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Circular RNA hsa_circ_103809 promotes cell migration and invasion of gastric cancer cells by binding to microRNA-101-3p

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Abstract. – OBJECTIVE: Studies have found that hsa_circ_103809, a newly discovered circRNA in recent years, can serve as an oncogene involved in the progression of hepatocellular carcinoma. However, its role in gastric cancer (GCa) remains elusive. The aim of this study was to reveal the molecular mechanism of hsa_circ_103809 affecting the process of GCa, thus providing new ideas for its treatment.

PATIENTS AND METHODS: Hsa_circ_103809 expression in GCa and adjacent tissues specimens were studied by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis, and its effect on the prognosis of GCa patients was analyzed. In GCa cells lines, hsa_circ_103809 was knocked down by small interfering RNA, and GCa cell metastasis ability was detected by cell wound healing test and transwell assay. Finally, the potential target gene of hsa_circ_103809 was predicted through bioinformatics website and verified by Luciferase assay.

RESULTS: Hsa_circ_103809 showed an increased expression both in GCa tissues and cell lines, predicting a poor prognosis of GCa patients. Meanwhile, the invasive and migration capacities of GCa cells were remarkably reduced after the knockdown of hsa_circ_103809. Bioinformatics website predicted that there existed binding sites of hsa_circ_103809 on microRNA-101-3p, and Luciferase assay verified that hsa_circ_103809 can adsorb microRNA-101-3p. In GCa tissues, qPCR detected a significantly reduced expression of microRNA-101-3p, which was negatively correlated with that of hsa_circ_103809. In addition, the knockdown of hsa_circ_103809 enhanced microRNA-101-3p expression in GCa cell lines. Subsequent *in vitro* experiments further detected that the overexpression of hsa_circ_103809 partially reversed the inhibitory effect of microRNA-101-3p overexpression on GCa cell migration ability and invasiveness.

CONCLUSIONS: Hsa_circ_103809, highly expressed in GCa, may promote the migration capacity of GCa cells by adsorbing microRNA-101-3p and thus become a new therapeutic target for GCa.

Key Words:
GCa, Hsa_circ_103809, MicroRNA-101-3p, Cell migration, Cell invasion.

Introduction

Gastric cancer (GCa) is one of the most common malignant tumors of the digestive system and the third most deadly malignant tumor in the world. There are more than 1 million new cases of GCa diagnosed worldwide every year, and about 783,000 people die of this cancer[1]. Epidemiological data show that men are more likely to develop GCa than women[2]. Although great progress has been made in the diagnosis and treatment of GCa with the continuous improvement of medical level, the overall survival rate of GCa patients is still unsatisfying. In most countries, the 5-year survival rate of GCa patients is less than 30%[3,4]. Therefore, there is a need to further explore the key molecular mechanisms underlying the occurrence of GCa, so as to provide new ideas and methods for the diagnosis and treatment of this cancer.

CircRNAs are a class of endogenous non-coding RNAs (ncRNAs) without protein-coding ability and produced by reverse mRNA splicing (ncRNAs), characterized by covalently closed ring structure[5]. Unlike long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), circRNAs do not have 5 'and 3' ends, showing high stability and sequence conservation in mammalian cells[6]. To date, more than 2000 different circRNAs have been discovered, but the functions of most circRNAs have not been fully clarified[7,8]. CircRNAs play different roles in various human malignancies. Consistently, circ-TADA2A regulates CREB3 expression and promotes the metastasis of osteosarcoma by combining with microRNA-203a-3p[9]. Circ-0001429 prompts the devel-

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opment of bladder cancer by modulating VEGFA expression by binding to microRNA-205-5p[10]. CircRNA-CER can be involved in the progression of breast cancer through microRNA-136/MMP13 pathway[11]. Nevertheless, the mechanism by which hsa_circ_103809 affects GCa development remains to be further investigated.

MiRNAs are endogenous ncRNAs with a short length of about 18-25 nucleotides that, by binding to the 3'-non-translational region (3'-UTR) of the target gene, regulate gene expression at the post-transcriptional level, promote mRNA degradation, and inhibit its translation[12,13]. Each miRNA precursor can be cleaved into two molecules, namely microRNA-5p and microRNA-3p, which have different recognition regions and play different functions[14]. They play an essential part in cell growth, proliferation, apoptosis, differentiation, and other physiological processes, and thus participate in the progression of various tumors[15,16]. Downregulating microRNA-205 can inhibit the proliferation and migration of hepatocyte cancer cells through targeted binding of vascular endothelial growth factor A[17]. MicroRNA-218 is capable of suppressing the proliferation and epithelial mesenchymal transformation of prostate cancer cells[18]. MicroRNA-503 enhances the invasiveness of colorectal cancer cells by modulating PDCD4[19]. However, the role of microRNA-101-3p in GCa remains unclear and needs further exploration.

It was speculated that hsa_circ_103809 might act as an oncogene in GCa as hsa_circ_103809 was highly expressed in GCa tissues and cell lines. The potential biological function and molecular mechanism of hsa_circ_103809 were investigated through a series of *in vitro* experiments in this study.

Patients and Methods

Sample Collection

A total of 60 pairs of postoperative tumor tissues and adjacent normal tissues from GCa patients in Dongying District Hospital were selected, and all tissues were confirmed by pathology. In this study, none of enrolled subjects were preoperatively treated with anti-tumor therapy. In addition, the tumor pathological classification and staging criteria of GCa were in accordance with the Union for International Cancer Control (UICC) Staging standard implementation. All participants in the study signed informed consent, and all research protocols were approved by the Ethics Committee of Dongying District Hospital.

Cell Culture

Human normal gastric mucosal epithelial cells (GES-1) and human GCa cell lines (AGS, MKN-45, SNU5, MK-2) provided by the Shanghai Institute of Cell Biology (Shanghai, China) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin and streptomycin (Lonza, Morristown, NJ, USA) in a cell incubator with 5% CO_2 at 37°C.

Cell Transfection

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was mixed with si-hsa_circ_103809 and microRNA-101-3p mimics and then added into cells when cell density reached to 60%. 48 hours later, the cells were collected for analysis.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Experiment

RNA extraction was performed by TRIzol method (Invitrogen, Carlsbad, CA, USA), and the extracted total RNA was reversely transcribed using a reverse transcription kit (TaKaRa, Dalian, China) according to the product manual. Then, SYBR Green Master reagent (Roche, Basel, Switzerland) was used for qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 are used as internal references. The primer sequence used were as follows: Hsa_circ_103809 Forward: 5′-AAAGAGTTTTTGGAAGACGTC-3′ and Reverse: 5′-TGGTGGCATGTTTTGTCATT-3′, microRNA-101-3p Forward: 5′-GCCGCCACCATGGTGAGCAAGG-3′ and Reverse: 5′-AATTGAAAAAAGTGATTTAATTT-3′, GAPDH Forward: 5′-TATGATGATATCAAGAGGGTAGT-3′ and Reverse: 5′-TGTATCCAAACTCATTGTCATAC-3 and U6 Forward: 5′-C8TCGCTTCGGCAGCACATA-3′ and Reverse: 5′-AACGATTCACGAATTTGCGT-3′.

Cell Wound Healing Test

When cells adhered to more than 80%, a single layer of cells was scratched with the tip of a 10 μL pipette, causing scratches. After rinsing, the 6-well plate was observed under a microscope and images were collected for quantitative analysis using Image-Pro Plus 6.0 software (Media Cybernetics, lnc. Silver Springs, MD, USA).

Transwell Experiment

Cell migration or invasion was tested using a 24-well plate cell pre-coated or not coated with matrix gel according to the manufacturer's instructions.

Luciferase Assay

Hsa_circ_103809-wt or hsa_circ_103809-mut and microRNA-101-3p mimics or microRNA-101-3p-NC were co-transfected into GCa cells using Lipofectamine 2000. 24 hours later, the Luciferase activity of each group was measured using a Luciferase reporting kit (Genecopoaie, Guangzhou, China).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp., Armonk, NY, USA) software, and graphs were plotted using GraphPad Prism (La Jolla, CA, USA). A paired *t*-test was used to compare the two sets of data. The correlation of hsa_circ_103809 and microRNA-101-3p expression levels was analyzed using linear regression. *p* less than 0.05 was statistically significant.

Results

Abnormally Increased Expression of hsa_circ_103809 In GCa

To search for the molecular mechanism of hsa_circ_103809 in GCa, tissue samples were collected from GCa patients, and it was found that hsa_circ_103809 was remarkably increased in tumor samples in comparison to the control ones (Figure 1A). Meanwhile, those patients in advanced stage had a higher expression of hsa_circ_103809 (Figure 1B). Consistently, qPCR detection revealed that hsa_circ_103809 was also highly expressed in human GCa cells as compared to GES-1 cells (Figure 1C). In addition, it could be seen from Figure 1D that the relative expression of hsa_circ_103809 was negatively correlated with the overall survival rate of GCa patients, indicating that hsa_circ_103809 may act as an oncogene in GCa.

Knockdown of Hsa_circ_103809 Suppressed the Migration and Invasiveness of GCa Cells

Two GCa cell lines (MKN45 and SNU5) with relatively high expression levels were selected for subsequent *in vitro* experiments. They were trans-

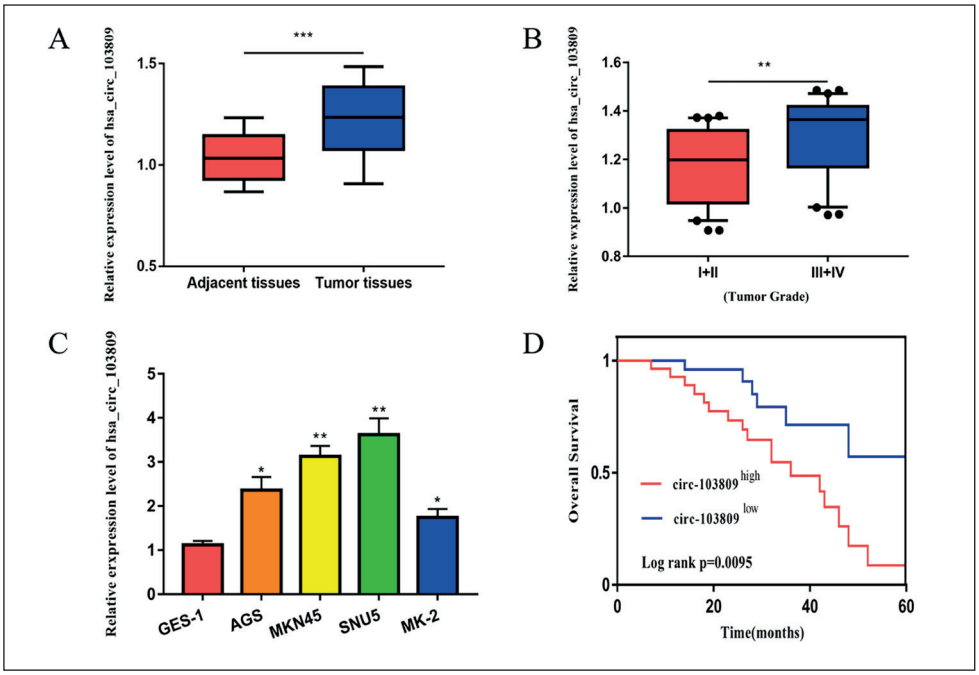

Figure 1. Hsa_circ_103809 was abnormally highly expressed in gastric cancer. **A,** The relative expression level of hsa_circ_103809 in gastric cancer tissues and normal control tissues was detected by qRT-PCR. **B,** Analysis of the correlation between the relative expression level of hsa_circ_103809 and the TNM stage of gastric cancer patients. **C,** The relative expression level of hsa_circ_103809 in gastric cancer cells and normal control cells was expressed by qRT-PCR. **D,** Analysis of the correlation between the relative expression level of hsa_circ_103809 and the overall survival rate of gastric cancer patients. **p*<0.05, ***p*<0.01.

fected with si-hsa_circ_103809 and negative control si-NC, and their expressions were detected using qPCR (Figure 2A). Next, the results of cell wound healing assay and transwell experiments revealed that the knockdown of hsa_circ_103809 markedly inhibited the healing ability and migration capacity of MKN45 and SNU5 cells (Figure 2B, 2C, 2D).

Hsa_circ_103809 Combined With MicroRNA-101-3p

Using the bioinformatics database CircInteractome (https://circinteractome.nia.nih.gov) to screen for miRNAs that may be bound by hsa_circ_103809, the highest binding score was found between hsa_circ_103809 and microRNA-101-3p (Figure 3A). Later, Luciferase assay indicated that microRNA-101-3p mimics markedly attenuated the Luciferase activity in hsa_circ_103809-wt group without affecting that in hsa_circ_103809-mut group (Figure 3B). Therefore, microRNA-101-3p level in GCa tissue samples was detected, and it was found to be remarkably reduced (Figure 3C). At the same time, hsa_circ_103809 and microRNA-101-3p showed a negative correlation in GCa tissues, measured by linear regression analysis (Figure 3D). In addition, Figure 3E showed that knocking down hsa_circ_103809 remarkably elevated microRNA-101-3p expression *in vitro*.

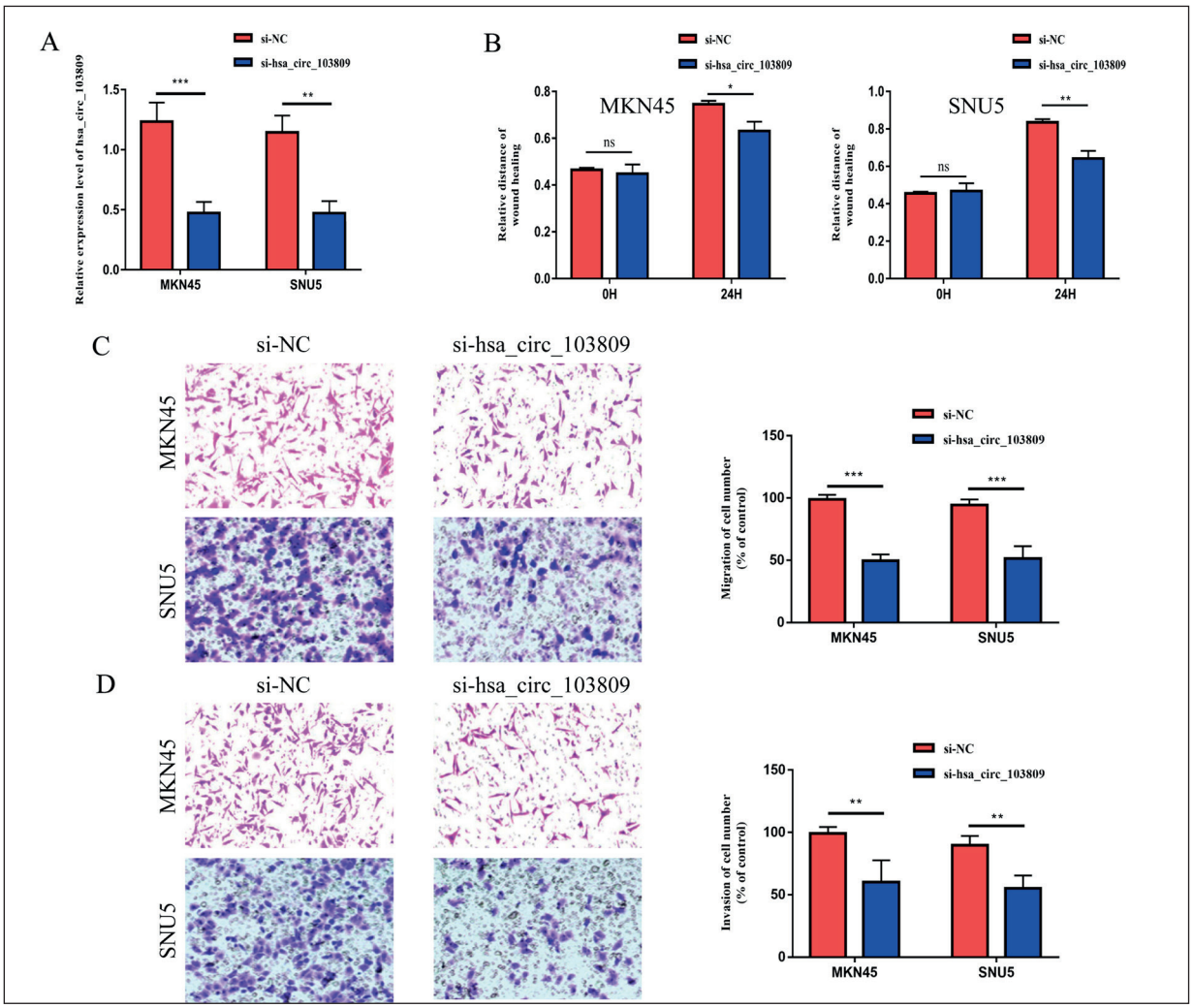

Figure 2. Downregulation of hsa_circ_103809 could inhibit the proliferation, migration and invasion of gastric cancer cells. **A,** The expression of hsa_circ_103809 in MKN45 and SNU5 cells transfected with si-NC and si-hsa_circ_103809 was detected by qRT-PCR. **B,** The effect of hsa_circ_103809 on the migration ability of MKN45 and SNU5 cells was indicated by a cell wound healing assay. **C,** The effect of hsa_circ_103809 on the migration of MKN45 and SNU5 cells was revealed by the transwell migration experiment (magnification: 200x). **D,** The effect of hsa_circ_103809 on the invasion of MKN45 and SNU5 cells was detected by transwell invasion experiments (magnification: 200×). *p<0.05, **p<0.01, ***p<0.001, ns: no significant difference.

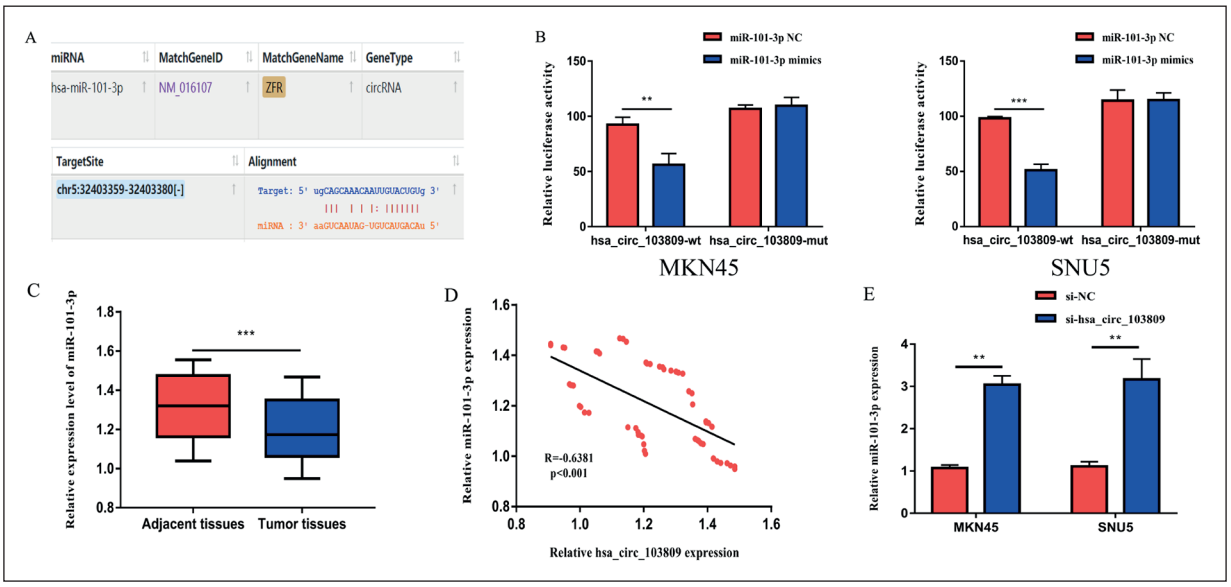

Figure 3. Hsa_circ_103809 was able to adsorb miR-101-3p. **A,** MiRNAs having binding sites on hsa_circ_103809 and miR-101-3p predicted by the bioinformatics website (http://starbase.sysu.edu.cn) were selected. **B,** The binding relationship between hsa_circ_103809 and miR-101-3p was detected by a dual luciferase reporter gene experiment. **C,** The expression of miR-101-3p in gastric cancer tissues and adjacent normal tissues was showed by qRT-PCR. **D,** Analysis of the correlation between hsa_circ_103809 and miR-101-3p expression levels in gastric cancer tissues. **E,** The expression of miR-101-3p in MKN45 and SNU5 cells transfected with si-NC and si-hsa_circ_103809 was revealed by qRT-PCR. $^{**}p<0.01$, $^{***}p<0.001$.

Overexpression of hsa_circ_103809 Could Partially Reverse the Inhibitory Effect of MicroRNA-101-3p Overexpression on GCa Cell Metastasis

To further clarify the role of the hsa_circ_103809/microRNA-101-3p axis in GCa, hsa_circ_103809 was overexpressed in MKN45 and SNU5 cells transfected with microRNA-101-3p mimics, and it was found that the overexpression of hsa_circ_103809 partially reversed the enhanced expression of microRNA-101-3p induced by its mimics (Figure 4A). Then, a series of *in vitro* cell function experiments were performed to verify the effects of simultaneous overexpression of microRNA-101-3p and hsa_circ_103809 on cell migration and invasion. Figure 4B-4D showed that the overexpression of hsa_circ_103809 reversed the inhibitory influence of microRNA-101-3p mimics on the migration and invasiveness of GCa cells.

Discussion

GCa is a heterogeneous and multi-factor disease, which endangers human physical and mental health and brings great pressure to human health and social economy[20]. Many risk factors, such as excessive salt intake, alcohol consumption, smoking, infection, heredity, etc. may be involved in the pathogenesis of this cancer[21]. At present, surgery is still the main treatment for patients in early stage, and postoperative adjuvant chemotherapy and other comprehensive treatments can be adopted to improve the prognosis of patients, but the overall prognosis of GCa is not optimistic[22]. Moreover, since the symptoms of early GCa are usually insidious and non-specific, most patients with GCa have progressed to late stage when a definite diagnosis is made[23]. Therefore, the purpose of this study was to provide some new ideas for the diagnosis and treatment of GCa.

Before 2012, circRNAs were considered to be the product of transcriptional errors. However, with the development of sequencing and bioinformatics, Salzman et al[24] conducted systematic and comprehensive studies on the characteristics of gene expression of circRNAs in human cells, making them widely used in the field of non-coding RNAs. A great number of studies have showed the potential clinical value of circRNAs in the diagnosis, treatment and prognosis of GCa. Notably, circ-0009910 can regulate the proliferation and migration of GCa and predict poor prognosis[25]. Circ_0027599/PHDLA1 inhib-

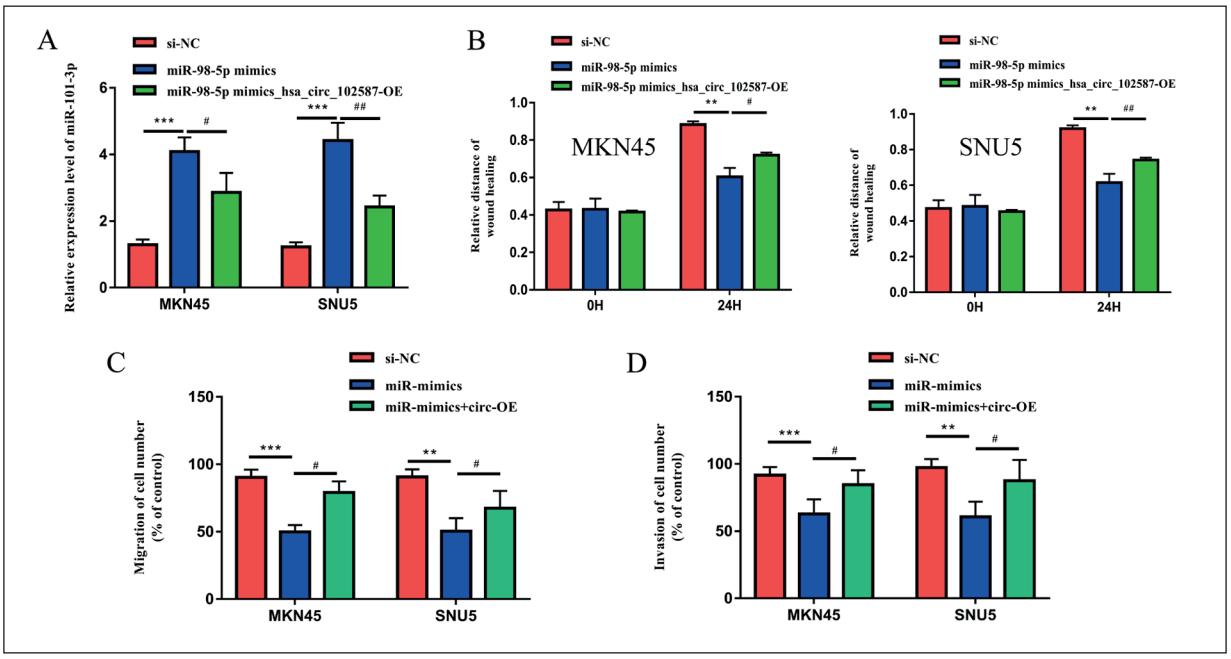

Figure 4. Overexpression of hsa_circ_103809 could partially reverse the inhibitory effect of miR-101-3p overexpression on gastric cancer cell migration and invasion. **A,** miR-101-3p expression was detected by qRT-PCR after simultaneous over-expression of miR-101-3p and hsa_circ_103809 in MKN45 and SNU5 cells. **B,** Cell migration ability was indicated by cell wound healing test after simultaneous over-expression of miR-101-3p and hsa_circ_103809 in MKN45 and SNU5 cells. **C,** Cell migration ability was determined by transwell migration experiments after simultaneous over-expression of miR-101-3p and hsa_circ_103809 in MKN45 and SNU5 cells. **D,** Transwell invasion assay was used to detect the effects of miR-101-3p and hsa_circ_103809 on cell invasion. **p<0.01, ***p<0.001, #p<0.01, ##p<0.001.

its the progression of GCa by adsorption of microRNA-101-3p[26]. Hsa_circ_0001368 inhibits the progression of GCa through the mir6505-5p/Fox O3 axis[27]. Thus, circRNAs may play a role as oncogenes or tumor suppressor genes in GCa. Hsa_circ_103809, located on chromosome 5p13.3 with a length of 693 nucleotides, is composed of 5 exons of the ZFR gene[28], and it is capable of prompting the progression of lung cancer by regulating the expression of ZNF121 through the adsorption of microRNA-4302[28]. In colorectal cancer, hsa_circ_103809 regulates the proliferation and migration of colorectal cancer cells through microRNA-532e3p /FOXO4 axis[29]. This study revealed a significantly increased expression of hsa_circ_103809 in GCa tissues and cell lines, and indicated the cancer-promoting role of hsa_circ_103809 in GCa through a series of *in vitro* experiments.

By searching for bioinformatics website, it was found that microRNA-101-3p could bind to hsa_circ_103809, and this binding relationship was detected through Luciferase assay. MicroRNA-101-3p plays a critical role in human nausea tumors, such as hepatocellular carcinoma[30], lung squamous cell carcinoma[31], breast cancer[32], and in the progression of GCa. LINC01303, as a competitive binding between ceRNA and microRNA-101-3p, can be engaged in the progression of GCa through the modulation of EZH2[33]. LncRNA SNHG6 promotes the proliferation and epithelial mesenchymal transformation of GCa cells through epigenetic silencing of p27 gene and adsorption of microRNA-101-3p[34]. MicroRNA-101-3p inhibits HOTAIR-induced proliferation and invasion of GCa cells by targeting SRF[35]. However, the binding association between hsa_circ_103809 and microRNA-101-3p has not been fully detected. In this investigation, linear analysis showed that the expression of the above two genes was negatively correlated and microRNA-101-3p may serve as a tumor suppressor gene in GCa. Moreover, *in vitro* overexpression of hsa_circ_103809 partially can partially reverse the inhibitory effect of overexpression of microRNA-101-3p on invasion of GCa cells. Therefore, hsa_circ_103809 might function as a ceRNA to exert a sponge effect on microRNA-101-3p, thus

promoting the malignant progression of GC. This study is expected to provide a new perspective for the occurrence and development mechanism of GC, and provide new ideas for the clinical diagnosis and treatment of GC.

Conclusions

In summary, this study found that hsa_circ_103809, highly expressed in GCa tissues, can be used as a marker for predicting a poor prognosis of GCa patients. In addition, hsa_circ_103809 is able to accelerate the migration of GCa cells by binding to microRNA-101-3p. It is suggested that hsa_circ_103809 may become a new target for the treatment of GCa and provide a new strategy for the clinical diagnosis of GCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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