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Down-regulation of hsa_circ_0006470 predicts tumor invasion: A new biomarker of gastric cancer

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Abstract

Background: Gastric cancer (GC) is a common cancer. Circular RNAs (circRNAs) regulate the pathogenesis of GC. This study aims to explore its potential as a GC biomarker.
Methods: The expression of hsa_circ_0006470 in GC tissues and GC cell lines was measured by quantitative reverse transcription-polymerase chain reaction. The diagnostic value of hsa_circ_0006470 was estimated by the receiver operating characteristic (ROC) curve.
Results: Compared with adjacent normal tissues, the expression of hsa_circ_0006470 in GC tissues was significantly lower. The expression levels of hsa_circ_0006470 in different TNM stages and different invasion degrees were significantly different. The area under the ROC curve was 0.783, with sensitivity and specificity 0.725 and 0.750, respectively.
Conclusions: Hsa_circ_0006470 has a high value as a diagnostic biomarker for GC.

KEYWORDS
biomarker, circular RNA, diagnosis, gastric cancer, hsa_circ_0006470

1 | INTRODUCTION

Gastric cancer (GC) is perceived to be one of the most commonly diagnosed tumor diseases in the world and is a major contributor to global tumor-related mortality.[1] In fact, the vast majority of patients are already advanced or have metastasized by the time they are diagnosed with GC. As the lack of efficient and simple screening methods, early detection and treatment of GC are difficult to achieve.[2,3] Studies have revealed that plenty of non-coding RNAs (ncRNAs) were involved in the pathogenesis of GC, especially in the early stage via playing a role of tumor initiation.[4–6] Therefore, identifying specific ncRNAs would provide novel strategies for early diagnosis of GC, as well as understanding the mechanism of its onset and progression.

Circular RNAs (circRNAs) possess a unique covalent loop structure, which is derived from exons, introns, or intergenic regions, leaving no free ends.[7] CircRNAs were first discovered as a product of splicing errors, and subsequent studies found that they exist widely in nature and are evolutionarily conserved among species. Growing evidence suggests that circRNAs could compete with mRNAs as sponges of microRNAs (miRNAs); interact with RNA binding protein and even have a translational function.[8–10] CircRNAs play remarkable regulatory parts in the majority of cellular physiological processes while deregulation of circRNAs may substantially contribute to a wide range of human diseases, especially in tumorigenesis and progression via regulation of cell activities.[11–16] Moreover, circRNAs are highly resistant to the enzymatic activity of exonucleases, resulting in them being generally more stable than the linear RNA in tissues and plasma.[17] Hence, some circRNAs that are stable and specifically expressed in organisms are considered as biomarkers for cancer diagnosis and early screening, or even therapeutic targets.[8,12–16]

In the past 10 years, high-throughput sequencing has paved the way for the identification of circRNAs, and an abundance of abnormal expression of circRNAs were identified in GC, which implies that they have an irreplaceable influence on the etiology of GC.[18,19] At present, some circRNAs have been proved to have high stability and specificity in GC diagnosis, and have excellent diagnostic value for distinguishing GC tissues from normal tissues.[20–22] Moreover, the combination of circRNAs and other markers can form more specific biomarkers. For example, the combination of circRNAs with carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) can better support the diagnosis of GC.[23,24]

A study on the expression profile of gastric cancer and circRNA in gastric cancer revealed the differentially expressed circRNA in gastric cancer.[25] Based on this result, we focused our attention on hsa_circ_0006470 and analyzed its diagnostic value in gastric cancer. This study is intended to verify the expression level of hsa_circ_0006470 in GC tissues and cell lines based on the previous microarray results,[26] and focuses on analyzing its value for GC diagnosis. Furthermore, we explored the expression difference of hsa_circ_0006470 under the different clinicopathological conditions of GC, laying a foundation for the further research on the early diagnosis of GC. Our findings support hsa_circ_0006470 as a considerable biomarker for GC diagnosis, and its expression is related to the tumor-node-metastasis (TNM) stage and invasion degree. The identification of this promising biomarker provided an essential strategy to improve the diagnostic level of GC.

2 | METHODS

2.1 | Sample collection

The lesion tissue samples of 80 GC patients confirmed by pathology section and the tissues without tumor cells confirmed by pathology about 5 cm away from the lesion sites were obtained (80 GC tissues vs. 80 adjacent non-tumor tissues). All samples were collected from the Affiliated People's Hospital of Ningbo University, from January 2013 to December 2018. The specimens taken from GC patients were immediately stored in RNA fixative reagent (Bioteke), and stored in the −80℃ ultra-low temperature refrigerator until RNA was extracted. The excluding criteria were as follows: cases with remnant GC, palliative surgery, combined with other visceral malignancies, and underwent preoperative chemotherapy or radiotherapy. The tissues underwent histopathological evaluation by at least two pathologists. Tumors were classified following the International Union Against Cancer's TNM staging system. All patients participating in this study signed an informed consent form. The study was approved by the Institutional Ethics Committee of Ningbo University.

2.2 | RNA extraction

Extraction of total RNA from tissues and cells is achieved with TRIzol reagent (Invitrogen). The quality of the extracted RNA was evaluated by the Full Wavelength Microplate Reader (Thermo Multiskan GO). The GO ScriptTM reverse transcription system (Promega) was used to convert RNA into stable cDNAs. The RNA purity was evaluated on the basis of the ratio of A260/280, which was between 1.8 and 2.0. In addition, we perform 1% agarose gel electrophoresis to ensure high-quality total RNA.

2.3 | qRT-PCR detection

The primer sequences of hsa_circ_0006470 were as follows: 5′-CGGGAGCAGCAGTGCG-3′ and 5′-CGTTGAGCACCTCCTTAGCA-3′. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the control: 5′-CCAGGTGGTCTCCTCTGA-3′ and 5′-GCCAAATCGTTGT-3′. Primer 3 was used for primer sequence design followed by BGI Tech (Shenzhen, China) synthesis. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was implemented by GoTaqqPCR Master Mix (Promega) on the Mx3005P real-time PCR system (Stratagene).

2.4 | Cell culture

Normal human gastric mucosal cell line GES-1 and GC cell lines (AGS, BGC-823 and MGC-803) were gained from the Cell Line Bank of the Chinese Academy of Sciences (Shanghai, China). The culture is free from mycoplasma contamination. GES-1 and GC cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium and Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin, respectively. All cell lines were cultured at 5% CO_2 and 37℃.

2.5 | Statistical analysis

All data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 and visualized by GraphPad Prism 7.0 (GraphPad Software). Continuous variables were described by the mean ± SD. The expression difference of hsa_circ_0000647 between GC and normal tissues was evaluated by the paired t test. The expression of the target circRNA under the different clinicopathological conditions was compared by one-way analysis of variance (ANOVA). The accuracy of diagnosis was measured by the receiver operating characteristic (ROC) curve. The cutoff value was analyzed by SigmaPlot 12.3 (Systat Software Inc.). Two-side p value ≤0.05 was adopted as statistically different.

3 | RESULTS

3.1 | Features of hsa_circ_0006470

Hsa_circ_0006470 gene is located on chr1:12061457-12062160, encoded by chromosome region 1p36.22, with genomic length of 703 bp. In this area, the typical transcript is mitofusin 2 (MFN2) mRNA. From exon 1 to exon 3, their three exons constitute hsa_circ_0006470. To identify the correction the amplification of qRT-PCR, we carried out Sanger sequencing to analyze the amplified products (Figure 1).

3.2 | Hsa_circ_0006470 expression in GC tissues and normal tissues

The expression of hsa_circ_0006470 in tissues was evaluated by qRT-PCR. The Cq value was applied to evaluate the relative expression of the target gene: $\Delta Cq = Cq$ (hsa_circ_0006470) – Cq (GAPDH). The relatively lower ΔCq value indicates the relatively higher expression. The results showed that in comparison with normal tissues, hsa_circ_0006470 in GC tissue was low expressed ($p < 0.001$, Figure 2A). Furthermore, the expression levels of hsa_circ_0006470 were significantly down-regulated in 85.0% (68/80) of gastric cancer tissues compared with paracancerous normal tissues (Figure 2B).

3.3 | Hsa_circ_0006470 expression in GC cell lines

The normal gastric epithelial cell line GES-1 was used as a control for exploring hsa_circ_0006470 expression level in GC cell lines (AGS, BGC-823, and MGC-803). As shown in Figure 3, compared with GES-1, hsa_circ_0006470 was down-regulated in AGS, BGC-823, and MGC-803. These results were consistent with the expression trend in GC tissues (Figure 2).

3.4 | Diagnostic value of hsa_circ_0006470 in GC

The area under the ROC curve (AUC) is an indicator of diagnostic accuracy and is positively correlated with the diagnostic value. As shown in Figure 4A, the AUC of hsa_circ_0006470 in GC tissues was 0.783 (95% CI 0.711–0.854). The cutoff value of hsa_circ_0006470 in GC diagnosis was 10.74. The corresponding specificity and sensitivity were 0.75 and 0.725, respectively (Figure 4B).

3.5 | Clinical relationship of decreased hsa_circ_0006470 in GC

As a further exploration, we evaluated the expression of hsa_circ_0006470 under the different clinicopathological characteristics. As shown in Table 1, the expression of hsa_circ_0006470 had
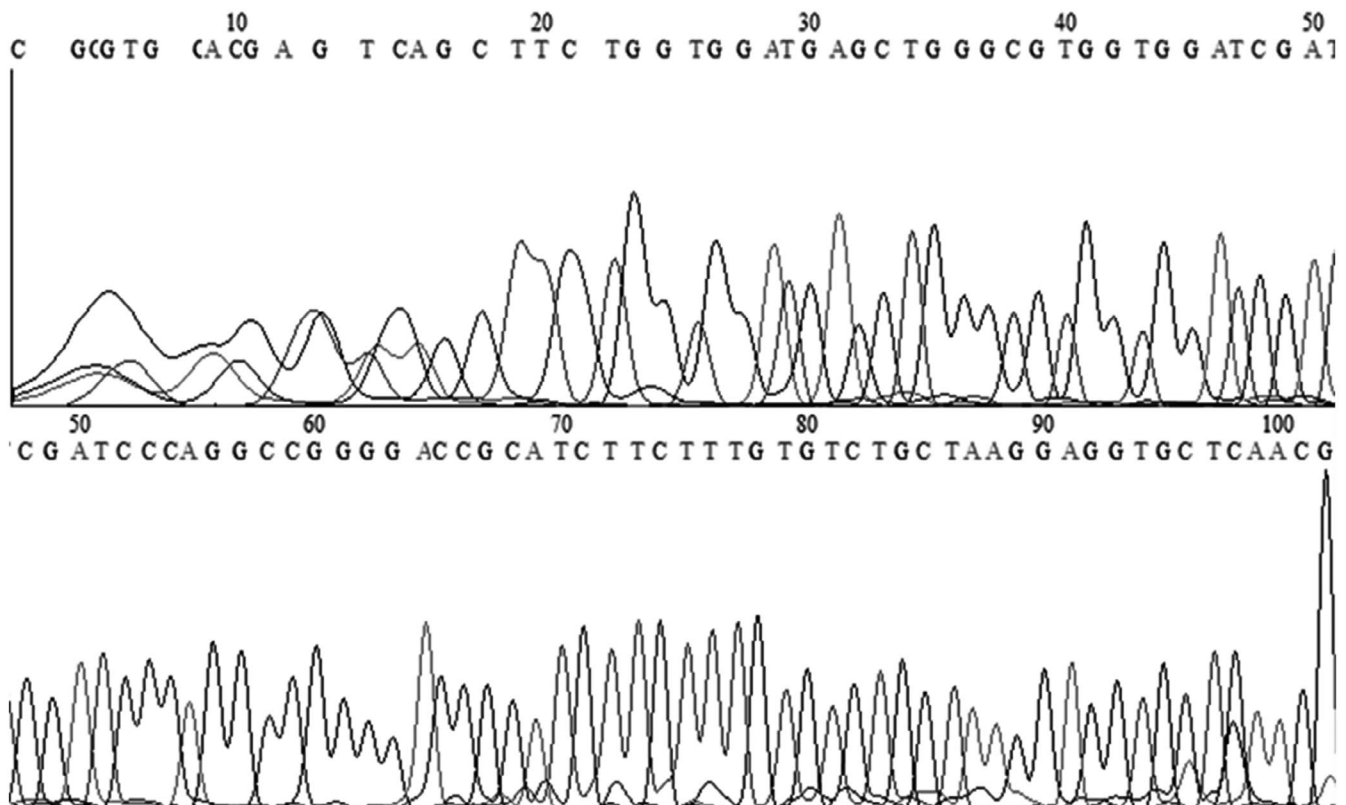

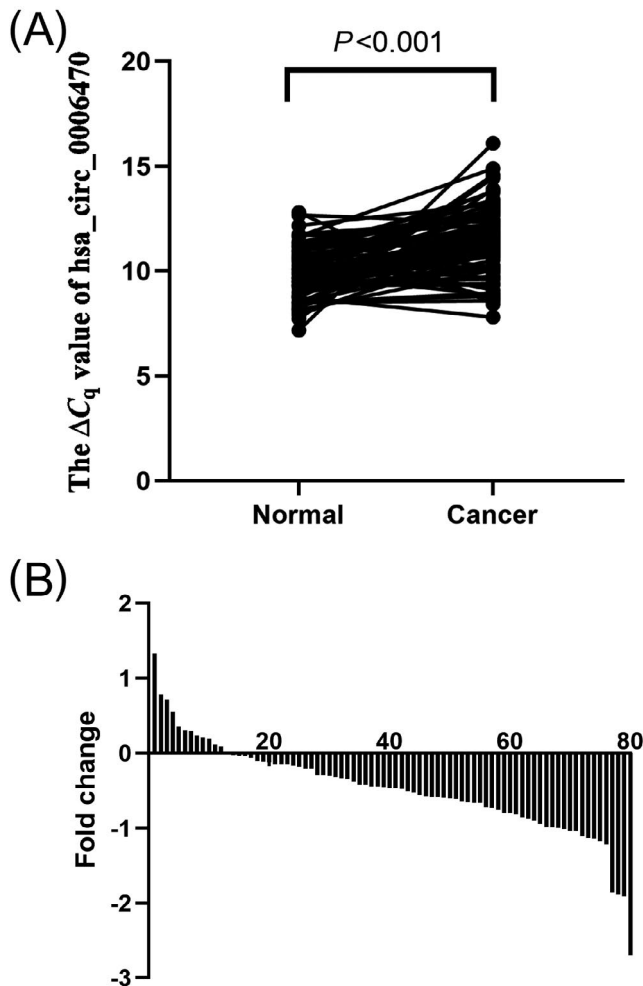**FIGURE 1** Sanger sequencing result of hsa_circ_0006470. Sequencing result of qRT-PCR product of hsa_circ_0006470 in GC tissues

(A)

(B)

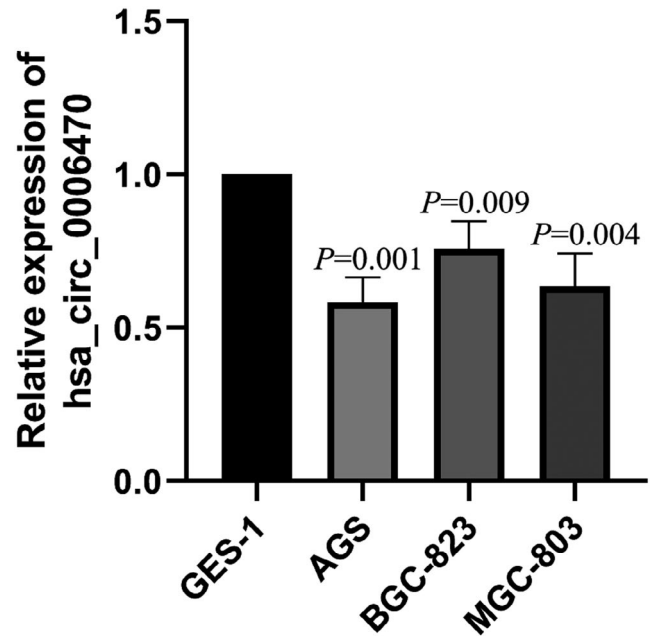**FIGURE 2** The expression levels of hsa_circ_0006470 in GC tissues and paracancerous normal tissues. (A) The expression levels of hsa_circ_0006470 in each GC and paracancerous tissues ($n = 80$, $p < 0.001$). Higher ΔCq value means the lower expression. (B) The expression levels of hsa_circ_0006470 were significantly down-regulated in 85.0% (68/80) of gastric cancer tissues compared with paracancerous normal tissues

FIGURE 3 Down-regulation of hsa_circ_0006470 in GC cell lines. Compared with normal gastric epithelial cells GES-1, hsa_circ_0006470 was lowly expressed in GC cell lines AGS, BGC-823, and MGC-803. The relative expressions were compared by using $2^{-\Delta\Delta Cq}$

in the complex biological courses and exhibit aberrant expression in a variety of cancers.[16,27] In view of the stability of circRNAs and its important role in cancer, the diagnostic significance of circRNAs is one of the main hotspots of current research. Here, we verified the relative expression of hsa_circ_0006470 in GC tissues and GC cell lines. Hsa_circ_0006470 was observed to be low expressed in GC tissues and cell lines for the first time (Figures 2 and 3). Our findings reveal a novel GC biomarker, which provide a direction for the diagnosis and prognosis of GC, and pave the way for further target exploration and early prediction.

CircRNAs have the following characteristics: (1) Due to their unique closed-loop structure, they are resistant to exonuclease RNase; (2) They have the features of tissue specificity and development stage specificity; and (3) They are highly conservative in evolution among different species.[17,28,29] Therefore, the possibility of circRNAs becoming biomarkers for human diseases is worth exploring. Regarding GC, a study found that the down-regulation of hsa_circ_002059 may indicate a poor prognosis of GC.[30] Hsa_circ_0000190 was significantly low expressed in plasma of GC patients and was associated with the pathological features of GC, including tumor size and lymphatic metastasis.[31] The diagnostic power of hsa_circ_0000190 far exceeds the common tumor markers CA19-9 and CEA. Also, the up-regulation of circPVT1 in osteosarcoma patients was a potential predictor of poor prognosis, and its diagnostic efficiency was even better than that of alkaline phosphate.[32] Meanwhile, the stability and abundance of circRNAs in exosomes further demonstrate their worth in the diagnosis of human disease.[24] More importantly, a plenty of circRNAs, such as

a strong correlation with the TNM stage ($p = 0.027$) and invasion degree ($p = 0.039$). Of the 80 patients, 11 had missing pathologic data. The differential expression of hsa_circ_0006470 between TNMI & II and III & IV may imply the evaluation of tumor staging. After the patients were grouped according to the degree of invasion, the expression of hsa_circ_0006470 showed significant differences between the T 3–4 group and the T 1–2 group. On the contrary, the expression of hsa_circ_0006470 has no obvious correlation with other indicators including gender, age, differentiation, tumor size, CEA, CA19-9, and distant metastasis.

4 | DISCUSSIONS

GC accounts for a large proportion of cancer-incurred mortality all over the world. Growing evidence reveals that circRNAs are involved

(A)

(B)
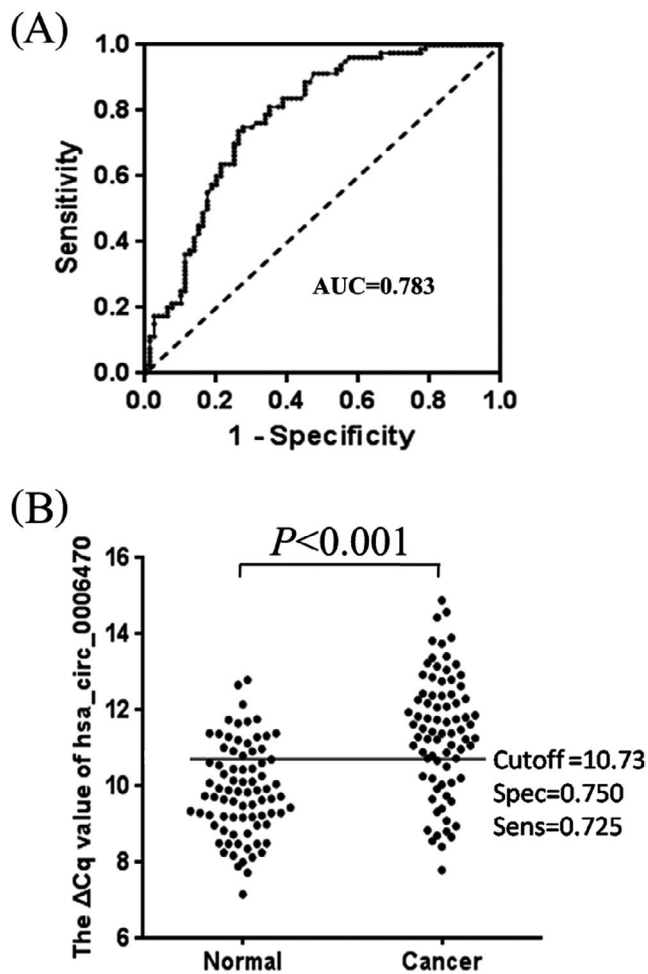
FIGURE 4 Diagnostic power of hsa_circ_0006470. (A) The ROC curve of hsa_circ_0006470. (B) The sensitivity, specificity, and cutoff value of hsa_circ_0006470

TABLE 1 The relationship between hsa_circ_0006470 expression level (ΔCq) and clinicopathological characteristics of patients with gastric cancer

Characteristics	No. of patients (%)	Mean ± SD	p Value
Age (year)			
≥60	50 (72.46)	11.31 ± 1.74	0.286
<60	19 (27.54)	11.44 ± 1.17	
Gender			
Male	46 (66.67)	11.20 ± 1.46	0.762
Female	23 (33.33)	11.64 ± 1.85	
CA19-9			
Positive	37 (53.62)	11.46 ± 1.80	0.504
Negative	32 (46.38)	11.20 ± 1.35	
CEA			
Positive	57 (82.61)	11.27 ± 1.65	0.416
Negative	12 (17.39)	11.69 ± 1.34	
Differentiation			
Well	6 (8.70)	12.25 ± 1.87	0.395
Moderate	37 (53.62)	11.33 ± 1.58	
Poor	26 (37.68)	11.16 ± 1.55	
Tumor size (cm)			
≥5	34 (49.28)	11.01 ± 1.67	0.090
<5	35 (50.72)	11.67 ± 1.48	
TNM stage			
I&II	31 (44.93)	11.81 ± 1.47	0.027
III&IV	38 (55.07)	10.96 ± 1.62	
Invasion			
Tis & T1&T2	28 (40.58)	11.82 ± 1.51	0.039
T3&T4	41 (59.42)	11.02 ± 1.60	
Distal metastasis			
M0	61 (88.41)	11.47 ± 1.50	0.077
M1	8 (11.59)	10.41 ± 2.08	
Lymphatic metastasis			
N0	29 (42.03)	11.90 ± 1.45	0.161
N1	11 (15.94)	11.12 ± 1.41	
N2	8 (11.59)	10.79 ± 2.14	
N3	21 (30.44)	10.90 ± 1.54	

hsa_circ_101308, hsa_circ_0001017, and circSMARCA5, have been identified as having the ability to be used as GC diagnostic and prognostic markers.[26,33,34] However, studies have shown that GC-related circRNAs still need to be verified.[35]

In our results, hsa_circ_0006470 was remarkably downregulated in GC tissues compared with the adjacent normal tissues (Figure 2). The same is true in GC cell lines (Figure 3). This study revealed the potential value of hsa_circ_0006470 in the diagnosis of GC with AUC reached 0.782 (Figure 4). In accordance with previous consensus, an AUC in the range 0.93–0.96 is considered excellent while a value in the range 0.75–0.92 is admissible.[36,37] CEA and CA19-9 are well known as common serum cancer markers in gastrointestinal cancer screening and auxiliary diagnosis; however, the low sensitivity and specificity make them lack of diagnostic power. As reported previously, the AUC of CEA and CA19-9 in GC diagnosis was 0.671 and 0.563, respectively.[38,39] According to reports, the sensitivity and specificity of traditional tumor markers CEA and CA19-9 are not more than 0.70.[40] The sensitivity and specificity of hsa_circ_0006470 are 0.750 and 0.725, respectively. In our results, hsa_circ_0006470 was superior to CA19-9 and CEA in the

sensitivity and specificity of GC diagnosis (Figure 4). Furthermore, hsa_circ_0006470 can provide a foundation for the identification of TNM stages of GC and the assessment of invasion (Table 1). TNM stages and invasion are extremely important factors affecting the prognosis of gastric cancer patients. These mean that hsa_circ_0006470 might be an important diagnostic biomarker for GC, and its diagnostic value exceeds the common serum tumor markers CEA and CA19-9.

In fact, this study still has several limitations. First, the size of sample was relatively small and there is a lack of follow-up for prognosis; thus, future long-term follow-up cohort studies should

be concentrated on the role of hsa_circ_0006470 in the prognosis of GC. Second, the diagnostic efficacy should be validated in plasma samples as it may have the potential as a noninvasive marker.

5 | CONCLUSIONS

In summary, our findings reveal that hsa_circ_0006470 is dramatically down-regulated in GC tissues and GC cell lines. The expression level of hsa_circ_0006470 is correlated to TNM stage and invasion. Hsa_circ_0006470 may serve as a considerable biomarker for GC diagnosis.

CONFLICTS OF INTEREST
No conflict of interest.

DATA AVAILABILITY STATEMENT
All data used to support the findings of this study are included in the article.

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