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Tumor suppressor role of hsa_circ_0035445 in gastric cancer

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Abstract

Circular RNAs (circRNAs) are closely related to the occurrence and development of cancers. However, the roles of circRNAs in gastric cancer are largely unknown. Total 104 pairs of gastric cancer tissues and non-cancer tissues, fasting plasma of 42 healthy people and 42 gastric cancer patients' one day before operation and 10 days after operation were collected. Quantitative reverse transcription-polymerase chain reaction was used to detect the expression level of hsa_circ_0035445. The receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) were used to analyze its diagnostic value. Small interfering RNA and overexpression vector were used to downregulate and upregulate the expression of hsa_circ_0035445, respectively. Cell Counting Kit-8 and colony formation assays were used to detect the proliferation ability. Trans-well assay and scratch assay were used to detect the migration ability. Finally, flow cytometry was used to detect the changes of cell cycle distribution and apoptosis. Hsa_circ_0035445 was lowly expressed in gastric cancer tissues, plasma of gastric cancer patients, and gastric cancer cells. The expression level of hsa_circ_0035445 in gastric cancer tissues was relationship with tumor size and distant metastasis. The AUC of hsa_circ_0035445 in tissues and plasma was 0.68 and 0.86, respectively. Upregulation of hsa_circ_0035445 suppressed the proliferation and migration, promoted apoptosis, and blocked cells at G0/G1 phase. Downregulation of hsa_circ_0035445 promoted the proliferation and migration, suppressed apoptosis, and blocked cells at S phase. In conclusion, hsa_circ_0035445 may become a new target for the treatment of gastric cancer.

KEYWORDS
biological function, circular RNAs, gastric cancer, hsa_circ_0035445, therapeutic target

1 | INTRODUCTION

Regard to malignant tumors, early detection, early diagnosis, and early treatment are the most effective ways to reduce deaths caused by cancer.[1] However, due to the lack of early diagnostic biomarkers, many cancer patients have reached the advanced stage and missed the best treatment opportunity. So, it is particularly important to find a suitable early diagnosis biomarker.[2] Gastric cancer is a malignant tumor of the digestive tract that originates in the epithelium of the gastric mucosa.[3]

The majority of gastric cancers are adenocarcinomas. There are no obvious symptoms in the early stage, and only non-specific symptoms such as upper abdominal discomfort and belching may occur.[4] Gastric cancer is easily confused with gastritis and gastric ulcers.[5] The early diagnosis rate of gastric cancer is still low.[6] Therefore, it is very important to find early specific markers of gastric cancer.

Circular RNAs (circRNAs) are a type of new endogenous RNAs produced by non-standard reverse splicing.[7] According to different sources, circRNAs can be divided into three different types: exon

circRNAs (EcRNAs),[8,9] intron circRNAs (CiRNAs),[10,11] and exon-intron circRNAs (EIcRNAs).[12,13] Most of the circRNAs found so far are derived from gene exons.[14] CircRNAs are mainly distributed in the cytoplasm and act as a sponge by binding to microRNAs (miRNAs), while a few circRNAs exist in the nucleus and mainly play a transcriptional regulation role.[15] They have certain tissue and temporal specificity and are closely related to physiological development and various tumors and other diseases.[6,9,14,15] CircRNAs have neither free 5′ end and 3′ end nor poly A tail structure, and form a ring structure with covalent bonds.[16] Its cyclization is not a passive effect caused by variable shear but is actively mediated by flanking sequences or RNA-binding protein (RBP) binding sites.[17] Therefore, compared with linear RNA, circRNAs are not easily degraded by RNase R and are highly conservative, indicating having the potential to become new diagnostic biomarkers.[18]

This study focused on the relationship between circRNAs and gastric cancer. Based on our previous circRNA microarray screening results,[18] hsa_circ_0035445 is one of the gastric cancer-associated circRNAs. However, its diagnostic value and roles in gastric cancer are unknown. Hsa_circ_0035445 is an EcRNA composed of three exons. Its gene is located at 15q21.3 (chr15:58302847-58306479). Its related gene symbol is aldehyde dehydrogenase 1 family member A2 (*ALDH1A2*). By detecting hsa_circ_0035445 expression levels in tissues and plasma from patients with gastric cancer and functional experiments, we found that hsa_circ_0035445 may become a new biomarker and potential therapeutic target of gastric cancer.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens and pathological data

The 104 pairs of gastric cancer tissues and paired non-cancer tissues were collected in the Affiliated People's Hospital, Ningbo University. The tumor staging is based on the International Cancer Alliance's Tumor-Node-Metastasis (TNM) staging system.[19] The histological grade was evaluated according to the National Comprehensive Cancer Network (NCCN) oncology clinical practice guidelines (V.1.2011).[20] Immediately after obtaining, the tissues were put into RNA-fixer Reagent (Kang Wei, Beijing, China) and then stored at −80℃ until use. The fasting plasma of 42 healthy volunteers and 42 gastric cancer patients one day before operation and 10 days after operation were also collected. All specimens have been diagnosed by two pathologists. All participants signed an informed consent form. All clinical data are collected by experienced clinicians. This study was approved by the Ethics Committee of Ningbo University School of Medicine (No. 2017022701).

2.2 | Cell lines and culture

The normal human gastric mucosal epithelial cells GES-1 were purchased from the Chinese Academy of Medical Sciences Cancer Hospital (Beijing, China). Gastric cancer cell lines, MGC-803, SGC-7901, AGS,

BGC-823, and HGC-27, were purchased from the Shanghai Institute of Life Sciences, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (HyClone) added with 10% fetal bovine serum (FBS; PAN-Biotech) and 1% penicillin/streptomycin (Beyotime) at 37℃ and 5% CO_2.[21]

2.3 | Total RNA extraction

The tissue and cell specimens were added with TRIzol reagent (Invitrogen) when extracting RNA, while the plasma specimens were added with TRIzol LS reagent (Invitrogen). Then, the concentration and purity of RNA extracted were measured via Smart Spec Plus Spectrophotometer (BioRad). Finally, the remaining RNA was stored at −20°C.

2.4 | Reverse transcription

Total 2 μg RNA was used for reverse transcription according to GoTaq® 2-Step RT-qPCR System (Promega).

2.5 | Real-time qPCR

According to the GoTaq qPCR Master Mix (Promega) instructions, hsa_circ_0035445 was amplified in the Mx3005P real-time PCR system (Stratagene). The specific divergent primer sequences of hsa_circ_0035445 are 5′-CTTTCGATATTACGCAGGCTG-3′ and 5′-GTTCTCCTGTGGCTGGATTA-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an external reference. Its primer sequences are 5′-TCGACAGTCAGCCGCATCTTCTTT-3′ and 5′-GTTCTCCTGTGGCTGGATTA-3′. These primers were synthesized by Sangon Biotech.

2.6 | Transfection

For downregulation of hsa_circ_0035445, two small interfering RNAs (siRNAs) were synthesized by GenePharma Co., Ltd. Their sequences are hsa_circ_0035445-1, 5′-CAUUCCUGUAGAUCUUUAUTT-3′, and 5′-AUAAAGAUCUACAGGAAUGTT-3′; hsa_circ_0035445-2, 5′-CCUGUAGAUCUUUAUAAACTT-3′, and 5′-GUUUAUAAAGAU CUACAGGTT-3′. Regarding the upregulation of hsa_circ_0035445, the overexpression vector was provided by GeneSeed Co., Ltd. According to the instructions, cells were transfected with Lipofectamine 2000 (Invitrogen).

2.7 | CCK-8 assay and Colony formation assay

Cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories) and colony formation assay. For CCK-8 assay,

5000 cells per well were first seeded into 96-well plates. Then, on day 0, 1, 2, 3, and 4, 10 ml of CCK-8 solution was added to each well. After incubating at 37°C for 3 h, cell growth was measured at 450 nm with Spectra-Max M5 multi-functional microplate reader (Synergy 4, Bio-Tek). For the colony formation assay, 1000 cells per well were first seeded in 6-well plates, then maintained in complete medium, and after incubation at 37°C for 14 days, cells were fixed with 4% paraform-aldehyde (Beyotime) for 30 min and stained with 0.1% crystal violet (Beyotime). After gently washing with PBS and air-drying, cell colonies were counted and analyzed. The experiment was repeated three times.

2.8 | Trans-well assay

Trans-well assay was used to detect cell migration ability. Total 750 μl complete culture medium was first added to the lower chamber, then 200 μl serum-free culture medium containing 4×10^4 transiently transfected cells were added to the upper chamber. After incubating at 37°C for 48 h, the upper chamber was removed and fixed with 4% paraformaldehyde (Beyotime) for 30 min and stained with 0.1% crystal violet (Beyotime). The dye was rinsed gently with water flow. After a little drying, it was photographed under an imaging microscope and counted for statistical analysis. Three independent experiments were performed.

2.9 | Cell apoptosis assay

After culturing the transiently transfected cells for 48 h, cells were collected, digested, and centrifuged at 668.57g for 5 min. Then 5 μl Annexin V-FITC and 10 μl PI (Lianke) were added. After incubating in the dark for 5 min, cell apoptosis was analyzed by flow cytometry (FACScan, BD Biosciences). This experiment was repeated three times.

2.10 | Cell cycle analysis

After transfection, cells were cultured for 24 h. Then, cells were starved with serum-free medium for 24 h. The digested and collected cells were centrifugated at 668.57g for 5 min. Cells were washed with 250 μl PBS and then fixed with 750 μl water-free ethanol at −20°C for at least 24 h. After centrifuged at 668.57g for 5 min, 1 ml PI/RNase Staining Buffer dye (Lianke) was added. After incubating in the dark for 30 min, cell cycle distribution was analyzed by flow cytometry (FACScan). This experiment was repeated three times.

2.11 | Statistical analysis

GraphPad Prism 6 (GraphPad Software) and SPSS 26.0 (SPSS) were used for statistical analysis. Various statistical methods include paired sample t test, independent sample t test, and chi-square test were chose according to specific circumstances. The experimental data statistics were expressed as mean ± SD. When $p < 0.05$, the difference was considered as statistically significant.

3 | RESULTS

### 3.1 | Determination of specific divergent primers for hsa_circ_0035445

qRT-PCR technology was used to detect the expression level, and the amplified products were subjected to sanger sequencing (BGI). Then, the sequencing results were compared with the original sequence. The results showed that the qRT-PCR product's sequence was consistent with the hsa_circ_0035445 sequence (Figure S1). This indicates that the primers we designed are specific primers that can be used to amplify hsa_circ_0035445.

### 3.2 | Hsa_circ_0035445 was lowly expressed in gastric cancer tissues, cells, and plasma

We detected the expression level of hsa_circ_0035445 in 104 pairs of gastric cancer tissues and paired non-cancer tissues by qRT-PCR. Among them, 40 pairs of tissues were used as testing cohort, and the rest samples were used to verify the results of testing cohort. As shown in Figure 1, hsa_circ_0035445 showed low expression in testing cohort (A), validating cohort (B), or all of them (C). Further as shown in Figure 2, the expression of hsa_circ_0035445 in plasma samples of gastric cancer patients one day before surgery was relatively low compared to the plasma samples of 10 days after surgery and healthy volunteers (A). Finally, compared with normal gastric mucosal epithelial cells GES-1, hsa_circ_0035445 was lowly expressed in gastric cancer cell lines, MGC-803, SGC-7901, AGS, BGC-823, and HGC-27, but not in SGC-7901 cells (B). Based on these results, in the next cell experiments, two representative gastric cancer cells, AGS and BGC-823, were selected.

3.3 | Potential diagnostic value of hsa_circ_0035445

To analyze the diagnostic value of hsa_circ_0035445, the receiver operating characteristic (ROC) curves were drawn. As shown in Figure 2C, the area under the ROC curve (AUC) of hsa_circ_0035445 in gastric cancer tissues was 0.68, while the AUC in plasma was 0.86. When combinative used tissues and plasma, the AUC increased to 0.88. Then, the relationships between the expression level of hsa_circ_0035445 in gastric cancer tissues (Table 1) or plasma (Table 2) and clinicopathological factors of gastric cancer patients were analyzed to further explore its diagnostic value. We found that the expression level of hsa_circ_0035445 in gastric cancer tissues was significantly associated with tumor size ($p = 0.0108$) and distant
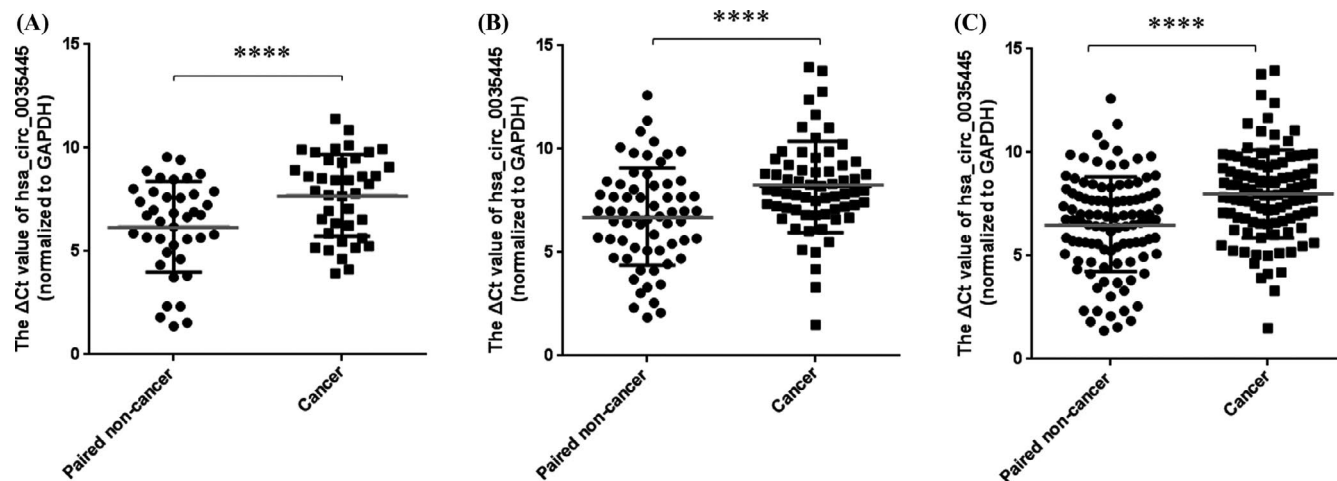
FIGURE 1 Expression levels of hsa_circ_0035445 in gastric cancer tissues. Hsa_circ_0035445 was significantly downregulated in gastric cancer tissues compared with the paired non-cancers tissues in testing cohort (A, $n = 40$), validating cohort (B, $n = 64$), and combined (C, $n = 104$). Larger ΔC_t value indicates lower expression. ****$p < 0.0001$
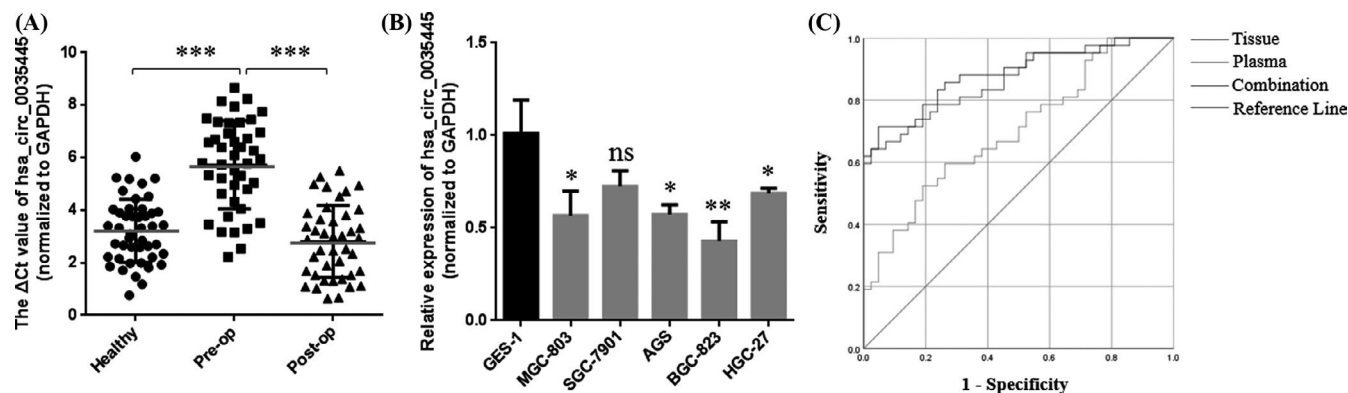
FIGURE 2 Hsa_circ_0035445 expression levels in plasma, cells, and its diagnostic value. A. Hsa_circ_0035445 levels among plasma samples from healthy volunteer, one day preoperation (Pre-op) and 10 days postoperation (Post-op) patients with gastric cancer. Larger ΔC value indicates lower expression. $n = 42$. B. Compared with the normal gastric mucosal epithelial cell line GES-1, hsa_circ_0035445 is lowly expressed in gastric cancer cell lines except SGC-7901. C. ROC curve. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, no significance

metastasis ($p = 0.0076$), providing a direction for subsequent cell experiments to study its effect on cell growth and metastasis. The hsa_circ_0035445 levels in plasma were significantly associated with carcinoembryonic antigen (CEA; $p = 0.0355$), carbohydrate antigen 19-9 (CA19-9) ($p = 0.0495$), and lymph node metastasis ($p = 0.0103$).

3.4 | Upregulation of hsa_circ_0035445 suppressed proliferation and migration of gastric cancer cells

To evaluate the effect of upregulated hsa_circ_0035445 on the biological behaviors of gastric cancer cells, we transfected its expressed vector into AGS and BGC-823 cells. The results showed that the overexpression vector effectively upregulated the expression of hsa_circ_0035445 (Figure S2A). After transfection of the overexpression vector, CCK-8 assay showed that cell proliferation was significantly suppressed (Figure 3), while the colony formation assay

showed that the colony formation viability of AGS and BGC-823 cells was significantly inhibited (Figure 4). Then, the Trans-well assay (Figure 5) showed that the migration ability of gastric cancer cells was significantly weakened. Afterward, we evaluated its effects on cell cycle and apoptosis. The results showed that upregulated expression of hsa_circ_0035445 significantly arrested cell cycle at G0/G1 phase (Figure S3) and promoted apoptosis of AGS and BGC-823 cells (Figure S5).

3.5 | Downregulation of hsa_circ_0035445 promoted proliferation and migration of gastric cancer cells

To evaluate the effect of downregulated hsa_circ_0035445 on the biological behaviors of gastric cancer cells, we designed two siRNAs. We transfected siRNA into AGS and BGC-823 cells. The

TABLE 1 The relationships between the expression levels of hsa_circ_0035445 (ΔC) in tissues and the clinicopathological factors of patients with gastric cancer

Characteristics	n	High (%)	Low (%)	p-Value
All cases	104 (100)	52	52	
Gender				
Male	80 (76.92)	34 (65.38)	46 (88.46)	**0.0052**
Female	24 (23.08)	18 (34.62)	6 (11.54)	
Age (years)				
<60	25 (24.04)	17 (32.69)	8 (15.38)	**0.0389**
≥60	79 (75.96)	35 (67.31)	44 (84.62)	
CEA				
Positive	91 (87.50)	45 (86.54)	46 (88.46)	0.7668
Negative	13 (12.50)	7 (13.46)	6 (11.54)	
CA19-9				
Positive	68 (65.38)	33 (63.46)	35 (67.31)	0.6802
Negative	36 (34.62)	19 (36.54)	17 (32.69)	
Differentiation				
Well	19 (18.27)	12 (23.08)	7 (13.46)	0.2045
Moderate-Poor	85 (81.73)	40 (76.92)	45 (86.54)	
TNM stage				
0 & I	19 (18.27)	11 (21.15)	8 (15.38)	0.7201
II	24 (23.08)	11 (21.15)	13 (25.00)	
III & IV	61 (58.65)	30 (57.70)	31 (59.62)	
Invasion				
Tis & T1	17 (16.35)	10 (19.23)	7 (13.46)	0.4716
T2 & T3	21 (20.19)	12 (23.08)	9 (17.31)	
T4	66 (63.46)	30 (57.69)	36 (69.23)	
Lymphatic metastasis				
N0	33 (31.73)	14 (26.92)	19 (36.54)	0.3546
N1 & N2	19 (18.27)	12 (23.08)	7 (13.46)	
N3	52 (50.00)	26 (50.00)	26 (50.00)	
Tumor size (cm)				
<5	32 (30.77)	22 (42.31)	10 (19.23)	**0.0108**
≥5	72 (69.21)	30 (57.69)	42 (80.77)	
Distal metastasis				
M1	91 (87.50)	41 (78.85)	50 (96.15)	**0.0076**
M0	13 (12.50)	11 (21.15)	2 (3.85)	

results showed that both siRNAs effectively downregulated the expression of hsa_circ_0035445 (Figure S2B). After transfection of siRNA, CCK-8 assay showed that cell proliferation was significantly increased (Figure 3); the colony formation assay showed that the colony formation viability of AGS and BGC-823 cells was significantly promoted (Figure 4). Then, the Trans-well assay showed that the migration ability of gastric cancer cells was significantly enhanced (Figure 5). After that, we assessed its effects on cell cycle and apoptosis. The results showed that downregulated expression of hsa_circ_0035445 significantly arrested cells at S phase (Figure S4) and suppressed apoptosis of AGS and BGC-823 cells (Figure S5).

4 | DISCUSSION

Tumors are a major problem that plagues human health and life.[22] As we all know, circRNAs are not easily degraded by RNase R.[23] They are extremely stable and can exist stably in tissues and plasma, so they have become diagnostic markers.[24] For gastric cancer, CEA is the most commonly used screening biomarkers. However, its sensitivity and specificity are not high enough.[25] If gastric cancer can be detected early and treated immediately, its 5-year survival rate can reach more than 90%.[26] Many studies have reported the possibility of circRNAs as diagnostic markers for tumors. For example, hsa_circ_002059, first discovered by our group, may be a diagnostic marker

| Characteristics	n	High (%)	Low (%)	p-Value
All cases	42 (100)	21	21	
Gender				
Male	32 (76.19)	15 (71.43)	17 (80.95)	0.4687
Female	10 (23.81)	6 (28.57)	4 (19.05)	
Age (years)				
<60	9 (21.43)	1 (4.76)	8 (38.10)	**0.0085**
≥60	33 (78.57)	20 (95.24)	13 (61.90)	
CEA				
Positive	38 (90.48)	21 (100)	17 (80.95)	**0.0355**
Negative	4 (9.52)	0 (0)	4 (19.05)	
CA19-9				
Positive	28 (66.67)	11 (52.38)	17 (80.95)	**0.0495**
Negative	14 (33.33)	10 (47.62)	4 (19.05)	
Differentiation				
Well	2 (4.76)	0 (0)	2 (9.52)	0.2997
Moderate-Poor	40 (95.24)	21 (100)	19 (90.48)	
TNM stage				
0 & I	9 (21.43)	5 (23.81)	4 (19.05)	0.3920
II	12 (28.57)	4 (19.05)	8 (38.10)	
III & IV	21 (50.00)	12 (57.14)	9 (42.85)	
Invasion				
Tis & T1	7 (16.67)	4 (19.05)	3 (14.29)	0.2101
T2 & T3	19 (45.24)	16 (76.19)	13 (61.90)	
T4	6 (38.09)	1 (4.76)	5 (23.81)	
Lymphatic metastasis				
N0	14 (33.33)	7 (33.33)	7 (33.33)	**0.0103**
N1 & N2	14 (33.33)	3 (14.29)	11 (52.38)	
N3	14 (33.34)	11 (52.38)	3 (14.29)	
Tumor size (cm)				
<5	10 (23.81)	4 (19.05)	6 (28.57)	0.4687
≥5	32 (76.19)	17 (80.95)	15 (71.43)	
Distal metastasis				
M0	32 (76.19)	17 (80.95)	15 (71.43)	0.4687
M1	10 (23.81)	4 (19.05)	6 (28.57)	

for gastric cancer,[27] and when hsa_circ_002059 was combined with hsa_circ_0000096, the diagnostic value was higher.[21] Another study reported that circ-SFMBT2 was highly expressed in gastric cancer and was related to the clinical stage of gastric cancer, indicating circ-SFMBT2 may be a target for gastric cancer treatment.[28] In addition, scRNA21, a circRNA synthesized in vitro, significantly inhibited the proliferation of gastric cancer cells.[29] It can be seen that circRNAs can not only be used as diagnostic markers but also as therapeutic targets.

The biological functions of circRNAs discovered today are mainly in three aspects: (1) circRNAs function as miRNA "sponges".[30] circRNAs contain different types of miRNA response elements (MREs),[31] and specifically bind to miRNA binding sites to adsorb specific miRNA competitively inhibits the binding of miRNA to the corresponding sites, thereby regulating the expression of downstream target genes.[32] (2) circRNAs regulate the expression of their parent genes in different ways: circRNAs bind to RBPs, which affect the expression of their parent genes.[33] During the formation of circRNAs, their balances with linear RNAs through competitive complementary pairing among introns, which in turn affect mRNA and protein expression.[34] (3) circRNAs may translate to proteins.[6,35] Recently, there has been increasing evidence that circRNAs can encode regulatory peptides as long as they have an internal ribosomal entry site (IRES)[36] and an open reading frame (ORF),[37] and there may be hidden proteins encoded by circRNAs.

FIGURE 3 The growth curves of gastric cancer cells of upregulation (A) or downregulation (B) of hsa_circ_0035445. Data are presented as mean ± SD, NC, negative control. $n = 5$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$
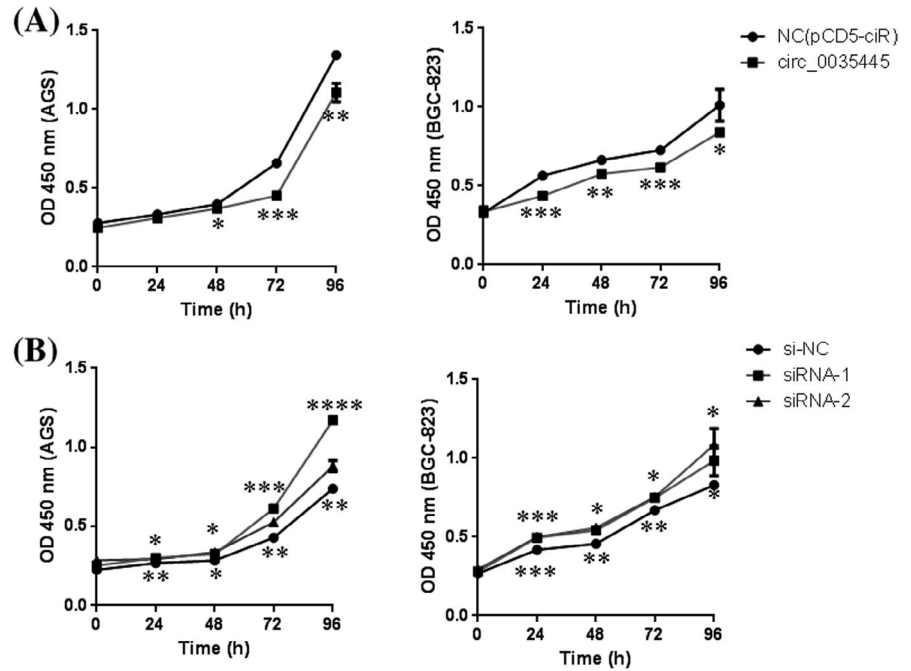
FIGURE 4 Cell cloning ability of gastric cancer cells of upregulation (A) or downregulation (B) of hsa_circ_0035445. Data are presented as mean ± SD, $n=3$, NC, negative control, $***p < 0.001$, $****p < 0.0001$
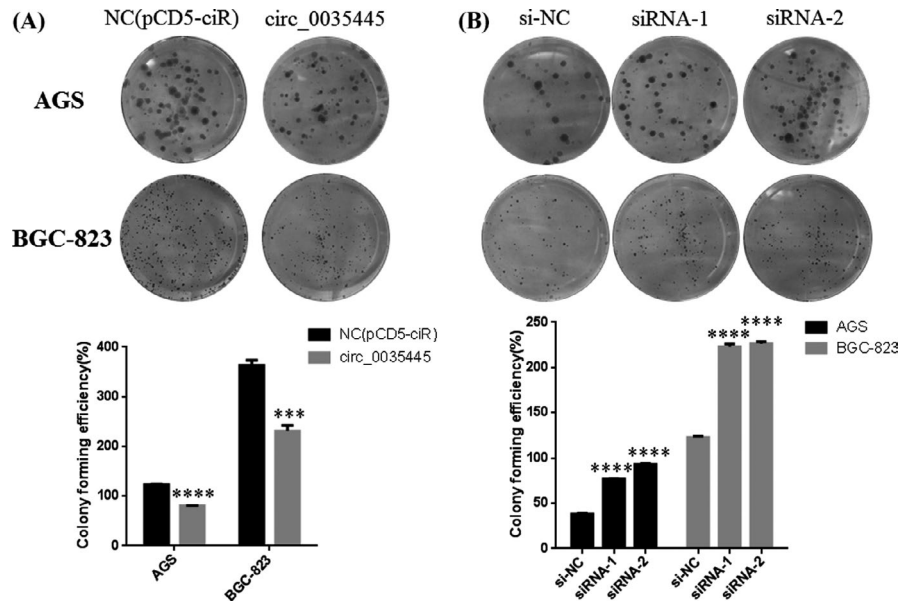

More and more studies have found that circRNAs are related to the occurrence and development of many human diseases and play an important role in the progress of diseases.[38-40] Among them, the circRNAs and malignant tumors are particularly worthy of attention by researchers. In this study, we studied the relationship between hsa_circ_0035445 and gastric cancer.

First, qRT-PCR results indicate that hsa_circ_0035445 was lowly expressed in gastric cancer tissues, plasma, and cell lines (Figures 1 and 2). The AUC of hsa_circ_0035445 in tissues and plasma was 0.68 and 0.86, respectively (Figure 2). The specificity and sensitivity are better than those of CEA. In addition, when the tissue and plasma hsa_circ_0035445 were combined, the AUC can be increased to 0.88 (Figure 2). These indicate that

hsa_circ_0035445 has the potential to become a new diagnostic marker for gastric cancer.

Second, to explore the effects of hsa_circ_0035445 on the biological functions of gastric cancer, we designed overexpression vectors and siRNA to upregulate and downregulate the expression of hsa_circ_0035445, respectively. The results showed that after downregulating the expression of hsa_circ_0035445, the proliferation and migration ability of gastric cancer cells were enhanced (Figure 3); the cell cycle was blocked in S phase (Figure S4), while the apoptosis was decreased (Figure S5). When it was upregulated, the proliferation and migration capacity of gastric cancer cells was reduced (Figures 4 and 5); the cell cycle was blocked at G0/G1 phase (Figure S3), while the apoptosis was increased (Figure S5).
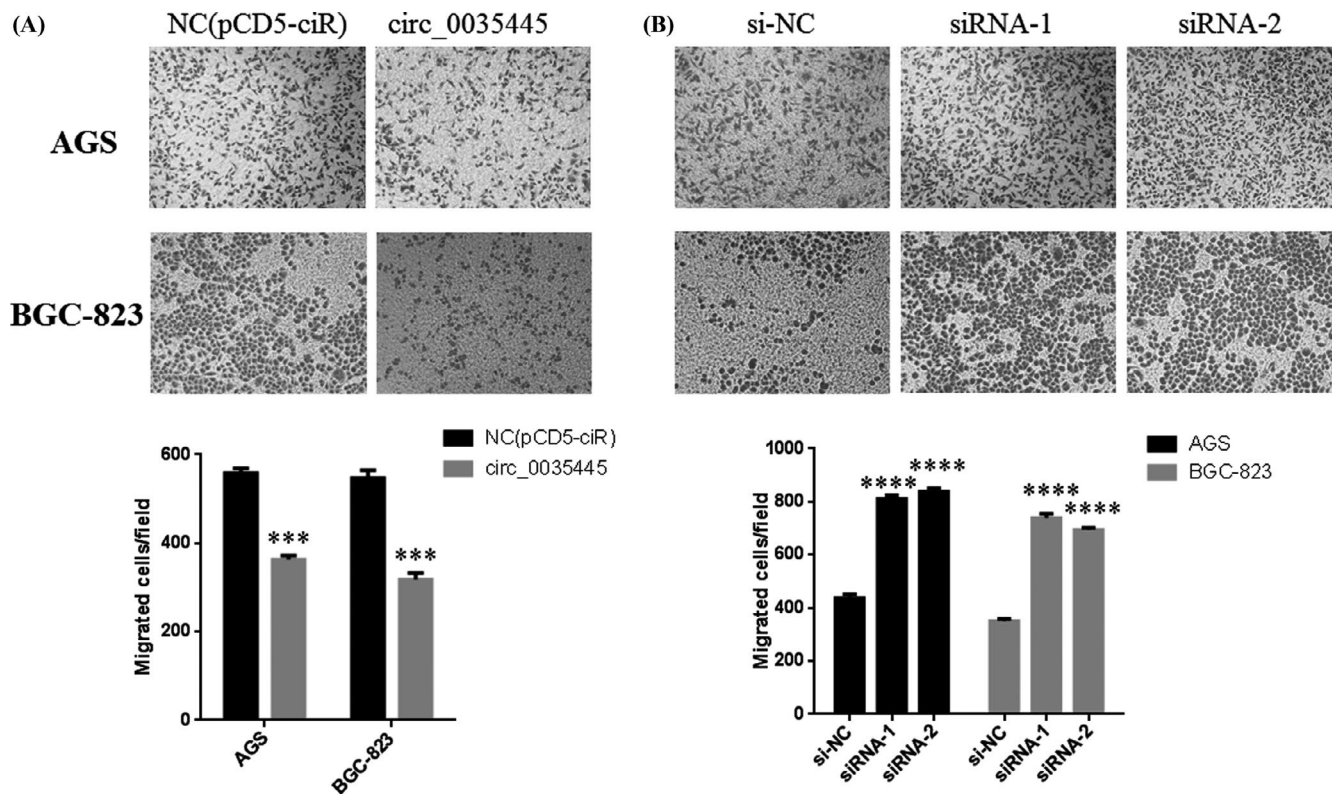
FIGURE 5 Cell migration ability of gastric cancer cells of upregulation (A) or downregulation (B) of hsa_circ_0035445. Data are presented as mean ± SD, $n = 3$, NC, negative control, ***$p < 0.001$, ****$p < 0.0001$

From the above results, it is not difficult to find that hsa_circ_0035445 functions as a tumor suppressor gene, which may provide guidance for the treatment of gastric cancer.

In conclusion, we found that hsa_circ_0035445 may become a new biomarker for clinical diagnosis of gastric cancer and may become a new target for the treatment of gastric cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are included in this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.