ABSTRACT

Purpose: Circular RNAs (circRNAs) are a new class of RNA molecules whose function is largely unknown. There is a growing evidence that circRNAs play an important regulatory role in the progression of a variety of human cancers. However, the exact roles and the mechanisms of circRNAs in gastric cancer are not clear. In this study, we aimed to elucidate the mechanism of hsa_circ_0005556.

Materials and Methods: Real-time quantitative polymerase chain reaction was used to detect the expression of hsa_circ_0005556, miR-4270, and matrix metalloproteinase-19 (MMP19) in gastric cancer tissues and cell lines. The expression of hsa_circ_0005556 in gastric cancer cells was silenced by lentivirus, and cell proliferation, invasion, migration, and tumorigenesis in nude mice were assessed to evaluate the function of hsa_circ_0005556 in gastric cancer.

Results: The expression of hsa_circ_0005556 in gastric cancer tissues and gastric cancer cell lines was higher compared to normal controls. In vitro, the downregulation of hsa_circ_0005556 significantly inhibited proliferation, migration, and invasion of gastric cancer cells. In vivo, the downregulation of hsa_circ_0005556 suppressed tumor growth in nude mice.

Conclusions: Our study shows that the hsa_circ_0005556/miR-4270/MMP19 axis is involved in proliferation, migration, and invasion of gastric cancer cells through the competing endogenous RNA (ceRNA) mechanism.

Keywords: CircRNAs; MMP19; miRNA sponge; Gastric cancer

INTRODUCTION

Gastric cancer is 1 of the 5 most common cancers worldwide and the third leading cause of cancer-related deaths [1]. Although much progress has been made in the surgical treatment, chemotherapy, and molecular targeted therapy for gastric cancer, the prognosis for patients with this type of cancer is often poor, and the 5-year survival rate in most countries is still less than 30% [2]. Therefore, it is very important to find effective molecular diagnostic markers and novel therapeutic targets by elucidating the molecular mechanisms involved in gastric cancer occurrence and development.
Circular RNAs (circRNAs) have a closed ring structure formed by covalent bonds at the 5’ and 3’ ends of linear RNA molecules and are not easily degraded by exonuclease [3]. CircRNAs are abundant in cancer tissues and cell lines, and their expression level is stable and conserved [4]. It has been reported that circRNAs play an important role in regulating gene expression in eukaryotic cells [5,6]. For example, it was found that circ-Donson was highly expressed in gastric cancer tissues, and silencing this circRNA significantly inhibited proliferation, migration, and invasion of gastric cancer cells [7]. Other studies have shown that overexpression of circPSMC3 inhibited the tumorigenicity of gastric cancer cells in vivo and in vitro and adsorbed miR-296-5p to regulate the expression of phosphatase and tensin homolog [8]. Furthermore, circOSBPL10 regulated the biological functions of gastric cancer cells through the miR-136-5p/Wnt2 axis in vitro and promoted tumor growth and metastasis in nude mice in vivo [9]. These results confirm that circRNAs play an important regulatory role in the occurrence and development of gastric cancer.

Previous studies have used circRNA microarray sequencing to show that the expression of hsa_circ_0005556 in gastric cancer tissues is higher compared to the parancancerous tissues [10]. In this study, we present evidence that hsa_circ_0005556 is highly expressed in gastric cancer tissues and cell lines. The downregulation of hsa_circ_0005556 weakens the carcinogenic effect by targeting the miR-4270/matrix metalloproteinase-19 (MMP19) axis.

**MATERIALS AND METHODS**

**Clinical sample collection and cell culture**

Gastric cancer tissues were collected from 32 patients who underwent surgical resection without chemoradiotherapy at the Second Hospital of Nanjing, China. The specimens were immediately snap-frozen and stored at –80°C in an ultralow temperature refrigerator until further analysis. The diagnosis of gastric adenocarcinoma for all patients was confirmed by pathology. Every patient provided prior written and informed consent, and the study (No. 2016-LS-ky014) was approved by the Ethics Review Board of the Second Hospital. Human gastric cancer cell lines (HGC-27, MKN-28 and MGC-803) and a normal gastric epithelium cell line (GES-1) were obtained from the Cell Bank of Chinese Academy of Medical Science (Shanghai, China). All cell lines were maintained in RPMI-1640 medium (Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 µg/ml streptomycin, and 100 U/mL penicillin (Gibco), and were cultured in a humidified atmosphere with 5% CO₂ in a 37°C incubator.

**RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. qRT-PCR for circ_0005556 and MMP19 was performed using the StepOne™ Real-Time PCR System and the SYBR® Green Mixture (Takara, Shanghai, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. qRT-PCR for miR-4270 was performed using a TaqMan MicroRNA Assays Kit (Applied Biosystems, Carlsbad, CA, USA). The relative expression of miR-4270 was normalized to U6. The primers were purchased from RiboBio (Guangzhou, China). The primer sequences were as follows: hsa_circ_0005556 (forward 5’-ACACCTTTTTTGCTCCCTGATG-3’ and reverse 5’-TTGTTGCCCTAGGCTGATT-3’), MMP19 (forward 5’-CTTCAGCAGCTACCCCAAC-3’ and reverse 5’-CCGTACCTGAGGGATGGTA-3’), and GAPDH (forward 5’-TCTCTGCTCCT-CCTGTTT-3’, and reverse 5’-GGTGTGACACAGGGTACTTTATGA-3’).
Transgenic transfections and the construction of stable cell lines
Small interference RNAs (siRNAs) targeting hsa_circ_0005556, the negative control, and the lentivirus were constructed by GenePharma (Shanghai, China). MiR-4270 mimic, inhibitor, and the relevant negative control were purchased from RiboBio. All the vectors were transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. After transfecting for 48 hours, the cells in each group were analyzed by qRT-PCR. The sequences for the siRNA hsa_circ_0005556 were as follows: siRNA-1: 5′-UGUGUGAAUAUCAGCUAGTT-3′; siRNA-2: 5′-GUGGAAUAUCAGCUCAGTT-3′; siRNA-3: 5′-GAAUCAGCCUA-GAGGTT-3′.

Cell proliferation assays
Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) was used to evaluate the cell proliferation. MGC-803 and MKN-28 cells in different groups were seeded into 96-well plates at 5,000 cells per well in quadruplicate and incubated with serum-free RPMI 1640. For the clonogenic assay, 500 cells were seeded into 6-well plates and cultured for 14 days. The colonies on the plates were fixed with 4% paraformaldehyde for 30 minutes, stained with 0.1% crystal violet, and the number of colonies was counted.

Cell migration and invasion assays
Transwell chambers were used for cell migration and invasion assays. After 24 hours of incubation, the cells on the upper side of the Transwell chamber were removed with cotton swabs. The cells on the lower side were then fixed with 4% paraformaldehyde for 30 minutes and stained with crystal violet for 15 minutes. In the invasion assay, cells were plated in the upper chamber of an insert coated with 50-µL Matrigel. To quantify cell invasion, images from 5 random fields of view were obtained.

Flow cytometry
For cell cycle analysis, the cells were washed with pre-chilled phosphate buffered saline (PBS) and fixed with 70% ice-cold ethanol overnight at 4°C. Next, the cells were stained with propidium iodide (PI) (Beyotime, Shanghai China) for 30 minutes in a 37°C incubator and protected from light before the flow cytometric analysis.

Western blotting
Total protein from gastric cancer cells and tumor tissues was extracted according to standard procedures (Beyotime, Shanghai, China) [11]. The primary antibodies used to detect the proteins of interest were anti-MMP19 (1:1,000, Abcam, Cambridge, UK), anti-E-cadherin (1:2,000, ProteinTech, Rosemont, IL, USA), anti-N-cadherin (1:2,000, ProteinTech), anti-Vimentin (1:2,000, ProteinTech), anti-GAPDH (1:20,000, ProteinTech), and anti-β-actin (1:1,000, Zoonbio Biotechnology, Nanjing, China). Proteins were separated on a 10% sodium dodecyl sulfate polyacrylamide gel, transferred to the polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), and then blocked in Tris-Buffered Saline, 0.1% Tween 20 (TBST) containing 5% milk. The membranes were incubated with specific primary antibodies in dilution buffer overnight at 4°C. Next, the membranes were washed with TBST and then incubated with goat-anti-rabbit immunoglobulin G (IgG) or goat-anti-mouse IgG (1:4,000) for 1 hour at room temperature. Specific protein expression levels were detected using ECL reagent (Thermo Fisher Scientific).
**Target prediction**
The miRNAs that interact with hsa_circ_0005556 were identified using TargetScan, miRanda and RNAhybrid predicting tools. The target gene of miR-4270 was predicted using TargetScan and miRanda.

**Dual-luciferase reporter assay**
The fragments of hsa_circ_0005556 and MMP19 containing the miR-4270 binding sites were synthesized by RiboBio and named WT-hsa_circ_0005556 and WT-MMP19. The corresponding mutated fragments, MUT-hsa_circ_0005556 and MUT-MMP19, were also synthesized. These wild type and mutated type fragments were subcloned into psi-CHECK-2 vector (Promega, China). HEK-293T cells (1×10^5 cells/well) were inoculated into 24-well plates and then co-transfected with miR-4270 mimic/mimic NC and WT-hsa_circ_0005556/MUT-hsa_circ_0005556 or WT-MMP19/MUT-MMP19. The firefly and Renilla luciferase activities were measured by dual-luciferase reporter assay (Promega, Madison, WI, USA) 48 hours after transfection. The ratio of the firefly luciferase activity to the Renilla luciferase activity was defined as the luciferase activity.

**In vivo tumor formation assay**
Male nude mice (BALB/c nude mice), 4–6 weeks old, were purchased from Slac Laboratories (Shanghai, China). The lentiviral vector carrying short hairpin RNA for hsa_circ_0005556 (sh-circ_0005556) or negative control (sh-NC) was generated by GenePharma and transfected into MGC-803 cells. MGC-803 cells stably transfected with sh-circ_0005556 or sh-NC were implanted by the subcutaneous injection of 4×10^6 cells in 100 μL of PBS into the flanks of mice to induce tumors. The size of the tumor was measured every 5 days, and the tumor volume was calculated by the following formula: (length×width^2)/2. The mice were sacrificed after 25 days and the tumors were harvested. Care of experimental animals was performed in accordance with the Southeast University Institutional Animal Care and Use Committee protocols.

**Statistical analysis**
All experimental data analyses were performed using SPSS 22.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). All data are expressed as the mean±standard deviation (SD) from at least three independent experiments. The in vitro and in vivo data were analyzed by independent samples t-test and 1-way analysis of variance. The expression differences between gastric cancer and its matched normal tissues were determined using paired sample t-test. A P-value <0.05 was considered significant for all statistical comparisons.

**RESULTS**

**Hsa_circ_0005556 is upregulated in gastric cancer tissues and cells**
To measure the expression of hsa_circ_0005556 in clinical gastric cancer tissue samples and cell lines, qRT-PCR was performed. We found that the levels of hsa_circ_0005556 were significantly higher in gastric cancer tissues (Fig. 1A). Furthermore, the expression of hsa_circ_0005556 was also increased in all tumor cell lines, namely, HGC-27, MGC-803, and MKN-28, compared to the normal gastric mucosal epithelial cell line (GES-1) (Fig. 1B).
Hsa_circ_0005556 facilitates the proliferation of gastric cancer cells

To investigate the role of hsa_circ_0005556 in the proliferation of gastric cancer cells, MGC803 and MKN28 cell lines were selected for the knockdown study. According to qRT-PCR results, si-hsa_circ_0005556-2 was selected for these experiments because it had a higher knockdown efficiency compared to si-hsa_circ_0005556-1 and si-hsa_circ_0005556-3 (Fig. 2A). The lentiviral vector carrying short hairpin RNA for hsa_circ_0005556 (sh-circ-2) or negative control (sh-NC) was generated by GenePharma and transfected into MGC-803 and MKN-28 cells. The Cell Counting Kit-8 (CCK-8) assay showed that the cell proliferation ability suppressed in MGC-803 and MKN-28 cells with hsa_circ_0005556 knockdown (Fig. 2B). The clonogenic assay showed that the number of clones in the hsa_circ_0005556-knockdown group was decreased (Fig. 2C). Flow cytometric analysis demonstrated that hsa_circ_0005556 silencing arrested the cell cycle in the G2/M phase (Fig. 2D). The wound closure assay showed that after 24 hours, the relative migration areas of MGC803 cells and MKN28 cells in the sh-hsa_circ_0005556-2 group were significantly decreased compared to the sh-NC group (Fig. 2E). The Transwell assay demonstrated that hsa_circ_0005556 knockdown inhibited the migration and invasion of gastric cancer cells compared to the control group (Fig. 2F).

In addition, we detected the epithelial-mesenchymal transition (EMT) related proteins by western blotting. Interestingly, we found that the downregulation of hsa_circ_0005556 increased the levels of E-cadherin and decreased the protein expression of N-cadherin and vimentin (Fig. 2G). These results suggest that hsa_circ_0005556 knockdown inhibits proliferation and the metastatic potential of gastric cancer cells.

Hsa_circ_0005556 targets miR-4270 as a miRNA sponge

TargetScan, miRanda, and RNAhybrid predicted that the miRNAs hsa-miR-4270, hsa-miR-4741, and hsa-miR-767-5p could be sponged by hsa_circ_0005556. The expression changes of miRNAs in response to hsa_circ_0005556 downregulation in MGC-803 and MKN28 cells were detected by qRT-PCR. The results showed that the expression of hsa-miR-4270 was the most affected by hsa_circ_0005556 (Fig. 3A). Furthermore, we identified complementary binding sites between miR-4270 and hsa_circ_0005556 (Fig. 3B). Luciferase activity assay confirmed the molecular binding interaction between hsa_circ_0005556 and miR-4270 (Fig. 3C). In gastric cancer tissues, the levels of miR-4270 were significantly
decreased (Fig. 3D). These results showed that hsa_circ_0005556 acted as a miRNA sponge for miRNA-4270.
Circ_0005556 Regulates miR-4270/MMP19 Axis

To demonstrate that hsa_circ_0005556 enhanced the progression of gastric cancer through miR-4270, we downregulated the expression of miR-4270 in MGC-803 and MKN-28 sh-circ_0005556 cells (Fig. 3E) and carried out a series of rescue experiments. Using CCK-8 and transwell assays, we confirmed that inhibition of miR-4270 could reverse the inhibitory effects of hsa_circ_0005556 knockdown on the proliferation and invasion of gastric cancer cells (Fig. 3F and G). Overall, the results suggested that the hsa_circ_0005556/miR-4270 axis could play a role in the progression of gastric cancer.

**MMP19 serves as the functional protein of hsa_circ_0005556/miR-4270**

Next, we investigated the downstream targets of hsa_circ_0005556 and miR-4270. The results showed that the MMP19 3′-untranslated region (UTR) contained complementary binding sites to miR-4270 (Fig. 4A). The luciferase activity assay confirmed that MMP19 mRNA bound to miR-4270 (Fig. 4B). The expression of MMP19 in the gastric cancer samples was higher compared to the normal tissues according to the The Cancer Genome Atlas (TCGA) sample analysis (Fig. 4C). qRT-PCR revealed that the expression of MMP19 was decreased in the miR-4270 upregulated group (Fig. 4D). Western blot analysis showed that the upregulation of miR-4270 silenced the expression of MMP19 (Fig. 4E). These results indicate that MMP19 acts as a functional protein of hsa_circ_0005556/miR-4270.

**Hsa_circ_0005556 knockdown suppresses gastric cancer growth in vivo**

To confirm our findings, next, we explored the effect of hsa_circ_0005556 on tumor progression in vivo. Stably downregulated hsa_circ_0005556 MGC-803 cells and their
Fig. 3. Hsa_circ_0005556 targets miR-4270 as a miRNA sponge. (A) Relative miRNA expression was measured after the transfection of MGC-803 cells. (B) Schematic representation of the binding sites for hsa_circ_0005556 and miR-4270. (C) The luciferase reporter assay was performed to evaluate the interaction between miR-4270 and hsa_circ_0005556. (D) The expression of miR-4270 in gastric cancer and para-carcinoma tissues was measured using qRT-PCR. (E) The expression of miR-4270 was measured using qRT-PCR in different groups. (F) The proliferation of MGC-803 and MKN-28 cells was tested by CCK-8 assay in different groups. (G) Cell invasion was measured in MGC-803 and MKN-28 cells after transfection (100×).

qRT-PCR = quantitative real-time polymerase chain reaction; CCK-8 = Cell Counting Kit-8; NC = negative control.

*P<0.05 and †P<0.01.
corresponding negative control cells were subcutaneously injected into nude mice (n=5 per group). Mice were sacrificed on day 25 after the inoculation and the xenograft tumors were extracted (Fig. 5A). Tumor sizes and weights were reduced in sh-circ_0005556 group compared to the sh-NC group (Fig. 5B and C). Moreover, hsa_circ_0005556 and MMP19 levels were markedly reduced, while miR-4270 levels were enhanced in tumor tissues from the sh-circ_0005556 group compared to the sh-NC group (Fig. 5D and E). In summary, these results confirmed that hsa_circ_0005556 is effective in promoting gastric cancer in vivo.

**DISCUSSION**

In recent years, with the rapid development of bioinformatics technology and high-throughput sequencing, circRNAs have become a hot topic in the field of tumor research. CircRNAs can function as a miRNA sponge and RNA binding protein sponge, interfere with splicing, regulate transcription, and even translate peptides [12-14]. There is growing evidence that circRNAs play an important role in the occurrence and development of human cancer [15-17].

Previous studies have shown by the circRNA microarray analysis that the expression of hsa_circ_0005556 in gastric cancer tissues and plasma was upregulated compared to normal controls [10,18]. The location of the hsa_circ_0005556 gene is chr2:15693549-15698758 (gene name: NBAS), and the splice length is 218 bp. However, the exact roles of this specific circRNA

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have not been investigated. In this study, qRT-PCR analysis confirmed that hsa_circ_0005556 was highly expressed in gastric cancer tissues and cell lines. In gastric cancer cells, knocking down the expression of hsa_circ_0005556 inhibited cell proliferation, invasion, and migration. Furthermore, dual luciferase reporter assay demonstrated that hsa_circ_0005556 targeted miR-4270, and miR-4270, in turn, targeted MMP19, confirming that hsa_circ_0005556 acted as a sponge to adsorb miR-4270, indirectly regulating the expression of MMP19.

MiR-4270 has been reported to be expressed in a variety of tumors. Some studies have shown that the expression of miR-4270 is low in hepatocellular carcinoma (HCC) tissues, with an area under the curve (AUC) value of 0.967. It is considered to be a promising marker for the diagnosis of HCC [19]. The expression of miR-4270 is also downregulated in patients with brain metastasis of lung adenocarcinoma and, according to the multivariate Cox proportional hazard regression analysis, is closely related to poor prognosis [20]. However, there are reports of miR-4270 expression upregulation in breast cancer [21]. Collectively, these results suggest that different levels of miR-4270 expression are associated with different cancers. In this study, qRT-PCR was used to demonstrate a decreased expression of miR-4270 in gastric cancer tissues compared with paracancerous tissues. Our cell function experiments showed that miR-4270 inhibitors could reverse the biological changes caused by hsa_circ_0005556 silencing.

To further explore the mechanism of hsa_circ_0005556/miR-4270-mediated gastric cancer progression, we used bioinformatics prediction tools and identified MMP19, a member of the matrix metalloprotein MMP family, as a downstream target of miR-4270. MMPs are associated with a variety of human physiological processes and are involved in cancer
invasion and metastasis [22,23]. There is growing evidence that MMP19 is involved in the progression and metastases of a variety of cancers. For example, the expression of MMP19 is upregulated in colon cancer; furthermore, the high expression levels of MMP19 are associated with tumor vascular invasion and lymph node metastases, and are also a factor for poor prognosis in colon cancer patients [24,25]. Furthermore, it has been confirmed that high expression of MMP19 can promote tumor metastases and increase mortality in non-small cell lung cancer [26]. MMP19 is also upregulated in melanoma and is involved in the invasion of tumor cells and the progression of melanoma [27]. The high expression levels of MMP19 in astrocytoma can promote the invasion of glioma cells [28].

However, the role of MMP19 in gastric cancer is not clear. In this study, MMP19 was shown to be the target of miR-4270 and to be a part of the hsa_circ_0005556/miR-4270/MMP19 axis. According to the TCGA, the expression of MMP19 in gastric cancer was significantly higher compared to the normal tissues. To further explore the function of hsa_circ_0005556, we carried out the experiments in vivo. Downregulation of hsa_circ_0005556 expression significantly inhibited the development of gastric cancer in mice. In our future experiments, we will further expand the clinical sample size, as well as collate and improve clinical data to explore the prognostic value of hsa_circ_0005556. Our study confirmed the high expression levels of hsa_circ_0005556 and the low expression levels of miR-4270 in gastric cancer. The experiments presented here confirm the existence of the hsa_circ_0005556/miR-4270/MMP19 axis in gastric cancer. According to our hypothesis, hsa_circ_0005556 promotes the progression of gastric cancer by upregulating the expression of MMP19 via the sponge adsorption of miR-4270.

In summary, this study is the first to explore the interactions between hsa_circ_0005556, miR-4270, and MMP19. Through the hsa_circ_0005556/miR-4270/MMP19 axis, hsa_circ_0005556 plays a specific regulatory role in the progression of gastric cancer. Therefore, hsa_circ_0005556 could be used as a potential drug target in the clinical treatment of patients with gastric cancer.

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