Fig. 4G). qPCR verified that circHAS2 and hsa-miR-944 were more abundant in hsa-miR-944 mimic-transfected MKN-45 cells than in the miR-NC group (Fig. 4H and I). The expression level of hsa-miR-944 was increased after knockdown of circHAS2 (Fig. 4J). In addition, there was a negative correlation between the expression of circHAS2 and hsa-miR-944 in 20 pairs of GC tissues (Fig. 4K), and the expression of hsa-miR-944 was downregulated in both GC tissues and GC cells (Fig. 4L and M), which was mainly distributed in the cytoplasm (Fig. 4N). Therefore, hsa-miR-944 might be the downstream target gene of circHAS2 in GC.

As a target gene of circHAS2, hsa-miR-944 can inhibit the proliferation, migration, and invasion of GC cells

The above experiments demonstrated that hsa-miR-944 can be downregulated in GC cells as a target gene of circHAS2. Thus, we hypothesized that hsa-miR-944 may inhibit the progression of GC. As shown in Fig. 5A–C, the proliferation ability of HGC-27 and MKN-45 cells with the addition of hsa-miR-944 mimics was inhibited, as shown by the CCK-8 and cell colony formation experiments. The EdU assay also demonstrated that the number...
of proliferating GC cells was significantly reduced after the addition of hasa-miR-944 mimics (Fig. 5D and E). The transwell assay demonstrated that HGC-27 and MKN-45 treated with hasa-miR-944 mimics had inhibited cell migration and invasion (Fig. 5F–I). Western blot analysis was used to detect the expression levels of key EMT proteins, and it was found that the expression of N-cadherin and vimentin in HGC-27 and MKN-45 cells treated with hasa-miR-944 mimics was decreased (Fig. 5J). These results suggest that as a target gene of circHAS2, hasa-miR-944 can inhibit the proliferation, migration, and invasion of GC cells.

As a target gene of hasa-miR-944, PPM1E is upregulated in GC

To further verify that circHAS2 may serve as a ceRNA in GC, we cross-analyzed the downstream target genes of hasa-miR-944 using the miRDB, miRWalk, miRpathDB, and TargetScan databases, and screened out eight mRNA molecules (Fig. 6A). To verify the binding relationship between these eight mRNAs and hasa-miR-944, only the expression of PPM1E was downregulated after the addition of hasa-miR-944 mimics to MKN-45 cells (Fig. 6B). Subsequently, we used luciferase gene reporter assays to confirm that the overexpression of hasa-miR-944 inhibited the luciferase activity of PPM1E, while MUT luciferase activity of PPM1E was not affected (Fig. 6C and D). The results suggest that PPM1E might be the downstream target gene of hasa-miR-944. PPM1E expression was also reduced by circHAS2 knockdown in HGC-27 and MKN-45 cells (Fig. 6E). Western blot analysis also showed that PPM1E protein was significantly reduced after circHAS2 knockdown or the addition of hasa-miR-944 mimics, but the inhibitor of hasa-miR-944 rescued the inhibition of PPM1E expression after circHAS2 knockdown (Fig. 6F). In addition, the expression of PPM1E was upregulated in GC tissues and cells (Fig. 6G and H), and the expression of PPM1E was negatively correlated with the expression of hasa-miR-944 (Fig. 6I) and positively correlated with the expression of circHAS2 (Fig. 6J). These results indicate that the PPM1E gene can be used as a target gene of hasa-miR-944 and is induced by circHAS2 to upregulate the expression of PPM1E through sponging with hasa-miR-944.

Hsa-miR-944 specifically affects the growth, migration, or invasion of GC cells through PPM1E targeting

To determine whether hasa-miR-944 can affect the growth, migration, or invasion of GC cells specifically through PPM1E targeting, we added hasa-miR-944 mimics to HGC-27 and MKN-45 cells overexpressing PPM1E (Fig. 7A). The results of the CCK-8 and
cell colony formation assays showed that the addition of hsa-miR-944 mimics rescued the proliferation of HGC-27 and MKN-45 cells overexpressing PPM1E (Fig. 7B–D). Meanwhile, the migration and invasion abilities of HGC-27 and MKN-45 cells overexpressing PPM1E were also rescued by adding hsa-miR-944 mimics as revealed by transwell assays (Fig. 7E–H). Western blot assays similarly demonstrated that the addition of hsa-miR-944 mimics rescued the proliferation effects on N-cadherin and vimentin after overexpression of PPM1E (Fig. 7I). These data suggest that hsa-miR-944 specifically affects the growth, migration, or invasion of GC cells through PPM1E targeting.

**PPM1E can reverse the effects of circHAS2 knockdown on GC cells**

To determine whether circHAS2 exerts its effects on GC through PPM1E, we restored PPM1E expression in circHAS2 knockdown HGC-27 and MKN-45 cells (Fig. 8A and B). The results of the CCK-8 and cell colony formation assays showed that PPM1E overexpression rescued the proliferation of circHAS2 knockdown HGC-27 and MKN-45 cells (Fig. 8C–E). At the same time, transwell experiments showed that the migration and invasion abilities of circHAS2 knockdown HGC-27 and MKN-45 cells were also rescued after PPM1E overexpression (Fig. 8F–I). Western blot analysis also demonstrated that PPM1E overexpression rescued the inhibition of N-cadherin and vimentin after circHAS2 knockdown (Fig. 8J). These data suggest that circHAS2 can regulate GC progression through PPM1E.

**DISCUSSION**

GC is the third leading cause of death worldwide [1]. Although great progress has been made in the treatment of GC tissues, the recurrence rate is still very high due to the strong tumor heterogeneity [29, 30], so it is urgent to identify biomarkers with strong sensitivity and specificity. In recent years, with the development of high-throughput sequencing, many noncoding RNAs have emerged. Among them, circRNAs have a stable structure, tumor-specific, and long half-life. Simultaneously, circRNA can act as circRNAs and also serve as miRNA sponges, RNA-binding protein scaffolds, transcriptional regulators, and potential translation templates [31, 32]. Thus, circRNAs may serve as potential biomarkers and therapeutic targets for GC.
In this study, three pairs of circRNAs of GSE121445 in the Gene Expression Omnibus database were analyzed for differential expression in GC tissues and corresponding paracancer tissues, and circHAS2 with the strongest correlation and upregulated expression in GC tissues was screened by qPCR. Subsequently, we found that the expression of circHAS2 increased with the degree of lymph node metastasis, and clinical data analyses showed that the expression of circHAS2 was correlated with T stage, TNM stage, lymph node metastasis, and nerve/vascular invasion. These studies suggest that circHAS2 can be a good biomarker for GC.

Survival curve analysis suggested that circHAS2 might be a good prognostic marker for GC. To verify the potential mechanism of circHAS2 in GC, we analyzed the effects of circHAS2 on the proliferation, migration, and invasion of GC cells. In vitro experiments showed that circHAS2 knockdown significantly inhibited the proliferation, migration, and invasion of GC cells and may be related to the EMT.

An increasing number of studies have revealed the potential mechanism of action of circRNAs. As miRNA sponges, circRNAs play a regulatory role in tumors [14, 21, 33]. Because circHAS2...
mainly exists in the cytoplasm, it may regulate the development of GC through the competitive binding of miRNA. We verified that hsa-miR-944 is a potential target gene of circHAS2 using bioinformatics prediction websites and double fluorescence gene reporter experiments. In vitro experiments showed that the overexpression of hsa-miR-944 inhibited the proliferation, migration, and invasion of GC cells. These results suggest that circHAS2 may promote the development of GC by competitively binding with hsa-miR-944. Previous studies have shown that hsa-miR-944 plays a potential tumor-suppressive role in colorectal cancer through the ubiquitin-proteasome system [34]. In addition, Xi et al. [35] found that circCSPP1 knockout attenuated adriamycin resistance and inhibited tumor progression in colorectal cancer via the miR-944/FZD7 axis. Pan et al. [36] also found that the downregulated expression level of miR-944 in GC may prevent the EMT through the MACC1/MET/Akt signaling pathway, thereby inhibiting the metastasis of GC, and also confirming the mechanism and function of circRNA-miRNA in GC.

To study the downstream target genes of circHAS2-hsa-miR-944, further prediction and experiments showed that the mimics of hsa-miR-944 can inhibit the luciferase activity of WT PPM1E. In addition, in vitro rescue experiments showed that hsa-miR-944 specifically affected the growth, migration, or invasion of GC cells through PPM1E targeting. These results suggest that hsa-miR-944 may regulate the progression of GC in combination with PPM1E. PPM1E is an AMP-activated protein kinase phosphatase [37]; PPM1E may induce AMPK activation, thus contributing to tumor progression. We found that the overexpression of hsa-miR-944 and knockdown of circHAS2 inhibited PPM1E mRNA and protein expression. The expression level of PPM1E was positively correlated with the expression level of circHAS2, and negatively correlated with the expression level of hsa-miR-944. Overexpression of PPM1E can restore the ability of circHAS2 knockdown to the proliferation, migration, and invasion of GC cells.

In summary, we identified a novel circRNA, circHAS2, which regulates PPM1E expression through hsa-miR-944. This axis plays a key role in regulating the occurrence and development of GC and provides a novel target for the treatment of GC, but the underlying mechanisms still need to be further explored.
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AUTHOR CONTRIBUTIONS

S.M. and X.L.G. drafted and revised the paper, designed the experiment, and selected the topic. L.S., Y.H.C., and C.Q. participated in the revision of the paper. X.J.S. and S.Q.J. provided resources and guidance for the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Ethics statement The ethics committee of the local hospital (ethical review report number: 2018-L055) approved the study.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Xianjuan Shen or Shaoqing Ju.

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