RESEARCH ARTICLE

Hsa_circ_0000467 promotes cancer progression and serves as a diagnostic and prognostic biomarker for gastric cancer

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Background: Emerging evidence indicates that dysregulation of circular RNAs (circRNAs) is implicated in the development of malignancies. However, the diagnostic value and functional role of circRNAs in gastric cancer (GC) remain largely elusive.

Methods: Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to detect the expression of hsa_circ_0000467 in tissues, cell lines, and plasma. A receiver operating characteristic (ROC) curve was constructed to estimate the diagnostic value of hsa_circ_0000467. The association between the expression level of hsa_circ_0000467 and the clinicopathological features was analyzed. Moreover, cell functional assays were performed.

Results: Hsa_circ_0000467 was significantly upregulated in GC tissue compared to adjacent nontumor tissue ($n = 51$, $P < 0.05$). Similar results were detected in the HGC-27, MGC-803, AGS, NUGC-3, GES-1 cell lines ($n = 15$, $P < 0.001$), and in the plasma samples from GC patients ($n = 20$, $P < 0.05$). The area under the ROC curve of hsa_circ_0000467 was 0.790, which is superior to commonly used biomarkers including CEA and CA-724. We found that the expression levels of hsa_circ_0000467 in the same patient were significantly lower after surgery ($n = 20$, $P < 0.05$). Moreover, the hsa_circ_0000467 expression level is closely associated with TNM stage. Additionally, Cox multivariate analysis showed that hsa_circ_0000467 is a novel independent prognostic factor. Furthermore, in vitro experiments demonstrated that knockdown of hsa_circ_0000467 markedly inhibited the proliferation, migration, and invasion of GC cells. Moreover, hsa_circ_0000467 silencing increased tumor apoptosis in vitro.

Conclusion: Hsa_circ_0000467 can act as a novel noninvasive biomarker for the diagnosis and prognosis of GC and may be a potential therapeutic target for GC.

KEYWORDS

biomarker, carcinogenesis, circular RNA, gastric cancer, hsa_circ_0000467

1 | INTRODUCTION

Gastric cancer (GC) is the fourth common gastrointestinal malignancy and the third leading cause of cancer-related death worldwide.¹ In addition, the existing bad phenomenon in Western and China is that many GC patients did not get accurate prognosis in the early stage of the disease, and a large proportion of patients are diagnosed only in the advanced stage. Despite recent developments in therapy, the prognosis of advanced gastric cancer patients remains poor.² Therefore, understanding the underlying molecular pathogenesis of GC progression and the development of novel therapeutic targets are extremely important.

Noninvasive blood-based biomarkers may provide some information on GC status.³ However, current serum tumor biomarkers including carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and CA72-4 have a low positive rate in GC diagnosis.⁴ Thus, it is necessary to find novel noninvasive biomarkers for diagnosing and monitoring patients with GC.

Circular RNA (circRNA) is a newly discovered class of relatively stable noncoding RNAs formed by back-splicing events with tissue and cell-type specificity.^{5,6} Recently, several circRNAs have been detected in the blood and were used as biomarkers for cancer diagnosis due to their high stability, high abundance, and tissue specificity.^{9,10} Furthermore, an increasing number of studies have shown that circRNAs play important roles in various cancer-related processes, including proliferation, migration, invasion, and apoptosis.^{14,15} However, the roles of circRNAs in GC are largely unknown.

Ska3 is a member of the spindle and kinetochore-associated (Ska) protein complex, which is required for accurate chromosome segregation during mitosis.¹⁸ Ska3 at chromosome 13q was recently identified as a gene involved in promoting malignant transformation in colorectal adenoma.¹⁹ Hsa_circ_0000467 was a circRNA which can be searched in circBase (<http://www.circbase.org>). Moreover, hsa_circ_0000467 were encoded by the Ska3 gene and located at chr13:21742126-21742538. However, there is no high-throughput sequencing data or databases revealed hsa_circ_0000467 expression level in gastric cancer and the roles of hsa_circ_0000467 in gastric cancer remain to be elucidated.

To the best of our knowledge, this is the first study to investigate the hsa_circ_0000467 expression in gastric cancer tissues, plasmas, and cell lines. We also analyzed the relationship between hsa_circ_0000467 expression and diagnosis and prognosis in patients. Finally, the biological functions of hsa_circ_0000467 were explored in vitro. Our study demonstrated that hsa_circ_0000467 may serve as a new kind of biomarker for GC diagnosis and prognosis and may play a role in proliferation, invasion, migration, and apoptosis of GC.

2 | MATERIAL AND METHODS

2.1 | Cell culture and transfection

Human GC cell lines, namely, HGC-27, MGC-803, AGS, NUGC-3, and GES-1, were obtained from the Cell Line Bank, Chinese Academy

of Sciences. The cell lines were confirmed by PCR, and the cultures were free from mycoplasma contamination. The species origin was confirmed by PCR. The cell lines were authenticated with short tandem repeat (STR) profiling. HGC-27, MGC-803, and NUGC-3 cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified atmosphere containing 5% CO₂. AGS cells were cultured in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified atmosphere containing 5% CO₂. GES-1 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified atmosphere containing 5% CO₂. HGC-27 and AGS cells were transfected with shRNA, including circRNAi (64736-1; shRNA1#) and circRNAi (64735-1; shRNA2#) and a control plasmid (Genechem, Shanghai, China) according to the manufacturer's protocol using Lipofectamine™3000 transfection reagent.

2.2 | Clinical specimens

A total of 51 pairs of gastric cancer tissue samples and adjacent non-tumor tissue samples were obtained from Fujian Medical University Union Hospital between May 2015 and May 2016. These tissue specimens were immediately preserved in RNA-fixer Reagent (Biotek, Beijing, China) after removal from patients' stomachs and were stored at -80°C until use. All patients underwent R0 gastrectomy. None of the patients underwent preoperative chemotherapy or radiotherapy. Cases with the following characteristics were also excluded: Remnant GC, treated with palliative surgery and concurrent malignancy in other organs. Postoperative adjuvant chemotherapy was performed with 5-fluorouracil-based drugs plus oxaliplatin in advanced cases. In addition, 20 paired preoperative and postoperative plasma samples from 20 gastric cancer patients were in a control group, and another 20 preoperative gastric cancer patients plasma samples and the control fasting plasma from 20 healthy volunteers (gender and age matched to patients) were in a control group. The above samples were obtained from Fujian Medical University Union Hospital between March 2018 and May 2018. The peripheral blood samples (5 mL) obtained from preoperative and postoperative patients (10 days after surgery) and controls were collected in BD vacutainer tubes (BD, New Jersey, USA) and centrifuged (3000 g for 10 minutes). The plasma samples were kept at -80°C. The pathologic stage of the tumor was reassessed according to the 2010 International Union Against Cancer (UICC) TNM classification on gastric cancer (seventh edition). This study was approved by the ethics committee of Fujian Medical University Union Hospital, and written consent was obtained from all patients involved.

2.3 | Cellular fractionation

The nuclear and cytoplasmic fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA). Total RNA from the nuclear and cytoplasmic

fractions was isolated using TRIzol (Life Technologies, Carlsbad, CA, USA).

2.4 | RNA isolation and real-time quantitative polymerase chain reaction

RNA isolation was performed using the TRIzol reagent according to the manufacturer's manual. Reverse transcription was performed with the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) using random primers and 1 µg of mRNA per reaction as a template. The thermocycling conditions for RT-PCR were as follows: 40 cycles at 95°C for 3 minutes, 95°C for 15 seconds, and 60°C for 30 seconds. RT-qPCR analysis was performed in triplicate on the ABI Prism 7500 Sequence Detection System (Thermo Fisher Scientific) using the Thunderbird SYBR qPCR mix (Toyobo) according to the manufacturer's recommendations. The primers' sequence of GAPDH was the control in tissue and plasma. Primer sequences were as follows: hsa_circ_0000467: 5'-AATGGGACTTAAAAATGCGAGG-3' (forward) and 5'-GTTGTGGACTACGTGGAGACT-3' (reverse); GAPDH: 5'-GGTCGGAGTCAACGGATTG-3' (forward) and 5'-ATGAGCCCCAGCCTTCTCCAT-3' (reverse). All experiments were performed three times independently.

2.5 | Cell proliferation, migration, and invasion assay

Cell proliferation was assayed by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) in octuplicate according to the manufacturer's instructions. A total of 5000 transfected cells were seeded into each well of a 96-well plate (Biosciences, Franklin, NY, USA) for 0, 24, 48, and 72 hours. CCK-8 was added at 10 µL per well and incubated at 37°C for 2 hours at each time point. Absorbance was measured at 450 nm with an ELx808 microplate reader (Bio-Tek, Winooski, VT, USA). Absorbance at 615 nm was used as a reference. Each experiment was performed at least in triplicate. Colony formation assays were carried out to evaluate clonogenic ability of transfected cells. The same number of cells was trypsinized into a single-cell suspension and seeded in 2.5-cm dishes. The cells were cultured for approximately 14 days, fixed with 4% paraformaldehyde, and stained with crystal violet (Beyotime, Beijing, China). Visible colonies were then counted from three randomly chosen fields.

For the invasion assay, the transwell system and Matrigel BD Biosciences (New York, NY, USA) were used according to the manufacturer's protocol. 1×10^5 cells were seeded into the upper chambers precoated with Matrigel and cultured in serum-free medium. The lower compartment was filled with medium supplemented with 10% FBS as a chemoattractant. After incubation for 48 hours, the cells remaining in the upper chamber were removed, and the cells at the bottom of the insert were fixed, stained with 0.5% crystal violet, and counted under a microscope (Olympus Corp., Tokyo, Japan). The results from three independent experiments were averaged. For the migration assay, the cells were seeded into the upper chambers

without Matrigel coating. The rest of the assay was conducted as described for the invasion assay.

2.6 | Cell apoptosis assays

For apoptosis analysis, cells were labeled with Annexin V-FITC and PI (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Cell apoptosis was assessed using FACS Calibur flow cytometry (BD Biosciences).

2.7 | Statistical analysis

The median expression was regarded as the cutoff value for hsa_circ_0000467 high and low expression, as previous described.²⁰ For comparisons, chi-squared test, Student's *t* test, log-rank test, and one-way ANOVA were performed, as appropriate. The receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value. The area under the curve (AUC) ≤ 0.5 represented no diagnostic value. Overall survival was estimated using the Kaplan-Meier method and log-rank test. Univariate analysis was performed to explore the associations of clinicopathological factors with overall survival. Multivariate analysis was performed using the Cox proportional hazards model to further evaluate all significant prognostic factors that were found in the univariate analysis. $P < 0.05$ was considered to be statistically significant, and all *P* values were two-sided. All statistical analyses were performed using SPSS software 22.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

3 | RESULTS

3.1 | Hsa_circ_0000467 is upregulated in GC tissues and cell lines

Hsa_circ_0000467 is located on chr13:21742126-21742538 and is derived from the exon 4 of its host gene (Figure 1A). To assay the expression level of hsa_circ_0000467 in gastric cancer, we performed real-time PCR to detect its expression in tumor samples and gastric cancer cell lines. We designed a set of specific divergent primers to examine hsa_circ_0000467 expression and performed Sanger sequencing of the amplified PCR product (Figure 1B). We found that hsa_circ_0000467 is upregulated in gastric cancer tissue samples vs corresponding adjacent tissue samples ($n = 51$, $P < 0.05$, Figure 1C). Moreover, hsa_circ_0000467 levels in plasma samples of patients with gastric cancer were higher than those in healthy controls ($n = 20$, $P < 0.05$, Figure 1D). Five cell lines including HGC-27, MGC-803, AGS, NUGC-3, and GES-1 were used in this study. In this study, GES-1 is the control normal cell line, as previous study described.¹⁴ Figure 1E showed that hsa_circ_0000467 expression was upregulated in HGC-27, MGC-803, AGS, and NUGC-3, compared with GES-1 cells. These results suggest that hsa_circ_0000467 may act as an oncogene.

FIGURE 1 Hsa_circ_0000467 is up-regulated in gastric cancer. A, hsa_circ_0000467 is circulated by exons; B, the Sanger sequencing of hsa_circ_0000467; C, the expression of hsa_circ_0000467 in gastric cancer tissue and corresponding adjacent tissues; D, the expression of hsa_circ_0000467 in plasma samples; E, the expression of hsa_circ_0000467 in cell lines; F, cytoscape was used to visualize hsa_circ_0000467-miRNA-mRNA; G, mRNA predicted participate in many pathways. H, Hsa_circ_0000467 was predominantly cytoplasmic. * $P < 0.05$, *** $P < 0.001$

Currently, the miRNA “sponge” is the most well-known regulatory mechanism for circRNA effects. Thus, we predicted the circRNA-miRNA-mRNA network using Arraystar’s homemade miRNA target prediction software and TargetScan and miRanda (Figure 1F). Figure 1G shows that predicted mRNAs may participate in numerous pathways; hence, further work is needed to uncover the mechanisms of hsa_circ_0000467 in GC progression.

To further investigate the role of hsa_circ_0000467 in gastric cancer, we fractionated the cells into nuclear and cytoplasmic fractions to detect the cellular localization of hsa_circ_0000467. We discovered that hsa_circ_0000467 was predominantly cytoplasmic in HGC-27 and AGS cells (Figure 1H). This result implies that endogenous hsa_circ_0000467 can work as a miRNA sponge to repress miRNA function.

3.2 | Potential diagnostic values of hsa_circ_0000467 in gastric cancer

To identify whether hsa_circ_0000467 can serve as a biomarker in gastric cancer, we collected 20 plasma samples of preoperative GC patients

and 20 healthy volunteers. Expression of hsa_circ_0000467 was measured via real-time PCR. Lower Δ CT values in GC patient samples indicated higher expression of hsa_circ_0000467 vs healthy volunteers (Figure 1E). ROC curves are used to evaluate potential diagnostic value. A larger area under the ROC curve corresponds to a higher diagnostic value. The area under the ROC curve (AUC) of hsa_circ_0000467 was 0.790; sensitivity and specificity were 0.705 and 0.648, respectively. At the same time, the ROC curve showed that AUC values for carcinoembryonic antigen (CEA) and carbohydrate antigen (CA72-4) were 0.560 and 0.670, respectively (Figure 2A). The results of the ROC curve analysis indicate that hsa_circ_0000467 had higher diagnostic efficiency than other known markers. Thus, hsa_circ_0000467 may be a promising biomarker for early detection of gastric cancer.

To validate the diagnostic value of hsa_circ_0000467, we assayed the plasma levels of hsa_circ_0000467 at preoperative and postoperative stage in another 20 gastric cancer patients. The plasma levels of hsa_circ_0000467 in postoperative patients were significantly decreased compared to preoperative patients ($P < 0.05$; Figure 2B).

FIGURE 2 Diagnostic value of hsa_circ_0000467 for GC patients. A, ROC curve of hsa_circ_0000467. B, The plasma levels of hsa_circ_0000467 in preoperative and postoperative gastric cancer patients. * $P < 0.05$

3.3 | Upregulated hsa_circ_0000467 expression predicts aggressive clinicopathological characteristics and poor prognosis in GC patients after R0 gastrectomy

The data demonstrated that hsa_circ_0000467 expression was significantly upregulated in gastric cancer tissue and plasma samples of patients with gastric cancer. Therefore, we analyzed the association between hsa_circ_0000467 expression and clinicopathological characteristics and long-term outcomes in GC patients after gastrectomy. As shown in Table 1, 32 of 51 (62.7%) patients had high hsa_circ_0000467 expression. We divided the patients

into two groups according to the median hsa_circ_0000467 expression. A strong association was observed between hsa_circ_0000467 expression and T classification, lymphatic invasion, and TNM stage (all $P < 0.001$; Table 1). The median follow-up of this study was 32 months; we found that patients with lower hsa_circ_0000467 expression have better prognoses than patients with higher expression (Figure 3A). Cox multivariate regression analysis showed that hsa_circ_0000467 is a novel independent prognostic factor (Table 2). The prognostic efficiency of the combined hsa_circ_0000467 TNM stage ($n = 51$, AUC = 0.779, 95% CI = 0.650–0.908) was higher when compared to that using TNM stage ($n = 51$, AUC = 0.708, 95% CI = 0.562–0.853) alone (Figure 3B).

FIGURE 3 Prognostic value of hsa_circ_0000467 for GC patients. A, The high expression of hsa_circ_0000467 predicts poor prognosis. B, The blue line was the ROC curve of TNM stage. The area under the curve was 0.708. The red line was the ROC curve of combine circ_0000467 and TNM stage. The area under the curve was 0.779. $P < 0.01$

TABLE 1 Correlation between hsa_circ_0000467 expression levels in gastric cancer tissues and clinicopathological parameters of patients with gastric cancer

Characteristics	hsa_circ_0000467 high expression cases (n = 32)	hsa_circ_0000467 low expression cases (n = 19)	P value
Age (years)			
<60	9 (28.1%)	5 (26.3%)	0.136
≥60	23 (71.9%)	14 (73.7%)	
Gender			
Male	24 (75.0%)	14 (73.7%)	0.418
Female	8 (25.0%)	5 (26.3%)	
Diameter (cm)			
<5	10 (31.3%)	6 (31.6%)	0.353
≥5	22 (68.7%)	13 (68.4%)	
Histologic type			
Differentiated	19 (59.4%)	10 (52.6%)	0.145
Undifferentiated	13 (40.6%)	9 (47.4%)	
T classification			
T1	4 (12.5%)	6 (31.6%)	<0.001
T2	3 (9.4%)	5 (26.3%)	
T3	18 (56.2%)	5 (26.3%)	
T4	7 (21.9%)	3 (15.8%)	
Lymphatic metastasis			
N0	2 (6.3%)	3 (15.8%)	<0.001
N1	5 (15.6%)	9 (47.3%)	
N2	10 (31.3%)	4 (21.1%)	
N3	15 (46.8%)	3 (15.8%)	
TNM stage			
I	7 (21.9%)	8 (42.1%)	<0.001
II	12 (37.5%)	6 (31.6%)	
III	13 (40.6%)	5 (26.4%)	
CEA (ng/mL)			
<5.0	27 (84.4%)	16 (84.2%)	0.752
≥5.0	5 (15.6%)	3 (15.8%)	
CA72-4 (U/mL)			
<6.9	25 (78.1%)	15 (78.9%)	0.561
≥6.9	7 (21.9%)	4 (21.1%)	

3.4 | Hsa_circ_0000467 induced GC proliferation, invasion, migration, and inhibited apoptosis in vitro

We transfected HGC-27 and AGS cells with shRNA1# (64736-1) and shRNA2# (64735-1; Figure 4A). Thus, we selected shRNA2# for further study. CCK-8 assay was performed to investigate the effect of hsa_circ_0000467 on GC cell proliferation. We observed that hsa_circ_0000467 silencing inhibits proliferation of the HGC-27 and AGS cells compared to the control groups (Figure 4B). Then, we performed the transwell assay using the HGC-27 and AGS cells and

found that hsa_circ_0000467 silencing inhibits invasion (Figure 4C) and migration (Figure 4D) of the cells. Thus, these data suggest that hsa_circ_0000467 plays a tumor-promoting role.

We also investigated the role of hsa_circ_0000467 in apoptosis in GC cells. HGC-27 and AGS cells were transfected with hsa_circ_0000467-silencing plasmid or scrambled nonspecific shRNA control. Knockdown of hsa_circ_0000467 in the cells increased the fraction of apoptotic cells (Figure 4E).

4 | DISCUSSION

Numerous studies have shown that circRNAs exhibit aberrant expression in many types of cancer, including NSCLC, hepatocellular carcinoma, colorectal cancer, breast cancer, and esophageal squamous cell carcinoma. In this study, we used RT-qPCR assays to verify that hsa_circ_0000467 was upregulated in GC tissue vs adjacent tissue. The function of hsa_circ_0000467 in tumor development and progression has not been investigated previously.

A number of circRNAs have been identified as ideal biomarkers for cancer diagnosis, due to their highly conserved and remarkably stable.^{7,11,21,22} However, their clinical diagnostic value in gastric cancer remains largely unknown. In this study, we found that hsa_circ_0000467 was upregulated in gastric cancer tissues, cell lines, and plasmas of patients with gastric cancer. Importantly, we are the first to determine the potential diagnostic value of hsa_circ_0000467. CEA, CA 19-9, and CA 72-4 are widely used in screening and auxiliary diagnosis of gastrointestinal malignancies. Chen et al reported that hsa_circ_0000190 is superior to commonly used serum cancer markers such as CEA and CA19-9 in gastric cancer.²³ Nonetheless, they had a low positive rate in early gastric cancer screening.²⁴ The sensitivity and specificity of CA19-9 were 0.605 and 0.559, respectively; the values for CEA were 0.686 and 0.593, respectively.²⁵ In this study, the sensitivity and specificity of CEA were 0.614 and 0.568, and for CA72-4, the sensitivity and specificity were 0.667 and 0.592, respectively. However, the sensitivity and specificity of plasma hsa_circ_0000467 were 0.705 and 0.648 indicating that hsa_circ_0000467 has higher sensitivity and specificity in the screening of gastric cancer. Apart from this, plasma hsa_circ_0000467 had a higher AUC value (0.790) than CEA (0.560) and CA72-4 (0.670). Our data indicate that hsa_circ_0000467 may be used as a potential noninvasive marker for the diagnosis of gastric cancer and is superior to commonly used gastric cancer biomarkers such as CEA and CA72-4; we thus suggest that a combination of plasma hsa_circ_0000467, CEA, and CA72-4 may increase the success rate of diagnostic procedures.

In subsequent studies, we have found that the plasma levels of hsa_circ_0000467 in postoperative patients are significantly decreased compared to those in preoperative patients ($P < 0.01$). This might be due mainly to a decrease in the release of tumor-derived nucleic acids after tumor excision.^{26,27} Thus, the peripheral blood levels of hsa_circ_0000467 in preoperative and postoperative patients are significantly changed. In summary, hsa_circ_0000467 may

TABLE 2 Univariate and multivariate survival analysis of patients following R0 resection for gastric cancer

Variable	Univariate analysis			Multivariate analysis		
	OR	95% CI	P	OR	95% CI	P
Age (<60 vs ≥60)	0.826	0.642-1.690	0.441	/	/	/
Gender (M vs F)	1.218	0.783-1.928	0.612	/	/	/
Tumor size (<5 cm vs ≥5 cm)	1.526	1.270-2.550	<0.041	1.139	0.977-1.719	0.085
Histologic type (Differentiated vs Un-)	0.792	0.605-1.528	0.280	/	/	/
Cancer stage			<0.001			<0.001
Stage II vs stage I	4.836	2.272-7.693	<0.001	3.284	1.859-6.729	<0.001
Stage III vs stage I	8.738	4.769-12.857	<0.001	7.189	4.020-9.893	<0.001
hsa_circ_0000467 expression (high vs low)	3.675	1.673-5.330	0.032	2.683	1.420-4.796	0.041
CEA (<5.0 vs ≥5.0)	0.892	0.703-1.772	0.593	/	/	/
CA72-4 (<6.9 vs ≥6.9)	0.811	0.612-1.527	0.329	/	/	/

represent a potential biomarker for assessing the risk of GC and may function as a diagnostic marker; our findings may provide a new avenue for early GC screening, which has been clinically difficult traditionally.

Increasing evidence uncovers a crucial role of circRNAs in the regulation of cancer cell proliferation, migration, and metastasis.^{16,28,29} Hsa_circ-Foxo3 could repress cell proliferation and regulate cell cycle progression.³⁰ ciRS-7 is one of the few known

FIGURE 4 Hsa_circ_0000467 induce GC malignant biological behavior. A, HGC-27 and AGS cells were transfected with shRNA, including circRNAi (64736-1; shRNA1#) and circRNAi (64735-1; shRNA2#) and control plasmid; B, the effect of hsa_circ_0000467 on GCC proliferation; C, hsa_circ_0000467 induced cell invasion; D, hsa_circ_0000467 induced cell migration; E, apoptosis measured by flow cytometry. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

circRNAs that has been proposed to inhibit tumor suppressor miR-7. Widespread expression of ciRS-7 in neuroblastomas, astrocytoma, renal cell, lung, and colorectal carcinomas implicates the role of ciRS-7 as a critical regulator in cancer.³¹ However, the functions of circRNAs in GC are emerging only recently. In our study, we identified a novel circRNA hsa_circ_0000467 as a tumor promoter. We found that higher expression of hsa_circ_0000467 correlates with multiple clinicopathological factors such as larger tumor size, advanced T-stage, and lymph node metastasis; hence, patients with high hsa_circ_0000467 expression had a worse prognosis than those with low expression; this finding prompted us to investigate the function of hsa_circ_0000467. Using CCK8 and transwell assays, we demonstrated that knockdown of hsa_circ_0000467 significantly inhibits proliferation, migration, and invasion of GC cells. These data indicate that hsa_circ_0000467 might participate in the occurrence and development of GC and might be a potential biomarker for prognosis and a therapeutic target for GC treatment.

Our results showed that hsa_circ_0000467 affects GC cell proliferation, apoptosis, and metastasis. However, the mechanisms of circRNA function in carcinogenesis and cancer progression have not been clearly elucidated. circRNAs may regulate the expression of oncogenic or tumor suppressor genes via different targets depending on the different types of cancer or even different stages of the disease. In most cases, circRNAs act as an miRNA sponge and form a circRNA-miRNA-mRNA axis.^{22,32,33}

There are some limitations to this study. First, the sample size was relatively small and the follow-up was relatively short; thus, these data have to be confirmed in a larger population with longer follow-up. Second, only one circRNA was investigated in this study. Third, we performed only loss-of-function biological experiments. Therefore, gain-of-function assays should be performed in the GC cells, and animal experiments are needed.

In conclusion, our study provides a novel potential circular RNA target in GC patients. To the best of our knowledge, this is the first study to investigate the expression, function, and diagnostic value of hsa_circ_0000467 in GC. Studies of the functions of hsa_circ_0000467 can improve our understanding of the mechanisms underlying gastric cancer occurrence and progression, and our findings may offer a potential therapeutic target for treating this fatal disease. However, further research in an external validation setting is warranted.

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CONFLICT OF INTEREST

The authors report no conflict of interests in this work.

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015;2:87-108.
2. Park JY, von Karsa L, Herrero R. Prevention strategies for gastric cancer: a global perspective. *Clin Endosc*. 2014;6:478-489.
3. Kalnina Z, Meistere I, Kikuste I, Tolmanis I, Zayakin P, Line A. Emerging blood-based biomarkers for detection of gastric cancer. *World J Gastroenterol*. 2015;41:11636-11653.
4. Shimada H, Noie T, Ohashi M, Oba K, Takahashi Y. Clinical significance of serum tumor markers for gastric cancer: a systematic review of literature by the task force of the Japanese Gastric Cancer Association. *Gastric Cancer*. 2014;1:26-33.
5. Salzman J, Gawad C, Wang PL, Lacayo N, Brown PO. Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. *PLoS ONE*. 2012;2:e30733.
6. Barrett SP, Salzman J. Circular RNAs: analysis, Expression and Potential Functions. *Development*. 2016;11:1838-1847.
7. Jeck WR, Sorrentino JA, Wang K, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA*. 2013;2:141-157.
8. Meng S, Zhou H, Feng Z, et al. CircRNA: functions and properties of a novel potential biomarker for cancer. *Mol Cancer*. 2017;1:94.
9. Preusser C, Hung LH, Schneider T, et al. Selective release of circRNAs in platelet-derived extracellular vesicles. *J Extracell Vesicles*. 2018;1:1424473.
10. Li J, Yang J, Zhou P, et al. Circular RNAs in cancer: novel insights into origins, properties, functions and implications. *Am J Cancer Res*. 2015;2:472-480.
11. Qin M, Liu G, Huo X, et al. Hsa_Circ_0001649: a circular RNA and potential novel biomarker for hepatocellular carcinoma. *Cancer Biomark*. 2016;1:161-169.
12. Abu N, Jamal R. Circular RNAs as promising biomarkers: a mini-review. *Front Physiol*. 2016;7:355.
13. Ji W, Qiu C, Wang M, Mao N, Wu S, Dai Y. Hsa_Circ_0001649: a circular RNA and potential novel biomarker for colorectal cancer. *Biochem Biophys Res Commun*. 2018;1:122-126.
14. Li P, Chen H, Chen S, et al. Circular RNA 0000096 affects cell growth and migration in gastric cancer. *Br J Cancer*. 2017;5:626-633.
15. Li GF, Li L, Yao ZQ, Zhuang SJ. Hsa_Circ_0007534/miR-761/ZIC5 regulatory loop modulates the proliferation and migration of glioma cells. *Biochem Biophys Res Commun*. 2018;4:765-771.
16. Xu Y, Yao Y, Zhong X, et al. Downregulated circular RNA Hsa_Circ_0001649 regulates proliferation, migration and invasion in cholangiocarcinoma cells. *Biochem Biophys Res Commun*. 2018;2:455-461.
17. Wang X, Zhu X, Zhang H, et al. Increased circular RNA Hsa_Circ_0012673 acts as a sponge of miR-22 to promote lung adenocarcinoma proliferation. *Biochem Biophys Res Commun*. 2018;4:1069-1075.
18. Zhang Q, Sivakumar S, Chen Y, et al. Ska3 phosphorylated by Cdk1 binds Ndc80 and recruits Ska to kinetochores to promote mitotic progression. *Curr Biol*. 2017;10:1477-1484.
19. Chuang TP, Wang JY, Jao SW, et al. Over-expression of AURKA, SKA3 and DSN1 contributes to colorectal adenoma to carcinoma progression. *Oncotarget*. 2016;29:45803-45818.
20. Li J, Li Z, Jiang P, et al. Circular RNA IARS (circ-IARS) secreted by pancreatic cancer cells and located within exosomes regulates endothelial monolayer permeability to promote tumor metastasis. *J Exp Clin Cancer Res*. 2018;1:177.

21. Li QF, Zhang YC, Chen YQ, Yu Y. Circular RNAs roll into the regulatory network of plants. *Biochem Biophys Res Commun*. 2017;2:382-386.
22. Hansen TB, Jensen TI, Clausen BH, et al. Natural RNA circles function as efficient microRNA sponges. *Nature*. 2013;7441:384-388.
23. Chen S, Li T, Zhao Q, Xiao B, Guo J. Using circular RNA Hsa_Circ_0000190 as a new biomarker in the diagnosis of gastric cancer. *Clin Chim Acta*. 2017;466:167-171.
24. Yang Y, Shao Y, Zhu M, et al. Using gastric juice lncRNA-ABHD11-AS1 as a novel type of biomarker in the screening of gastric cancer. *Tumour Biol*. 2016;1:1183-1188.
25. Wu J, Li G, Wang Z, et al. Circulating microRNA-21 is a potential diagnostic biomarker in gastric cancer. *Dis Markers*. 2015;2015:1-8.
26. Nilsson RJ, Balaj L, Hulleman E, et al. Blood platelets contain tumor-derived RNA biomarkers. *Blood*. 2011;13:3680-3683.
27. Zheng X, Koropatnick J, Li M, et al. Reinstalling antitumor immunity by inhibiting tumor-derived immunosuppressive molecule IDO through RNA interference. *J Immunol*. 2006;8:5639-5646.
28. Ma HB, Yao YN, Yu JJ, Chen XX, Li HF. Extensive profiling of circular RNAs and the potential regulatory role of circRNA-000284 in cell proliferation and invasion of cervical cancer via sponging miR-506. *Am J Transl Res*. 2018;2:592-604.
29. Zhang R, Xu J, Zhao J, Wang X. Silencing of Hsa_Circ_0007534 suppresses proliferation and induces apoptosis in colorectal cancer cells. *Eur Rev Med Pharmacol Sci*. 2018;1:118-126.
30. Du WW, Yang W, Liu E, Yang Z, Dhaliwal P, Yang BB. Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with P21 and CDK2. *Nucleic Acids Res*. 2016;6:2846-2858.
31. Weng W, Wei Q, Todén S, et al. Circular RNA ciRS-7-A promising prognostic biomarker and a potential therapeutic target in colorectal cancer. *Clin Cancer Res*. 2017;14:3918-3928.
32. Kristensen LS, Hansen TB, Veno MT, Kjems J. Circular RNAs in cancer: opportunities and challenges in the field. *Oncogene*. 2018;5:555-565.
33. Huang WJ, Wang Y, Liu S, et al. Silencing circular RNA Hsa_Circ_0000977 suppresses pancreatic ductal adenocarcinoma progression by stimulating miR-874-3p and inhibiting PLK1 expression. *Cancer Lett*. 2018;422:70-80.

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