INTRODUCTION

Gastric cancer (GC) is prevalent throughout the world and its incidence appears to be rising. The situation is similar in China. As the 5-year overall survival rate for the cancer in most countries is below 30%, elucidation of its underlying mechanisms is critical.

There is increasing documentation of the roles of non-coding RNAs (ncRNAs) in various cancers, including GC. Of these, circular RNAs (circRNAs) are especially involved in the early stages of tumor development. CircRNAs are found in eukaryotic cells and, in the past, have been regarded as the “noise” in gene shearing. Developments in high-throughput techniques and bioinformatics have led to the identification of numerous circRNAs. These RNAs derive from the exons of protein-coding genes and have highly stable closed-loop structures. Research has shown that circRNAs are especially abundant and specifically expressed in tumor tissues. The stability of the ring structure facilitates their use as biomarkers for disease. In addition, circRNAs may act as microRNA (miRNA) sponges to modulate the expression of target genes.

Hsa_circ_0005529 promotes ZEB1 expression by regulating miR-873-5p and enhancing proliferation, invasion, and migration in gastric cancer cell lines

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Abstract

Background: Gastric cancer is a relatively common tumor. As circular RNAs (circRNAs) are documented to modulate proliferation and metastasis in various cancers, we evaluated the functions of circRNAs, in particular, hsa_circ_0005529, in gastric cancer cells.

Methods: Levels of hsa_circ_0005529 and miR-873-5p were examined by qRT-PCR, and the presence of hsa_circ_0005529 was confirmed by RNase R treatment. CCK-8, wound-healing, and Transwell assays were used to assess proliferation, migration, and invasion, respectively, while Western blotting was used to determine levels of zinc finger E-box-binding homeobox 1 (ZEB1) and dual-luciferase reporter assays to examine relationships between hsa_circ_0005529 and miR-873-5p.

Results: hsa_circ_0005529 was strongly expressed in gastric cancer where it stimulated tumorigenic behavior. Furthermore, hsa_circ_0005529 was shown to promote ZEB1 expression by sponging miR-873-5p, an inhibitor of ZEB1 expression.

Conclusion: Our research showed that hsa_circ_0005529 promoted tumorigenic behavior in gastric cancer cells by adsorbing miR-873-5p to modulate ZEB1 levels. This suggests that hsa_circ_0005529 may be useful as a biomarker and target for diagnosing and treating gastric cancer.

KEYWORDS
CircRNA, gastric cancer, miR-873-5p, ZEB1
sponges. miRNAs bind the 3'UTRs of mRNAs, preventing translation and thus silencing the gene. Many RNAs, including miRNAs, have common binding sites and can thus compete for binding, functioning as competing endogenous RNAs (ceRNAs) and modulating tumor development. For example, modification of circNSUN2 with N6--methyladenosine leads to increased cytoplasmic export and stabilization of HMGA2 to promote the development of liver metastases in colorectal cancer, while circNRIP1 sponges miRNA-149-5p, leading to GC progression through the AKT1/mTOR pathway.

Studies have demonstrated involvement of the zinc finger E-box-binding homeobox 1 (ZEB1) transcription factor in the control of the epithelial-mesenchymal transition (EMT). The EMT is controls cellular transformation from an epithelial to a mesenchymal phenotype. ZEB family proteins, including ZEB1 and ZEB2, have a characteristic series of domains and interact with various proteins such as SMAD and CtBP to modulate their transcriptional function. Therefore, while ZEB1 and ZEB2 were originally considered to be repressors of transcription due to their ability to interact with CtBP co-repressors, they are also able to promote transcription by interacting with co-activators such as p300 and P/CAF. These dual functions are essential in controlling the EMT. ZEB1 has been linked to tumorigenesis in, for example, lung and colorectal cancer cells where it promotes the cells’ ability to migrate and invade in vitro. ZEB1 can also promote the transformation of lung cancer epithelial cells and is involved in tumorigenesis in the KRasv12 mouse model of lung cancer in addition, ZEB1 modulates K-Ras “addiction” in pancreatic cancer cells.

Here, we identified several circRNAs documented to be differentially expressed in GC using data mining and sorting on GC gene chips. It was also found that hsa_circ_0005529 was strongly expressed in GC and that it controlled ZEB1 levels through sponging miR-873-5p, leading to the promotion of the tumor phenotype. This research provides a new direction in the understanding of GC pathogenesis.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The GC cell lines HGC-27 and AGS, as well as the human normal gastric mucosal cell line GES-1, were cultured in DMEM containing 10% fetal bovine serum (FBS) in a 5%-CO₂ incubator at 37 °C.

2.2 | Cell transfection

HGC-27 and AGS cells were inoculated in six-well plates. RNA (0.67 μg) and PCD5 plasmid (1 μg) were mixed in 100μl opti-MEM. A further 100μl of opti-MEM was mixed with Lipofectamine 2000 and left at 37 °C for 5 min. The two solutions were then mixed gently and allowed to stand for 20min before addition to the cells and incubation under normal conditions for 5 h. After removal of the solution, the cells were grown in normal medium for 24h before use.

2.3 | qRT-PCR

Total RNA was extracted using the TRIzol reagent, and the NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix was used to reverse-transcribe the RNA into cDNA. NovoStart® SYBR qPCR SuperMix Plus was used for qRT-PCR detection. Relative gene expression was calculated with the $2^{-\Delta\Delta C_T}$ method from triplicate experiments. The primer sequences are provided in Table S1.

Recombinant vectors expressing hsa_circ_0005529, siRNA, miRNA inhibitors and mimics were synthesized by GenePharm. The sequences are given in Table S2. Transfection was performed using Lipofectamine 2000.

2.4 | CCK-8 assay

CCK-8 assays were used to examine proliferation. Cells (2×10³ per well) in 100μl medium were inoculated into the wells of 96-well plates and grown for 0, 24, 48, or 72h. Media were replaced with medium containing 10% CCK-8 reagent and absorbances at 450nm were read after 1 h in a microplate reader.

2.5 | Wound-healing experiment

Cells were grown in 6-well plates to cover the bottom of the well. The monolayer was scratched with a 200-μl pipette tip. After rinsing the cells in PBS, 2 ml of medium with 2% FBS was placed in each well. The cells were photographed immediately and after 24h and the extent of migration was calculated.

2.6 | Transwell assay

Two hundred microliters of medium without FBS were included in the upper chamber of the Transwell apparatus, with 1×10⁵ HGC-27 or AGS cells in 500μl of medium with 15% FBS in the lower chamber and incubated for 24h at 37°C and 5% CO₂. The cells were subsequently fixed with 4% paraformaldehyde for 20min, stained with 0.1% crystal violet for 5 min, and photographed for analysis.

2.7 | Luciferase reporter gene assay

Wild-type (WT) or mutant (MUT) hsa_circ_0005529 containing the miR-873-5p-binding site were cloned into luciferase reporter vectors to construct the WT-hsa_circ_0005529 and MUT-hsa_circ_0005529 reporter plasmids, respectively. Lipofectamine 2000 was used to transfect the cells with the plasmids and miR-873-5p mimics. After incubation for 48h, the luciferase activity relative to the controls was evaluated.
2.8 | Western blotting

Cells \((2 \times 10^5 / \text{ml})\) were seeded in 6-well plates and grown until 90% confluent, after which, they were removed with EDTA, washed twice, homogenized by ultrasonication, and centrifuged. The BCA method was used for protein quantification. The proteins were separated on SDS-PAGE and electroblotted onto PVDF. After blocking (5% fat-free milk, 2 h, room temperature), the blots were probed the primary antibodies rabbit anti-ZEB1 (1:1000) and anti-GAPDH (1:1500) overnight at 4°C. The following day, the blots were probed with HRP-conjugated goat anti-rabbit (1:2000, 1 h, room temperature), developed with ECL chemiluminescent solution, and photographed. Images were analyzed with Image J software using GAPDH as loading control.

2.9 | Statistical analysis

Data were analyzed with SPSS 11.5 (SPSS Inc.). Data were expressed as means ± standard deviation (x ± s). Between-group differences were analyzed by t-tests with \( p \leq 0.05 \) considered statistically significant.

3 | RESULTS

3.1 | CircRNA hsa_circ_0005529 is upregulated in gastric cancer

We designed qRT-PCR primers spanning the specific linker sequence of hsa_circ_0005529. The amplification results showed that the dissociation curve of the primer comprised a single peak and the melting temperature value was within the normal range (Figure S1A,B).

Hsa_circ_0005529 expression was remarkably over-expressed in GC cell lines or tissues compared with adjacent normal tissues or GES-1, an immortalized, but non-cancerous gastric mucosal cell line (Figure 1A,B). As circRNAs may have different subcellular locations and thus different functions in cells, we investigated the distribution of hsa_circ_0005529 in HGC-27 and AGS cells using nucleoplasmic separation experiments. These showed a predominantly cytoplasmic distribution for hsa_circ_0005529 (Figure 1C,D), suggesting that hsa_circ_0005529 may function by adsorbing miRNAs. circRNAs are renowned for their stability. To examine this in hsa_circ_0005529, RNase R was used for processing RNA from HGC-27 and AGS cells. As shown by qRT-PCR, while RNase R treatment produced a sharp drop in the linear VPS33B levels, the levels of hsa_circ_0005529 remained unchanged, confirming the stability of hsa_circ_0005529 (Figure 1E,F).

3.2 | hsa_circ_0005529 promotes GC tumorigenesis in vitro

To study the function of hsa_circ_0005529 in GC, we designed three siRNAs that specifically targeted the reverse-splicing junction region of hsa_circ_0005529. Of these, si-hsa_circ_0005529 #1 was found to significantly down-regulate the expression of hsa_circ_0005529 by 50%-55% (Figure 2A). In addition, we constructed a hsa_circ_0005529 overexpression plasmid (Figure 3B) which upregulated hsa_circ_0005529 expression 30-36-fold. As shown by the CCK-8 assays, hsa_circ_0005529 silencing slowed the proliferation of both cell lines while overexpression of hsa_circ_0005529 had the reverse effect (Figure 2C,D), indicating the promotion of proliferation by hsa_circ_0005529 in cancerous cells. The invasion experiment showed that knocking down hsa_circ_0005529 significantly reduced invasion in both cell lines,
in contrast to hsa_circ_0005529-overexpressing cells where invasion was enhanced (Figure 2E). Similarly, migration was prevented by hsa_circ_0005529 silencing but enhanced by hsa_circ_0005529 overexpression (Figure 2F).

### 3.3 | Hsa_circ_0005529 sponges miR-873-5p

The cell phenotyping experiments showed that hsa_circ_0005529 regulated tumorigenic behavior in GC cells. It is well known that circRNAs in the cytoplasm can modulate tumor development by sponging miRNAs and thus altering the expression of downstream target genes. Using CircInteractome and miRanda software, we found that hsa_circ_0005529 contains multiple miRNA-binding sites. Using the intersecting information from these two databases (Figure 3A), five potential interacting miRNAs were identified, namely miR-873-5p, miR-518a-5p, miR-224, miR-671-5p, and miR-527-5p. Overexpression of hsa_circ_0005529 in the GC cells and comparison with the empty plasmid control group indicated significant regulation of miR-873-5p by hsa_circ_0005529 (Figure 3B). To verify binding between miR-873-5p and hsa_circ_0005529 (Figure 3C), we designed wild-type and mutant plasmids of hsa_circ_0005529, and then performed dual-luciferase reporter gene experiments. These indicated a significant reduction
hsa_circ_0005529 targets miR-873-5p. (A) Database prediction of miRNA and hsa_circ_0005529 interactions. (B) qRT-PCR measurement of expression of predicted miRNAs in GC cells transfected with PCD5-hsa_circ_0005529 or control; (C) Interaction site between miR-873-5p and hsa_circ_0005529; (D) Luciferase activity of hsa_circ_0005529 wild-type and mutant vectors in GC cells transfected with miR-873-5p; (E) qRT-PCR measurement of ZEB1 and miR-873-5p in GC cells transfected with PCD5-hsa_circ_0005529 or control; (F) qRT-PCR measurement of ZEB1 and miR-873-5p in GC cells transfected with si-hsa_circ_0005529 or control (G) ZEB1 protein levels in cells expressing miR-873-5p mimics and controls. (H) ZEB1 mRNA levels in cells expressing miR-873-5p mimics and controls.

**p < 0.01, ***p < 0.001
in luciferase activity in cells expressing both miR-873-5p and the hsa_circ_0005529 wild-type, while after co-transfection with the hsa_circ_0005529 mutant sequence, the luciferase activity remained essentially unchanged (Figure 3D). This confirms that miR-873-5p and hsa_circ_0005529 can interact. In addition, binding between miR-873-5p and the ZEB1 3'UTR is documented. Further verification showed that up-regulation of hsa_circ_0005529 expression on both GC cell lines significantly promoted ZEB1 expression while inhibiting that of miR-873-5p (Figure 3E). Silencing of hsa_circ_0005529 lowered ZEB1 expression while enhancing that of miR-873-5p (Figure 3F). In addition, Western blotting and qRT-PCR results showed that ZEB1 levels were reduced miR-873-5p-overexpressing cells (Figure 3G,H). This indicates that hsa_circ_0005529 negatively regulates miR-873-5p while positively modulating ZEB1, suggesting that the hsa_circ_0005529/miR-873-5p/ZEB1 axis promotes GC progression.

3.4 Overexpression of hsa_circ_0005529 reverses miR-873-5p effects

We then investigated the possible promotion of GC by hsa_circ_0005529 through controlling miR-873-5p, thus influencing the ZEB1 level. Using cells expressing both miR-873-5p mimic and hsa_circ_0005529 and comparing them with those transfected with the hsa_circ_0005529 plasmid alone, it was found that hsa_circ_0005529 restored the impact of miR-873-5p on the tumorigenic behavior of GC cells (Figure 4A-D). These findings indicate

**FIGURE 4** hsa_circ_0005529 overexpression reverses miR-873-5p inhibitory effects in GC cells. Cells were with both miR-873-5p mimics and plasmids inducing hsa_circ_0005529 overexpression (A) Proliferation, measured by CCK-8 assays; (B) Invasion, measured by Transwell assays; (C) Migration, measured by wound-healing assays; (D) Western blot showing expression of ZEB1 protein. *p < 0.05, **p < 0.01, ***p < 0.001
that hsa_circ_0005529 promotes GC development via the miR-873-5p/ZEB1 axis.

4 | DISCUSSION

CircRNAs are a subset of abundant and stably expressed endogenous RNAs.\textsuperscript{21} Recent developments in high-throughput sequencing and bioinformatics have led to the elucidation of the expression patterns and functions of numerous circRNAs.\textsuperscript{8} However, there are still many biological functions of circRNAs that remain poorly understood. Here, we identified a circRNA, hsa_circ_0005529, that is strongly expressed in GC, suggestive of involvement in GC tumorigenesis.

We next investigated the specific molecular role of hsa_circ_0005529 in GC. It was observed that hsa_circ_0005529 was strongly expressed in the GC cell lines HGC and AGS. We, therefore, speculated that it may be involved in the targeting of certain miRNAs in GC. The databases predicted that hsa_circ_0005529 and miR-873-5p could interact. The role of miR-873-5p in tumorigenesis is well documented. For example, it has been found that the miR-873-5p/PTD-1-L axis modulates the stemness in breast cells,\textsuperscript{22} while inhibiting tumorigenesis papillary thyroid carcinoma through CXCL16.\textsuperscript{23} Together with reducing cervical cancer growth and invasion through negative regulation of ULBP2.\textsuperscript{24} Here, miR-873-5p and hsa_circ_0005529 were shown to be negatively associated. We used dual-luciferase experiments to verify this potential interaction, observing that hsa_circ_0005529 can target miR-873-5p. Therefore, we hypothesize that hsa_circ_0005529 promotes GC development and progression by adsorbing miR-873-5p.

We then examined the potential means by which hsa_circ_0005529/miR-873-5p promoted GC tumorigenesis. We used bioinformatics analysis to confirm that ZEB1 is targeted by miR-873-5p. ZEB1 is known to modulate the EMT and is differentially expressed in various malignancies, including breast,\textsuperscript{25} lung,\textsuperscript{26} and pancreatic cancer,\textsuperscript{27} and leukemia.\textsuperscript{28} Our results showed that ZEB1 levels were negatively correlated with those of miR-873-5p and that miR-873-5p bound to the ZEB1 3’UTR to reduce its expression. Therefore, we have confirmed that the hsa_circ_0005529/miR-873-5p axis targets ZEB1, and that hsa_circ_0005529 promotes ZEB1 expression by adsorbing miR-873-5p.

Although we have clarified the promotion of ZEB1 expression by hsa_circ_0005529 adsorption of miR-873-5p, and its influence on GC development, there are still several aspects that need to be improved. First, it would be recommended to verify the levels of hsa_circ_0005529 in the sera of GC patients to determine its differential expression in GC. This could be analyzed in relation to clinical data to investigate potential associations between hsa_circ_0005529 and the staging and prognosis of the cancer. Secondly, although we verified the part played by the hsa_circ_0005529/miR-873-5p/ZEB1 axis in cell lines, in vivo animal data is lacking. Therefore, by constructing animal models, we can better verify the regulatory mechanism of hsa_circ_0005529 in GC. Finally, ZEB1 is closely involved in the regulation of the EMT, suggesting this as a potential avenue to explore.

In conclusion, our research showed that hsa_circ_0005529 competed with miR-873-5p to counteract the negative influence of miR-873-5p on ZEB1, thus enhancing cell invasion, proliferation, and migration in GC. These findings add to our knowledge of GC pathogenesis and suggest new avenues for treating the disease.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data included in this study are available upon request by contact with the corresponding author.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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