Hsa_circ_0007967 promotes gastric cancer proliferation through the miR-411-5p/MAML3 axis

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INTRODUCTION

Gastric cancer (GC) is a significant public health problem because of its high morbidity and mortality. Across the world, the prevalence is much higher in Asia, Africa, South America, and Eastern Europe [1]. With the development of advanced technology, targeted drugs, and immune checkpoint inhibitors are emerging, but their effect on advanced GC patients is unsatisfactory, and the 5-year survival rate of such patients is <10% [2], which is mainly caused by the recurrence and metastasis. It is important for patients to accept early diagnosis and prevent metastasis. However, the specificity and sensitivity of clinical traditional test like serum tumor markers is low [3], so it is meaningful to further investigate mechanisms behind GC progression and provide new sights for diagnosis and treatment.

As an important kind of non-coding RNAs, circular RNAs (circRNAs) exhibit closed circular structures without free 3' and 5' tails, which are resistant to nuclease and make circRNAs stable [4]. Owing to the development of high-throughput sequencing and bio-informatics, more knowledge about circRNAs is being discovered and understood. circRNAs can not only function as miRNA sponges [5], but also act as transcription and translation regulators [6-9], as well as become scaffolds to facilitate the interaction between protein [10-13]. What's more, specific circRNAs could encoding protein [14-16]. Emerging studies suggest that the aberrant expression of circRNAs may lead to a variety of diseases, such as cancers, cardiovascular system diseases, and nervous system diseases [17].

In our study, we performed RNA-seq between GC tissues and matched normal tissues and identified hsa_circ_0007967. We demonstrated that hsa_circ_0007967 promotes GC proliferation through miR-411-5p/MAML3 axis in vitro and in vivo.

RESULTS

Identification and characterization of hsa_circ_0007967

To study how circRNAs involved in GC progression, 5 pairs of GC tissues and normal tissues were collected for RNA sequencing. A total of 20 circRNAs were differently expressed in GC tissues (fold change > 5), among which 10 were upregulated and 10 were downregulated. We evaluated the expression of top 5 upregulated and downregulated circRNAs in formentioned 5 pairs of tissues. It was found that only hsa_circ_0007967 exhibited the most significant change (Fig. 1A). Then we evaluated the expression of hsa_circ_0007967 in another 47 pairs of GC tissues and matched normal tissues by RT-qPCR, and the expression of hsa_circ_0007967 was significantly higher in GC tissues (Fig. 1B). Consistently, hsa_circ_0007967 was higher expressed in GC cell lines (SGC7901, MGC803, BGC823, HGC27, AGS, and MKN87) than in the normal gastric epithelial cell lineGES1 (Fig. 1C).

hsa_circ_0007967 (chr2:175976295-175986268) was back spliced from 5 to 10 exons of protein-coding gene ATF2 (Fig. 1D). Its circular structure was confirmed by sanger sequencing (Fig. 1E). As well, RNase R digestion assays and the use of act-D (1 μg/μl) suggested that hsa_circ_0007967 was more stable and had a lower degradation rate compared with the corresponding mRNA (Fig. 1F, G). Additionally, hsa_circ_0007967 could be amplified from cDNA and gDNA by convergent primers, but only cDNA by divergent primers (Fig. 1H).

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Fig. 1  Identification and characterization of hsa_circ_0007967.  A Expression of five most upregulated and downregulated circRNAs in five paired GC tumors and normal tissues.  B Expression of hsa_circ_0007967 in 47 pairs of GC tissues and normal tissues.  C Expression of hsa_circ_0007967 in GC cell lines and normal gastric epithelial cell line.  D The location of hsa_circ_0007967 in chromatin and its structure.  E Sanger sequencing confirming the back splicing junction site.  F The RNase R digestion assay suggesting hsa_circ_0007967 is resistant to RNase R.  G The use of act-D (1 μg/μl) demonstrating that hsa_circ_0007967 is stable than its linear form.  H Northern blotting of hsa_circ_0007967 and its linear form in cDNA and gDNA amplified by convergent and divergent primers.  I Location of hsa_circ_0007967 in BGC823 cells detected by FISH.  J QPCR analysis of nuclear and cytoplasmic fractions conforming that hsa_circ_0007967 is located in cytoplasm.
Fig. 2 Overexpressing hsa_circ_0007967 promotes GC proliferation in vitro and in vivo. A, B The expression of hsa_circ_0007967 in BGC823 and SGC7901 cells transfected with vector and hsa_circ_0007967 plasmids. C, D The growth rate of BGC823 and SGC7901 cells transfected with vector and hsa_circ-0007967 by cck8 assays. E EdU assays of BGC823 and SGC7901 cells transfected with vector and hsa_circ-0007967 plasmids. F, G DNA positive cells of BGC823 and SGC7901 cells transfected with vector and hsa_circ-0007967 by EdU assays. H, I The assessment of proliferation of BGC823 and SGC7901 cells transfected with vector and hsa_circ_0007967 plasmid by colony formation assays. J-L Tumors from mice in two different groups, and the weight and volume of tumors at the end point. M The weight of tumors measured every 5 days until the end point.
Fig. 3  Silencing hsa_circ_0007967 inhibits GC proliferation in vitro and in vivo. A, B The expression of hsa_circ_0007967 in BGC823 and SGC7901 cells transfected with sinc, si-hsa_circ_0007967#1, and si-hsa_circ_0007967#2. C, D The growth rate of BGC823 and SGC7901 cells transfected with sinc, si-hsa_circ_0007967#1, and si-hsa_circ_0007967#2 by cck8 assays. E, F DNA positive cells of BGC823 and SGC7901 cells transfected with sinc, si-hsa_circ_0007967#1, and si-hsa_circ_0007967#2 by EdU assays. G–I The assessment of proliferation of BGC823 and SGC7901 cells transfected with sinc, si-hsa_circ_0007967#1, and si-hsa_circ_0007967#2 by colony formation assays. J–L Tumors from mice in two different groups, and the weight and volume of tumors at the end point. M The weight of tumors measured every 5 days until the end point.
Fig. 4  **Hsa_circ_0007967 serves as the sponge of miR-411-5p.**

A, B Level of hsa_circ_0007967 enriched for Ago2 by RIP assays in BGC823 and SGC7901 cells. C, D The expression of selected miRNAs in BGC823 and SGC7901 cells after overexpressing hsa_circ_0007967. E The potential binding site between miR-411-5p and hsa_circ_0007967. F The relative luciferase activity in HEK-293T cells cotransfected with miR-411-5p mimics and hsa_circ_0007967 plasmids with WT/MUT binding site by Dual luciferase reporter assays. G, H The growth rate of BGC823 and SGC7901 cells transfected with vector, hsa_circ_0007967 plasmids, miR-411-5p mimics or cotransfected with hsa_circ_0007967 plasmids and miR-411-5p mimics by cck8 assays. I–K DNA positive cells of BGC823 and SGC7901 cells transfected with vector, hsa_circ_0007967 plasmids, miR-411-5p mimics or cotransfected with hsa_circ_0007967 plasmids and miR-411-5p mimics by EdU assays. L, M The assessment of proliferation of BGC823 and SGC7901 cells transfected with vector and hsa_circ_0007967 plasmids by colony formation assays.
hybridization (FISH) and RT-qPCR assays of nuclear and cytoplasmic fractions confirmed that hsa_circ_0007967 mainly located in cytoplasm (Fig. 1I, J). These results suggested that hsa_circ_0007967 was upregulated in GC tissues, and was a stable circRNA in cytoplasm.

Hsa_circ_0007967 promotes GC cell proliferation in vitro and in vivo

To explore whether hsa_circ_0007967 involves in the progression of GC, we first overexpressed hsa_circ_0007967 in BGC823 and SGC7901 cells (Fig. 2A, B). Cck8 assays showed that cells with
hsa_circ_0007967 overexpressed had a faster growth rate (Fig. 2C, D). 5-Ethynyl-2′-deoxyuridine (EdU) assays suggested that the percent of DNA positive cells was higher in hsa_circ_0007967-overexpressed cells (Fig. 2E-G). Consistently, colony formation assays showed that hsa_circ_0007967-overexpressed cells grew into bigger clones (Fig. 2H, I). Then, we downregulated the expression of hsa_circ_0007967 in BGC823 and SGC7901 with 2 siRNAs (Fig. 3A, B). On the contrary, Cck8, EdU, and colony formation assays revealed that silencing hsa_circ_0007967 could inhibit GC cell proliferation (Fig. 3C-I). To further investigate whether hsa_circ_0007967 could accelerate the proliferation of GC cell in vivo, twenty BALB/c/nude mice were divided randomly into 4 groups and mice in different groups were subcutaneously injected with differently treated BGC823 cells (hsa_circ_0007967-overexpressed, hsa_circ_0007967-silenced, and the correspond control ones). Mice that were injected with hsa_circ_0007967-overexpressed BGC823 cells bore bigger and heavier tumors than those in the control group (Fig. 2J-M). And the weight and volume of tumors in the mice that were injected with hsa_circ_0007967-downregulated BGC823 cells were significantly lighter and smaller than those in the control group (Fig. 3J-M). These data suggested that hsa_circ_0007967 facilitated GC cells proliferation in vitro and in vivo.

Hsa_circ_0007967 serves as the sponge of miR-411-5p As hsa_circ_0007967 is mainly located in the cytoplasm, we speculated that hsa_circ_0007967 may act as miRNA sponges. We performed RNA-protein immunoprecipitation (RIP) assays and found that more hsa_circ_0007967 was enriched with anti-AGO2 antibody than IgG, which suggested that hsa_circ_0007967 bound well to miRNAs (Fig. 4A, B). Then we searched Circinteractome (circinteractome.nia.nih.gov) and chose six potential miRNAs with highest score (miR-127-5p, miR-411-5p, miR-495, miR-611, miR-513, and miR-877). Overexpressing hsa_circ_0007967 only caused a significant downregulation of miR-411-5p in both BGC823 and SGC7901 cells, suggesting that miR-411-5p may be the downstream molecule of hsa_circ_0007967 (Fig. 4C, D). We predicted the binding site between hsa_circ_0007967 and miR-411-5p (Fig. 4E). Dual luciferase reporter assays showed that miR-411-5p mimics only caused a significant reduction of the relative luciferase activity in hsa_circ_0007967-WT group (Fig. 4F). To further study whether hsa_circ_0007967 promoted GC proliferation by regulating miR-411-5p, we performed rescue experiments. Cck8, EdU, and colony formation assays showed that miR-411-5p mimics inhibited GC cell proliferation, and suppressed GC cell proliferation caused by hsa_circ_0007967 overexpression (Fig. 4G-M).

MAML3 is the target gene of miR-411-5p and promotes GC progression MiRNAs generally bind to the 3′ untranslated region of mRNAs and lead to mRNA degradation [18]. After searching three websites (TargetScan, TargetMiner, and miRDB), we found 12 mutual protein candidates that miR-411-5p targets. While miR-411-5p mimics only caused MAML3 downregulate in both BGC823 and SGC7901 cells (Fig. 5A, B), MAML3 is encoded by Mastermind like (MAML) family genes and is an important transcriptional co-activator in the Notch signaling pathway. The aberrant expression of MAML3 may lead to different diseases, like cancers [19]. Then, we conducted dual luciferase reporter assays with mutant and wild-type MAML3 plasmids (Fig. 5C). Dual Luciferase reporter assays showed that miR-411-5p mimics reduced the activity of luciferase in the WT group, which suggested that miR-411-5p bound to the 3′UTR of MAML3 mRNA (Fig. 5D). To further investigate whether MAML3 was involved in GC proliferation, we silenced MAML3 in both BCG823 and SGC7901 cells, which was confirmed in mRNA and protein level (Fig. 5E-G). Cck8, EdU, and colony formation assays suggested that silencing MAML3 inhibited GC proliferation (Fig. 5H-N). What’s more, WB assays suggested that overexpressing hsa_circ_0007967 could cause the high expression of MAML3 and miR-411-5p mimics downregulated the expression of MAML3. While miR-411-5p mimics suppressed the upregulation of MAML3 caused by hsa_circ_0007967 overexpression (Fig. 5O). Overall, hsa_circ_0007967 promoted GC proliferation through miR-411-5p/MAML3 axis.

DISCUSSION
In recent years, emerging studies suggested that circRNAs play an important role in cancerogenesis. Here, we identified the oncogenic circRNA, hsa_circ_0007967, through RNA-seq, which was highly expressed in GC tissues than in matched normal tissues. It was back spliced from ATF2 gene and mainly located in cytoplasm. Cck8, EdU, and colony formation assays demonstrated that overexpressing hsa_circ_0007967 promoted GC proliferation in vitro. Consistently, overexpressing hsa_circ_0007967 promoted subcutaneously injected GC cells proliferation. Hsa_circ_0007967 was the sponge of miR-411-5p and finally upregulated MAML3 expression. Overexpression miR-411-5p suppressed GC proliferation and weakened the oncogenic effect of hsa_circ_0007967. MAML3 is an important transcriptional co-activator in the Notch signaling pathway and silencing MAML3 suppressed GC proliferation. Overexpression hsa_circ_0007967 caused the upregulation of MAML3, which was resecured by overexpression miR-411-5p. It was suggested that hsa_circ_0007967/miR-411-5p/MAML3 axis involved in GC progression. Consequently, hsa_circ_0007967 is a promising biomarker for GC diagnosis and prognosis, and is a potential therapeutic target for GC treatment.

Though our study demonstrated that hsa_circ_0007967 played an oncogenic role in GC, we only investigated that hsa_circ_0007967 functioned as an miRNA sponge and just focused on the malignant oncogenic role in GC, we only investigated that hsa_circ_0007967/miR-411-5p/MAML3 axis involved in GC progression. Consequently, hsa_circ_0007967 is a promising biomarker for GC diagnosis and prognosis, and is a potential therapeutic target for GC treatment.

MATERIALS AND METHODS
Patient samples A total of 47 pairs of GC tissues and adjacent normal tissues were collected from the hospital of Zhenjiang according to institutional protocols and this
study was approved by the Medical Ethics Committee of First Affiliated Hospital of Nanjing Medical University. Informed consent form was signed by every patient.

Cell culture
The HEK-293T, MGC803, BGC823, HGC27, SGC7901, AGS, and GE1 cell lines were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The HEK-293T, MGC803, HGC27, BGC823, SGC7901, and GE1 cells were cultured in RPMI 1640 medium (Gibco, USA). The AGS cells were cultured in F12k medium (Wisent, Canada). All the cell lines were cultured in a 37 °C, 5% CO₂ incubator (Thermo Fisher, USA), and were provided with 100 µl/ml streptomycin (Gibco), 100 U/ml penicillin (Gibco), and 10% fetal bovine serum (BI, Iseral).

RNA extraction and quantitative real-time polymerase chain reaction
Total RNA was extracted from the cells or tissues using TRIzol reagent (Ambion, USA). The nuclear and cytoplasmic RNAs were extracted with PARIS Kit (Thermo Fisher, USA). Isolated RNAs were reversely transcribed into cDNAs with HiScript Q RT SuperMix for qPCR (Vazyme, China) on the Applied Biosystems steponeplus (USA) Real Time PCR system. GAPDH and U6 were used as internal controls, and expressions of all samples were normalized to GAPDH and U6. The primers are shown in Table 1.

Table 1. Primers used in this study.

| Names          | Sequences (5’-3’) |
|----------------|------------------|
| Hsa_circ_0007967: forward | CCGTTCACGAGGCCCATTTC |
| Hsa_circ_0007967: reverse  | TGGGACTGCAGCTGGAACCA |
| MAML3: forward          | CCTACACACCAACGGAAATGTA |
| MAML3: reverse           | ATGCTCTGACCAAAGCCACTCAC |
| miR-411-5p: forward      | GGCCGGTCTAGTAGCCCTATAG |
| miR-411-5p: reverse      | ACGTCAAGGTCGCAATGATT |
| GAPDH: forward           | GGGAGCAGCAAAAAGGCTCAT |
| GAPDH: reverse            | GAGTCCTTCCAGGATACCA |
| U6: forward              | CTGCCTTCGGACGACCA |
| U6: reverse              | AAGCTTCAAGAATTTCGAGG |

Cell counting kit-8 assay
After 48 h of transfection, cells (2 × 10³/well) were seeded into 96-well plates (Corning, USA). Then 100 µl of 10% Cell counting kit-8 (CCK8; Beyotime, China) solution was added to each well at appointed time (8 h, 24 h, 48 h, 72 h, 96 h). After 2 h of incubation at 37 °C, the absorbance at 450 nm was measured with a microplate reader (Pro-11 multiskan FC, Thermo Fisher, USA).

Colonies formation assay
After 48 h of transfection, cells (1 × 10³/well) were seeded into six-well plates (Corning). After incubation for 10 days at 37 °C, cells were fixed with methyl alcohol and stained with crystal violet solution.

5-Ethynyl-2′- deoxyuridine incorporation assay
EdU assays were performed with the Cell-Light EdU DNA Cell Proliferation Kit (RiboBio) according to the manufacturer’s instructions. Images were obtained with a Nikon Ti microscope (Nikon, Tokyo, Japan), and the number of EdU positive cells was counted.

Western blotting
Cells were lysed in RIPA buffer (Beyotime, China) with 1% PMSF (BioSharp, China). The protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Epizyme, China) and transferred onto PVDF membrane (Millipore, USA). Primary antibodies were applied at 4 °C overnight and HRP-conjugated secondary antibodies were applied for an hour at room temperature. The immunocomplexes were detected with ECL Western Blotting Substrate (NCM Biotech, China), visualized with Tanon (5200multi 46093, Tanon, USA). GAPDH was used as the internal control. Primary Antibodies included rabbit antiMAML3 (1:500, Biorbyt, UK), mouse antiGAPDH (1:20000, Beyotime, China). Secondary antibodies (A0208 and A0216, Beyotime, China) were diluted in 1:1000.

Northern blotting
DNA was separated using 1% agarose gel electrophoresis for 20 min under 110 v and was detected by BIO-RAD (BIO-RAD Gel Doc XR+, USA).

RNA stability assay
Cells were seeded into 6-well plates for 12 h incubation and grew to 50% confluence. Then cells were treated with 1 µg/ml actinomycin D and total RNAs were collected at 0, 6, 12 h. RNA levels were detected using RT-qPCR, and the half-life of circRNAs and miRNAs was evaluated.

Dual luciferase reporter assay
The wild-type sequence of hsa_circ_0007967 and the 3’UTR of MAML3 which containing predicted binding site of miR-411-5p were subcloned into the luciferase reporter vector GV272 (GenePharma, China). The corresponding ones containing mutant predicted binding site of miR-411-5p were subcloned into the luciferase reporter vector GV272 (GenePharma). HEK-293T cells were seeded in 24-well plate (6 × 10⁴ cells/well) for 24 h before transfection. Cells were co-transfected with a mixture of luciferase reporter vectors containing wild-type sequence or mutant sequence along with miRNA mimics. After 24 h incubation, the luciferase activity was measured with a specific microplate reader (Synergy H1, USA). Dual-Luciferase® Reporter (DLR™) Assay System was used according to the manufacturer’s instructions.

RNA-protein immunoprecipitation
The MagnaRIP RNA-Binding Protein Immunoprecipitation Kit (Merk, USA) was employed according to the manufacturer’s instructions. The cell lysate was incubated with beads coated with 5 µg of antibody against Argonaute-2 (AGO2) (Abcam, USA), and control IgG with rotation at 4 °C overnight. Total RNA was extracted for the evaluation of circRNA expression by RT-qPCR.

Animal studies
All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Twenty BALB/c-nude mice (female, 4-week-old) were divided randomly into 4 groups with online tool (Each was given a random number) and mice in different groups were injected with differently treated BGC823 cells (hsa_circ_0007967-over-expressed, hsa_circ_0007967-silenced and the correspond control ones).
Cells (5 × 10^5) were injected into the left back subcutaneously. The body weight and tumor volume (volume = length × width^2/2) were measured every 5 days after injection until mice were killed. At the end of experiments, the mice were killed, and the tumors were dissected and weighed.

Statistical analysis
GraphPad Prism software was used for statistical analysis. The data were presented as the mean ± standard deviation. Student’s t test was used for the determination of the statistical significance. A p value that <0.05 was considered statistically significant. Each experiment was repeated for three times with similar results.

DATA AVAILABILITY
The data supporting the conclusion of this article are presented within the article and its additional files.

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AUTHOR CONTRIBUTIONS
Study design: Q.Z.; cellular experiments, data collection, and analysis: X.W.; manuscript preparation: J.Z.; figures and manuscript proofreading: T.X., Y.S., Y.S., Y.F., and Y.G.; project administration: P.M., Y.S., and S.T.

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CONFLICT OF INTEREST
The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Human tissue study was approved by the Medical Ethics Committee of First Affiliated Hospital of Nanjing Medical University.

INFORMED CONSENT
Written informed consent was obtained from all participants.

ADDITIONAL INFORMATION
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