CircRNA_0005075 suppresses carcinogenesis via regulating miR-431/p53/epithelial-mesenchymal transition axis in gastric cancer

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This study was aimed to explore the expression and biological function of circRNA_0005075 in gastric cancer (GC) progression and its underlying mechanism. First, the expression level of circRNA_0005075 and microRNA-431 (miR-431) in GC tissues were detected with the quantitative real-time polymerase chain reaction. In addition, after down-regulated the circRNA_0005075 expression by plasmid transfection in GC cells, the Cell Counting Kit-8 (CCK-8), EDU, transwell assay were conducted to evaluate the function of circRNA_0005075 or miR-431 on cell proliferation, metastasis in vitro. Moreover, p53 and Epithelial-mesenchymal transition (EMT) pathway related proteins were also measured with western blotting. Then, our data revealed that CircRNA_0005075 was found to be significantly up-regulated in GC tissues as well as GC cell lines, and the GC patients with higher CircRNA_0005075 expression were more likely to have poor outcomes. Down-regulation of CircRNA_0005075 could significantly suppress the GC cell proliferation and cell metastasis ability, while the addition of miR-431 inhibitors could counteract this effect. Importantly, we discovered that the silencing of circRNA_0005075 could weaken the micro-RNA sponge function for miR-431, and then upregulate the expression of p53 and forbid the EMT signalling pathway, and finally suppress the tumourigenesis of GC. To sum up, CircRNA_0005075 could inhibit cell growth and metastasis of GC through regulating the miR-431/p53/EMT axis.

Significance of the study: The research clearly elucidated the potential role and relative regulatory mechanism of circRNA_0005075 in gastric cancer (GC) progression. Briefly, circRNA_0005075 could directly inhibit the expression level of miR-431, then regulate the p53/Epithelial-mesenchymal transition axis, and finally inhibit cell growth and metastasis in GC. Consequently, circRNA_0005075 might act as an oncogene in the GC procession, which provides a promising way for the treatment of GC.

KEYWORDS

cell proliferation and metastasis, circRNA_0005075, EMT, gastric cancer, P53
1 | INTRODUCTION

Gastric cancer (GC) is the fifth most frequently diagnosed cancer and ranks third in terms of mortality in the world. Due to the shortage of early diagnostic methods, numerous patients were diagnosed with advanced GC. At present, the radical gastrectomy combined with system chemotherapy was the standardized treatment for patients with the advanced GC, but the total survival time was still at a low level. Therefore, it is urgent and important to explore novel bio-markers for GC and make clear its underlying mechanism.

Circular RNAs (circRNAs) are a newly discovered noncoding RNAs with closed loops structure, but without 5’ to 3’ polarity and polyadenylated tail. In recent years, accumulating studies demonstrated that circRNAs played a significant role in the development of various cancer, including colorectal cancer, ovarian cancer and hepatocellular carcinoma. Furthermore, emerging evidences proved that circRNAs could serve as a micro-RNA (miRNA) sponge, then regulate the biological function of miRNA and participate in the tumour progression. For example, circ_0091570 had been identified as a tumour inhibitor of hepatocellular cancer via sponging miR-1307. In addition, Gao et al detected that circ-PKD2 sponged miR-204-3p, and then suppressed carcinogenesis in oral squamous cell carcinoma. However, the biological function of circRNA as miRNA sponges in GC has not been clearly elucidated.

CircRNA_0005075 is a noncoding RNA oriented from chr1:21377358-21415706. To date, circRNA_0005075 is identified to serve as an oncogene in colorectal cancer and hepatocellular cancer. For example, Yang et al provided evidence that circRNA_0005075 could improve cell growth and metastases in hepatocellular cancer via regulating miRNA-335/MAPK1 axis. However, it is still lack of understanding of the role and potential regulator mechanism of circRNA_0005075 in GC. Epithelial-mesenchymal transition (EMT) is a biological process in which epithelioid cells transform into mesenchymal cells and then acquire enhanced migratory and invasive properties. Increasing studies have suggested that EMT is a significant mechanism in the metastasis of malignant tumours, and this signalling pathway could be regulated by circRNAs. Although circRNA_0005075 has been implicated to be an important IncRNA oncogene in various cancers, its role in EMT remains unclear.

In the present study, we first explored the relationship between the clinicopathological characteristic of patients with GC and the expression level of circRNA_0005075. Subsequently, we performed in vitro experiments to detect the biological function of circRNA_0005075 in GC progression. Moreover, based on the results of bio-informatics analysis, we hypothesized that circRNA_0005075 could sponge miR-431, and further enhance the tumourigensis of GC, which was confirmed by the dual luciferase reporter assays. In summary, our data consistently revealed that circRNA_0005075 would be a novel diagnostic and therapeutic bio-marker for GC patients.

2 | METHOD

2.1 | Patient tissue samples

In the present study, the GC tissue samples and their adjacent normal tissues were obtained from 70 GC patients who suffered from gastrectomy in the First Affiliated Hospital of Jiaxing University from 2015 to 2018 and signed the informed consent voluntarily. In addition, all of the GC tissues were confirmed as gastric adenocarcinoma via two experienced pathologists. All of the tissue samples were stored in liquid nitrogen before used. Furthermore, this study had gained the approval from the First Affiliated Hospital of Jiaxing University.

2.2 | Cell culture and RNA transfection

The human GC cell lines (MNK45, AGS, MGC-803, BGC-823 and HGC-27) and the normal gastric epithelial cell lines (GES-1) were bought from American Type Culture Collection (ATCC, Virginia). In addition, these cells were treated with DMEM (Invitrogen, California) contained 10% FBS (GIBCO, Brazil) and cultured in the incubator with 37°C and 5% CO₂. Moreover, based on the manufacturer’s protocol, the Lipofectamine 3000 (Invitrogen) were used to transfect these small interfered RNAs (including si-circRNA_0005075-1, si-circRNA_0005075-2, miR-431 mimics, miR-431 inhibitors and their negative controls) into GC cells, then regulate the expression level of these target genes in GC cells.

2.3 | Quantitative real-time polymerase chain reaction

In brief, the total RNA of the GC tissue samples and GC cells were extracted with Trizol reagent (Invitrogen) based on the manufacturer’s instructions, and their concentrations were detected with the Beckman Coulter. On the one hand, the expression level of circRNA_0005075 were measured with the One Step SYBR PrimeScript RT-PCR Kit II (Takara, Kusatsu, Japan) via real-time polymerase chain reaction (RT-qPCR) assays, and its expression levels was normalized with GAPDH. On the other hand, the expression level of miR-431 were measured with the TaqMan MicroRNA Assays Kit (Applied Biosystems, Carlsbad, California) via RT-qPCR assays, and its expression levels was normalized with U6. Finally, we performed 2−ΔΔCt method to evaluate the expression levels of the target genes. Moreover, The following primers were used: CircRNA_0005075 (forward: 5’-CAA ATC TTG CGG CAA CGC-3’, reverse: 5’-GCG GGA GTG AAG ATT CGA-3’), miR-431 (forward: 5’-CCA GGT CTT GCA GG-3’, reverse: 5’-CCT GCC GTG TGG TTG GTT CT-3’), GAPDH (forward: 5’-TCC GTG GCC ACC ACC TTC-3’).
2.4 | In vitro cell growth assay

In order to evaluate the GC cells proliferation ability, the Cell Counting-8 (CCK-8) assay and EDU assay were performed in this research. For CCK-8 assay, the GC cells viability were detected and evaluated with the Cell Counting Kit (CCK-8, Dojindo) at the designated time interval. For EDU assay, the GC cells were fixed with PBST, the protein band were detected and evaluated through Genetools (GeneSnap using SynGene systems).

2.5 | Transwell assay

The Transwell chambers (0.8 μm; Corning, New York) with or without Matrigel coating were used to evaluate the cell migration and invasion ability, respectively. The cell precipitations were suspended with the serum-free medium and added into the upper chamber, while the 500 μL DMEM medium contained 20% FBS were transferred into the lower chamber. After 24 hours incubation, the cells were fixed with paraformaldehyde, then stained with crystal violet and washed with PBS. Finally, the migratory and invasive cells were imaged and counted with microscope.

2.6 | Western blotting

The RIPA Lysis buffer (Beyotime, Shanghai, China) was used to treated the GC cell precipitations for protein extraction. Then, the BCA protein Assays Kit (Beyotime, Shanghai, China) was used to calculate the concentration of the protein. The equal amounts of protein were separated and transferred into the polyvinylidene fluoride (PVDF) membranes. Subsequently, in order to block the unspecific antibody, the 10% BSA (Bovine Serum Albumin) solutions were performed to incubate the PVDF membranes. Afterward, the PVDF membranes were incubated with primary antibody, and follow with secondary antibody. Finally, after three time washing with PBST, the protein band were detected and evaluated through GeneSnap using SynGene systems.

2.7 | Prediction of downstream molecules regulated by circRNA_103809

A publicly available bioinformatic algorithm (Starbase 2.0) was utilized to predict the downstream microRNAs of circRNA_103809.

2.8 | Dual-luciferase reporter assays

First, the 293 T cells were collected and placed in the 24-well plates. Twenty-four hours later, these cells were transfected with pmirGLO-circRNA_0005075-WT or pmirGLO-circRNA_0005075-MUT plasmid, with miR-431 mimics or miR-431 NC. Forty-eight hours after that, the dual-luciferase reporter assay system (Promega, Madison, Wisconsin) was performed to detect and evaluate the relative luciferase activity.

2.9 | Statistics

All data are shown as mean ± SD. In addition, the student’s t test and χ² test were used to analysis these data with IBM SPSS 20.0 software. All the experiments were performed three times. When P < .05, the significant differences were considered.

3 | RESULTS

3.1 | Over-expression of CircRNA_0005075 predicted poor prognosis of GC

As shown in Figure 1A, CircRNA_0005075 was remarkably up-regulated in GC tissue samples. Meanwhile, the upregulation of CircRNA_0005075 accounted for 72.8% (51/70) of GC tissue samples (Figure 1B). Subsequently, based on the median of the relative circRNA_0005075 expression, the total GC patients were divided into two groups, including high-expression group and low-expression group. In addition, the data also indicated that the circRNA_0005075 expression was highly associated with vascular invasion (P = .000), T stage (P = .013), N stage (P = .003) and TNM stage (P = .016) (Table 1). Similarly, our results also showed that the GC patients with circRNA_0005075 expression were more likely to have higher T stage (Figure 1C, P = .0057), lymph node metastasis (Figure 1D, P = .0187), advanced TNM stage (Figure 1E, P = .0204), vascular invasion (Figure 1F, P = .0079) and shorter overall survival time (Figure 1G, P = .0352). Taken together, the results consistently showed that circRNA_0005075 was closely related with poor clinical outcome.

To further explore the biological function of circRNA_0005075 in GC progression, the construction of circRNA_0005075 knocked-down cell models are of great important. At first, we detect the expression level of circRNA_0005075 in a panel of GC cell lines via RT-qPCR assay. The results showed that compared with the GES-1 cell lines, circRNA_0005075 expression was upregulated in the GC cell lines, and MNK-45 and AGS cells showed the higher expression (Figure 1H). Subsequently, we established the circRNA_0005075 silenced MNK-45 and AGS cells with the transfection of CircRNA_0005075 specific siRNAs, which was confirmed with RT-qPCR (Figure 1I and J).

3.2 | Downregulation of CircRNA_0005075 suppresses cell growth rate via activating p53 pathway

Afterward, these Circ_0005075-knocked down cells were collected for proliferation experiments. As the results of the CCK-8 had shown, the OD value in si-Circ_0005075-1 and si-Circ_0005075-2 groups were significantly lower than that in control group, indicating that
downregulation of circRNA_0005075 could suppress the proliferation of MNK-45 and AGS cells (Figure 2A and B). Consistent with the previous results, the EDU assay showed that there were less EdU-positive cells in the si-CircRNA_0005075 groups than in the control groups (Figure 2C and D). Taken together, these results consistently revealed that CircRNA_0005075 was highly participated in the regulation of cell proliferation in GC cells.

To further verify regulatory mechanism on suppressing cell proliferation induced by the silencing of Circ_0005075, we further detected the expression level of the anti-oncogene p53 through western blot. From the western blot results, we found that compared with the control group, the silencing of CircRNA_0005075 could upregulate the p53 expression level in GC cells, accompanied with the elevated accumulation of Bcl-2 and the repressed accumulation of Bax (Figure 2E and F). Taken together, these data revealed that the downregulation of CircRNA_0005075 inhibited the proliferation via regulating p53 pathway in GC.

3.3 Downregulation of CircRNA_0005075 inhibits the metastasis abilities of GC cells via regulating EMT pathway

From the relationship results between of CircRNA_0005075 and clinic-pathological parameter, we have concluded that the patients with CircRNA_0005075 upregulation were more likely to have lymph node metastasis. Therefore, we performed the Transwell assay to detect the migration and invasion ability of the CircRNA_0005075 knocked-down GC cells. Our data showed that after the depletion of CircRNA_0005075 in GC cells, the migration and invasion potentials of MNK45 and AGS cells were significantly inhibited (Figure 3A-D). However, there was still not clear how CircRNA_0005075 regulate the metastasis abilities in GC cells. Therefore, in this study, we further detected the EMT-related markers of GC cells via western blot assay. Interestingly, we discovered that the silencing of CircRNA_0005075 could
upregulate the expression of epithelial-like phenotypic characteristic substance (E-cadherin), accompanied with the down-regulation of mesenchymal phenotypic characteristic substances (N-cadherin and Vimentin) (Figure 3C and D). Taken together, these data revealed that the downregulation of CircRNA_0005075 inhibited the metastasis abilities via regulating EMT pathway in GC.

3.4 | MiR-431 would be a direct downstream target of CircRNA_0005075 in GC

Based on the results of the available bioinformatic algorithms (Starbase 2), we predicted that miR-431 would be a downstream target for Circ_0005075. Consequently, we evaluated miR-431’s expression pattern of in GC samples. Our data showed that miR-431 was dramatically downregulated in GC tumour samples relative to normal samples (Figure 4B). In addition, the low expression of miR-431 accounted for 81.4% (57/70) of GC specimens (Figure 5C). Meanwhile, the miR-431 was negatively correlated with the expression of CircRNA_0005075 in GC (Figure 4D).

3.5 | Knock-down of miR-431 could reverse the cell proliferation inhibited by the depletion of CircRNA_0005075 in GC

To further explore their association, we subsequently predicted their potential binding site, which was shown in Figure 4A. To date, the luciferase reporter assay was employed, which suggested that the luciferase activity was obviously lower in wild-type cells treated with miR-431 mimics (Figure 4A). Taken together, these results showed that miR-135a-5p might be the direct target of CircRNA_0005075 in GC.

| TABLE 1 Correlation between the clinicopathological data and Circ_0005075 expression of in gastric cancer |
|---|---|---|---|
| Characteristics | N | High | Low | P-value |
| Sex | | | | .339 |
| Male | 36 | 16 | 20 | |
| Female | 34 | 19 | 15 | |
| Age | | | | .138 |
| <60 y | 44 | 25 | 19 | |
| 60 y | 26 | 10 | 16 | |
| Differential status | | | | .607 |
| Moderate/well | 22 | 12 | 10 | |
| Undifferentiated/poorly | 48 | 23 | 25 | |
| Vascular invasion | | | | .467 |
| Negative | 29 | 13 | 16 | |
| Positive | 41 | 22 | 19 | |
| T stage | | | | .013 |
| T1 | 26 | 8 | 18 | |
| T2-T4 | 44 | 27 | 17 | |
| N stage | | | | .003 |
| N0 | 26 | 7 | 19 | |
| N1-N3 | 44 | 28 | 16 | |
| TNM stage | | | | .016 |
| I-II | 30 | 10 | 20 | |
| III-IV | 40 | 25 | 15 | |

To further explore their association, we subsequently predicted their potential binding site, which was shown in Figure 4A. To date, the luciferase reporter assay was employed, which suggested that the luciferase activity was obviously lower in wild-type cells treated with miR-431 mimics (Figure 4A). Taken together, these results showed that miR-135a-5p might be the direct target of CircRNA_0005075 in GC.
CCK-8 assay, the down-regulation of miR-431 consistently reduced cell proliferation abilities compared with that in miR-431 NC group (Figure 5C). Similarly, the results of EDU assays also indicated that the addition of miR-431 inhibitors could increase the EdU-positive cells in the si-Circ_0005075-1 groups (Figure 5D). Furthermore, the quantified bands of western blotting (Figure 5E) also showed the decrease of p53 and Bax, accompanied with the increase of Bcl-2 in si-Circ_0005075-1 MNK45 cells after incubating with miR-431 mimics.

From the Transwell assays, the down-regulation of miR-431 consistently reduced cell migration and invasion abilities compared with that in miR-431 NC group (Figure 5F and G). We further detected the EMT-related markers of GC cells, after the depletion of miR-431. From the western blot results, we found the decrease of epithelial-like
phenotypic characteristic substance (E-cadherin), and the increase of mesenchymal phenotypic characteristic substances (N-cadherin and Vimentin) (Figure 5H). Taken together, these data revealed that the downregulation of CircRNA_0005075 inhibited the proliferation and metastasis abilities, via sponging to microRNA-135a-5p in GC.

4 | DISCUSSION

GC is one of the most common malignant tumours in the world. Recently, the treatment for the post-operated GC patients, including molecule-targeted therapy and immunotherapy, had made a big through. However, due to the lack of the typical symptoms and effectively screening methods in the early stage, numerous patients were diagnosed with advanced GC and their 5-year survival rate was not satisfactory. Interestingly, based on the high-throughput sequencing and bioinformatics technologies, increasing attention had been paid for verifying the biological role of CircRNAs in the development of GC. For instance, Huang et al demonstrated that circRNA AKT3 could augment cell sensitivity to cisplatin in GC through regulating miR-198/PIK3R1 axis. Therefore, identifying a novel circRNA related to GC progression is of great important.

FIGURE 3  Downregulation of CircRNA_0005075 inhibit cell metastasis through suppressing EMT signalling pathway. A and C, Cell migration and invasion images and the statistics analysis of migrated and invaded cells for the Circ_0005075-downregulated MKN45 cells. B and D, Cell migration and invasion images and the statistics analysis of migrated and invaded cells for the Circ_0005075-downregulated AGS cells. E and F, Protein expression of E-cadherin, N-cadherin and Vimentin. **P < .001
Actually, this study was conducted to elucidate the biological role of circRNA_0005075 and the possible mechanism. First, the RT-qPCR results showed that the expression level of circRNA_0005075 in the GC tissues were higher than that in the normal group. Importantly, the patients with circRNA_0005075 overexpression were more likely to higher T stage, lymph node metastasis, advanced TNM stage, vascular invasion and shorter overall survival time. Previous study identified that circRNA_0005075 acted as an oncogene in the development of colorectal cancer. Similarly, we discovered that silencing of circRNA_0005075 could significantly suppress the GC cell growth rate based on the results of CCK-8 and EDU assay. However, the precise mechanism of how circRNA_0005075 regulate GC cell proliferation was poorly understood.

In recent year, p53 had gained increasing attention for its tumour inhibitory effect, including cell-cycle arrest, cell death, DNA repair and others. Previous study demonstrated that the deletion of p53 could promote liver cancer progression via activating mevalonate pathway. In this study, the Transwell results revealed that after the depletion of CircRNA_0005075 in GC cells, the migration and invasion potentials of GC cells were significantly inhibited. Nevertheless, whether EMT signalling pathway was involved in the regulation of cell metastasis ability for CircRNA_0005075 downregulated cell was particularly unclear. Based on the function of p53-mediated pathway suppression, consistent with the previous studies, our data showed that the silencing of circRNA_0005075 was coupled with the p53 and Bax expression increased, as well as the Bcl-2 expression reduced. Taken together, these data revealed that the downregulation of CircRNA_0005075 could inhibit p53-mediated pathway, which resulted in the suppression of GC cell proliferation.

In fact, the cell migration and invasion ability acted as an important index to measure the malignant degree of various cancers. Interestingly, accumulating studies demonstrated that the activation of EMT signalling pathway contributed more to the enhancement of cell metastasis ability, which could transform more epithelioid cells into mesenchymal cells. For example, Wei S et al revealed that circPTPRA could inhibit NSCLC cells metastasis through suppressing EMT signalling pathway. In this study, the Transwell results revealed that after the depletion of CircRNA_0005075 in GC cells, the migration and invasion potentials of GC cells were significantly inhibited. Nevertheless, whether EMT signalling pathway was involved in the regulation of cell metastasis ability for CircRNA_0005075 downregulated cell was particularly unclear.
Therefore, we further detected the EMT related proteins with western blot, and found that the silencing of circRNA_0005075 could up-regulate the epithelial-like phenotypic characteristic substance (E-cadherin) and down-regulate the mesenchymal phenotypic characteristic substance (N-cadherin and Vimentin). Taken together, the cell metastasis ability of CircRNA_0005075 downregulated cells might be alleviated due to the suppression of EMT signalling pathway.

Increasing studies demonstrated that due to the cavernous mechanism, the circ_RNAs could directly inhibit the function of miRNAs, and then exert tumour suppression or cancer.
promotion effect.\textsuperscript{26,27} For example, circ-UBE2D2 had been confirmed to promote the breast cancer progression via Sponging miR-1236 and miR-1287.\textsuperscript{28} Therefore, we further screened that miR-431 might be the downstream target gene of circRNA\textsubscript{0005075} based on the results of bioinformatics analysis, and their relationships were further identified with the dual-luciferase reporter assay.

Recently, increasing attentions were paid in elucidating the biological function of miRNAs, and miR-431 played a pivotal role in the development of various cancer, including melanoma, pancreatic cancer, colorectal cancer and others.\textsuperscript{29-31} In the present study, the RT-qPCR results showed that the miR-431 expression level in tumour tissues was more higher relatively than that in normal tissues, and its expression was negatively related with circRNA\textsubscript{0005075}. Moreover, our data revealed that the addition of miR-431 inhibitors could reverse the p53/EMT-mediated signalling pathway and the promotion of p53-mediated pathway induced by the silencing of circRNA\textsubscript{0005075}. Taken together, circRNA\textsubscript{0005075} was highly participated in GC progression through miRNA sponge function of miR-431.

5 CONCLUSION

To sum up, our data revealed that CircRNA\textsubscript{0005075} was upregulated in GC tissues, and which contributed to the GC progression. Importantly, the depletion of CircRNA\textsubscript{0005075} could directly regulate the function of miR-431, then interfere the p53/EMT-mediated signalling pathway, followed by inhibit cell proliferation, cell migration and invasion in GC. However, there are still some limitations in our research, as the clinical samples were too small to evaluate prognosis value of circRNA\textsubscript{0005075} in GC. Meanwhile, the understanding for the potential downstream of circRNA\textsubscript{0005075}/miR-431 axis was particularly poor, and in vivo experiments need further exploration. Taken together, circRNA\textsubscript{0005075} might act as an oncogene in the GC process, which provide a promising way for the treatment of GC.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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