INTRODUCTION

Gastric carcinoma (GC) is a familiar malignant carcinoma all over the world, which has high malignancy and poor prognosis. Patients with early GC have a higher 5-year survival rate after surgery, but most patients are diagnosed with advanced GC at the time of admission. With further research on the mechanism of GC, molecular therapy has become a hot spot in the field of GC. Furthermore, a number of researches have shown that various genes are associated with GC. Therefore, further research of the mechanism in GC was helpful to improve the diagnosis and treatment of GC.

Circular RNAs (CircRNAs) are a type of non-coding RNAs with high tissue specificity and stable structure. CircRNAs regulate the gene expression by interacting with microRNAs (miRNAs) or other molecules.

Hsa_circ_0001649 restrains gastric carcinoma growth and metastasis by downregulation of miR-20a

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Abstract

Background: Gastric carcinoma (GC) is a familiar carcinoma and serious threat to human health. We investigated the efficacy and mechanism of circular RNA hsa_circ_0001649 on the growth, migration, and invasion of GC cells.

Methods: microRNA (miR)-20a and hsa_circ_0001649 expression was investigated by RT-qPCR and was changed by cell transfection. CCK-8, flow cytometry, and BrdU assays were, respectively, used to investigate the efficacies of hsa_circ_0001649 and miR-20a on cell viability, apoptosis, and proliferation. Transwell assay was used to investigate the efficacies of hsa_circ_0001649 and miR-20a on cell migration and invasion. Moreover, the levels of cyclin D1, Bax, cleaved caspase-3, and signal pathway-related proteins were investigated by Western blot.

Results: Hsa_circ_0001649 was downregulated in GC cells and tissues. Upregulation of hsa_circ_0001649 restrained viability, proliferation, migration, and invasion, while promoted apoptosis. Furthermore, miR-20a was negatively regulated by hsa_circ_0001649 and miR-20a overexpression reversed the efficacy of hsa_circ_0001649 upregulation. Finally, upregulation of hsa_circ_0001649 restrained ERK and Wnt/β-catenin pathways while miR-20a overexpression reversed these progresses.

Conclusion: Upregulation of hsa_circ_0001649 restrained GC cell growth and metastasis by downregulating miR-20a and thereby inactivated ERK and Wnt/β-catenin pathways.

Keywords: gastric carcinoma, growth, hsa_circ_0001649, invasion, migration, miR-20a
CircRNAs have been found to play a vital efficacy in many biological processes such as cell growth, migration, and invasion. Moreover, circRNAs have been shown to be potential markers for many diseases and carcinomas, including GC. It was documented that hsa_circ_000181 and hsa_circ_0003159 were notably decreased in the tissues and plasma of patients with GC, and their expression was closely associated with the clinical stages of patients with GC. In recent years, researches have displayed that the hsa_circ_0001649 was downregulated in liver carcinoma and upregulation of hsa_circ_0001649 could restrain the progress of liver carcinoma. Further researches have shown that the hsa_circ_0001649 was downregulated in GC tissues and cells. Then, the efficacies of hsa_circ_0001649 and miR-20a in GC remains unclear.

2.3 | Reverse transcription quantitative PCR (RT-qPCR)

Trizol was used to extract RNA (Invitrogen). The concentrations of the extracted RNAs were determined by the Nanodrop 2000 system (Thermo Fisher Scientific). MultiScribe™ RT kit (Applied Biosystems) was used to transcribe RNA into cDNA. Fast SYBR™ Green Master Mix (Thermo Fisher Scientific) and random hexamers or oligo (dT) were used to investigate the levels of miR-20a and hsa_circ_0001649. U6 and β-actin were, respectively, used as the internal parameters for miR-20a and hsa_circ_0001649. Samples were run in triplicate. The $2^{-\Delta\Delta Ct}$ equation was used to quantify the data.

2.4 | Transfection

The sequences of miR-20a mimic and its control oligonucleotide (NC mimic), as well as the overexpression vector of hsa_circ_0001649 and the empty vector, were all synthesized by GenePharma (Shanghai, China). These vectors were, respectively, transfected into cells for miR-20a and hsa_circ_0001649 overexpression. Lipofectamine 3000 (Invitrogen) was employed for performing cell transfection. The transfection concentration was 50 nmol/L, and cells were harvested 48 hours after transfection. The transfection efficiency of miR-20a mimic was displayed below: miR-20a mimic: mimic sense 5'-UAAGUGCUUAAGUGCAGGUAG-3'; mimic antisense 5'-ACCUGCACUAUGACACUUAUU-3'.

2.5 | Cell counting kit-8 (CCK-8) assay

Cells were cultured in the 96-well plates (5 × 10^5 cells/well). CCK-8 kit (Beyotime) was used to investigate cell viability. After transfection, CCK-8 solution (10 μL) was added to each well and reacted for 1 hour. Finally, the absorbance was quantitated at 450 nm.

2.6 | Flow cytometry

Cell apoptosis analysis was carried out with the help of PI and FITC-conjugated Annexin V staining (Beyotime). In short, after transfection, cells were collected and centrifuged and then were resuspended in 195 μL of Annexin V-FITC binding buffer. Afterward, 5 μL of Annexin V-FITC and 10 μL of PI were, respectively, added and reacted at 25°C in dark condition for 15 minutes.
Thereafter, the results were measured by utilizing FACS can (Beckman Coulter). The data were analyzed by FlowJo software (Tree Star Software).

2.7 | BrdU assay

After transfection, cells were plated in 96-well plate (2500 cells per well). Then, BrdU reagent (Cell Signaling Technology) was added to each well at a final concentration of 1 × BrdU and cells were incubated for 1 hour. Afterward, cells were resuspended in 100 μL of fixing/denaturing solution and maintained at 25°C for 30 minutes. After discarding the solution, 100 μL of 1x detection antibody was supplied and the mixture was reacted at 25°C for 1 hour. Thereafter, 100 μL of 1x HRP-labeled secondary antibody was provided and reacted for 30 minutes at 25°C. After that, TMB substrate was provided and incubated at 25°C for 30 minutes. Finally, stop solution was added and the absorbance (450 nm) was detected.

2.8 | Migration and invasion assay

The transwell (BD Biosciences) assay was used to analyze cell migration and invasion. The only difference between migration and invasion assay was that a layer of matrigel was placed on the upper side to mimic the extracellular matrix in vivo in invasion assay. The cells cultured in serum-free medium were supplied to the upper chamber, while medium replenished with 20% FBS was added to the lower chamber as a chemoattractant. After cultured in a 37°C and 5% CO₂ incubator for 24 hours, cells in lower chamber were fixed with methanol and then stained with 0.2% crystal violet. Thereafter, the migrated and invaded cells were counted by utilizing a light microscope (Zeiss).

2.9 | Western blot

Protein was extracted from cells by RIPA lysis buffer (Beyotime) with the existence of protease inhibitors (Beyotime). 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins. After that, proteins were transferred onto the PVDF membrane (Millipore). The PVDF membrane was blocked with 5% bovine serum albumin (BSA; Biowest) for 2 hours at 25°C. Then, the membrane was incubated with primary antibody (1:1000, Abcam) at 4°C. After overnight incubation, goat anti-rabbit IgG (ab6721, Abcam, 1:5000) was added onto the membrane. Signals were captured and quantified by Image Lab™ Software (Bio-Rad). β-actin was used as internal parameter in all above experiments.

Primary antibody contained cyclin D1 (ab16663), Bax (ab32503), β-actin (ab8227), cleaved caspase-3 (ab2302), Wnt3a (ab219412), β-catenin (ab16051), t-ERK (ab184699), p-ERK (ab76299), and β-actin (ab227387).

2.10 | Statistical analysis

All experiments were run in triplicate. Data were displayed as mean ± standard deviation (SD). Statistical analyses were displayed by GraphPad 6.0 (GraphPad Software). The P-values were calculated by a one-way analysis of variance (ANOVA) and Student’s t test. P < .05 was considered statistically significant.

3 | RESULTS

3.1 | Hsa_circ_0001649 was downregulated in GC cells and tissues

For determination of the expression pattern of hsa_circ_0001649 in GC, we, respectively, tested its expression in GC tissues and cells. Figure 1A revealed hsa_circ_0001649 was prominently lower expressed in GC tissues relative to normal tissues (P < .01). Figure 1B revealed that hsa_circ_0001649 was notably downregulated in SGC-7901, MKN45, AGS, and MKN28 cells (P < .05 or P < .01 or P < .001). Moreover, we chose MKN28 and MKN45 cells which had the lowest hsa_circ_0001649 expression to investigate the efficacy of hsa_circ_0001649 in following experiments.

3.2 | Hsa_circ_0001649 was upregulated in GC cells

In order to investigate the efficacy of hsa_circ_0001649 in GC, we overexpressed hsa_circ_0001649 in MKN28 and MKN45 cells. The

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**FIGURE 1** Hsa_circ_0001649 was downregulated in Gastric carcinoma (GC) cells and tissues. (NT = non-carcinoma, T = carcinoma). RT-qPCR was used to investigate expression of hsa_circ_0001649 in A, GC tissues, corresponding adjacent tissues, and B, GC cells. (*P < .05; **P < .01; ***P < .001)
result displayed that hsa_circ_0001649 expression was dramatically elevated by threefold after transfected with the recombined overexpression vector hsa_circ_0001649 in MKN28 and MKN45 cells (both $P < .001$, Figure 2). This implied that the transfection efficiency was high.

3.3 | Upregulation of hsa_circ_0001649 restrained cell proliferation while facilitated apoptosis

Based on Figure 2 result, we investigated the efficacy of hsa_circ_0001649 upregulation in MKN28 and MKN45 cells. Data displayed that upregulation of hsa_circ_0001649 conspicuously restrained cell viability ($P < .05$ or $P < .01$, Figure 3A) and proliferation ($P < .01$ or $P < .001$, Figure 3B) of these two cell lines. The results of cell proliferation were further validated by the declined expression of cyclinD1 (both $P < .001$, Figure 3C-E). In addition, flow cytometry analysis disclosed that upregulation of hsa_circ_0001649 dramatically stimulated cell apoptosis in MKN28 and MKN45 cells (both $P < .001$, Figure 3F). These outcomes were consistent with the alteration of apoptosis-related Bax and cleaved caspase-3, which expression was both remarkably enhanced (all $P < .001$, Figure 3G-I). This implied that upregulation of hsa_circ_0001649 restrained the growth of GC cells.

3.4 | Upregulation of hsa_circ_0001649 restrained the cell metastasis

Additionally, we investigated the influences of hsa_circ_0001649 upregulation on the metastasis of MKN28 and MKN45 cells. Results displayed that upregulation of hsa_circ_0001649 conspicuously declined the migratory and invasive capacities of MKN28 and MKN45 cells ($P < .01$ or $P < .001$, Figure 4A and B). This implied upregulation of hsa_circ_0001649 restrained migration and invasion of MKN28 and MKN45 cells.

3.5 | miR-20a was negatively regulated by hsa_circ_0001649

It was documented that miR-20a-3p was highly expressed in GC tissues and miR-20a could be a potential diagnostic biomarker for GC. Therefore, we, respectively, tested its expression in GC tissues and cells. Results showed that miR-20a was both highly expressed in GC tissues and cells relative to control ($P < .05$ or $P < .01$, Figure 5A and B). In order to investigate the relationship between hsa_circ_0001649 and miR-20a, we tested the expression of miR-20a in hsa_circ_0001649 upregulated MKN28 and MKN45 cells. The result displayed that miR-20a expression was conspicuously declined after upregulation of hsa_circ_0001649 in GC cells MKN28 and MKN45 ($P < .01$ or $P < .001$, Figure 5C). This implied that miR-20a was negatively regulated by hsa_circ_0001649.

3.6 | Upregulation of hsa_circ_0001649 restrained cell growth by downregulating miR-20a

For further determining whether upregulation of hsa_circ_0001649 achieved its efficacy via regulating miR-20a expression, we overexpressed miR-20a in MKN28 and MKN45 cells. Data shown in Figure 6A demonstrated that miR-20a expression notably upregulated by fourfold after transfected with miR-20a mimic both in MKN28 and MKN45 cells (both $P < .01$). This implied that we successfully transfected miR-20a mimic into MKN28 and MKN45 cells. Results displayed in Figure 6B-F revealed that miR-20a overexpression distinctly reversed upregulation of hsa_circ_0001649-induced inhibitory impacts on viability (both $P < .05$, Figure 6B) and proliferation (all $P < .05$, Figure 6C-F) of MKN28 and MKN45 cells. Besides, detection of cell apoptosis and expression of apoptosis-related proteins also disclosed that upregulation of hsa_circ_0001649-induced promoting impacts were all remarkably remitted by miR-20a overexpression ($P < .05$ or $P < .01$, Figure 6G-J). In short, these results implied that upregulation of hsa_circ_0001649 restrained cell growth by downregulating miR-20a in MKN28 and MKN45 cells.

3.7 | Upregulation of hsa_circ_0001649 restrained metastasis by downregulating miR-20a

Additionally, we determined the influences of miR-20a overexpression on the migratory and invasive capacities of hsa_circ_0001649 upregulated MKN28 and MKN45 cells. Outcomes showed that upregulation of hsa_circ_0001649-induced suppressive impacts on the migration and invasion were all noticeably mitigated by miR-20a overexpression (all $P < .05$, Figure 7A and B). Above results implied that upregulation of hsa_circ_0001649 restrained metastasis by downregulating miR-20a.
FIGURE 3  Upregulation of hsa_circ_0001649 restrained cell growth in MKN28 and MKN45 cells. A, CCK8 assay was used to investigate efficacies of hsa_circ_0001649 upregulation on cell viability. B, BrdU assay was used to investigate efficacies of hsa_circ_0001649 upregulation on proliferation. C-E, Western blot was used to investigate the influences of hsa_circ_0001649 upregulation on expression of proliferation-associated cyclinD1. F, Flow cytometry was used to investigate efficacies of hsa_circ_0001649 upregulation on cell apoptosis. G-I, Western blot was used to investigate efficacies of hsa_circ_0001649 upregulation on expression of apoptosis-associated Bax and cleaved caspase-3. (*P < .05; **P < .01; ***P < .001)

FIGURE 4  Upregulation of hsa_circ_0001649 restrained the cell migration and invasion in MKN28 and MKN45 cells. Transwell assay was used to investigate the efficacies of hsa_circ_0001649 upregulation on A, cell migration and B, invasion. (***P < .01; ****P < .001)
Upregulation of hsa_circ_0001649 restrained the ERK and Wnt/β-catenin pathways via downregulating miR-20a

For disclosing the underlying mechanism of upregulation of hsa_circ_0001649 suppressing cell growth and metastasis of GC cells, we tested the expression of Wnt/β-catenin and ERK pathway-related proteins. Results showed that upregulation of hsa_circ_0001649 noticeably repressed the expression of Wnt/β-catenin pathway-related Wnt3a (**P < .01 or ***P < .001) and β-catenin (both ***P < .001, Figure 8A-C) in both MKN28 and MKN45 cells. Besides, detection of ERK pathway-related ERK expression demonstrated that the ratio of p/t-ERK was dramatically depressed by upregulation of hsa_circ_0001649 (both P < .01, Figure 8D-F),

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**FIGURE 5** miR-20a was negatively regulated by hsa_circ_0001649 in MKN28 and MKN45 cells. RT-qPCR was used to investigate expression of miR-20a in A, Gastric carcinoma (GC) tissues and B, GC cells, as well as its expression in C, hsa_circ_0001649-upregulated MKN28 and MKN45 cells. (*P < .05; **P < .01; and ***P < .001)

**FIGURE 6** Upregulation of hsa_circ_0001649 restrained cell growth by downregulating miR-20a in MKN28 and MKN45 cells. The sequences of miR-20a mimic/NC mimic were, respectively, co-transfected with the recombined overexpression vector hsa_circ_0001649/empty vector. A, RT-qPCR was used to investigate transfection efficiency of miR-20a mimic in MKN28 and MKN45 cells. B, CCK8 assay was used to investigate efficacies of miR-20a overexpression on cell viability. C, BrdU assay was used to investigate efficacies of miR-20a overexpression on proliferation. D-F, Western blot was used to investigate the efficacies of miR-20a overexpression on expression of proliferation-associated cyclinD1. G, Flow cytometry was used to investigate efficacies of miR-20a overexpression on cell apoptosis. H-J, Western blot was used to investigate efficacies of miR-20a overexpression on expression of apoptosis-associated Bax and cleaved caspase-3. (*P < .05; **P < .01; and ***P < .001)
whereas further research disclosed that the above mentioned impacts were all noticeably abolished by miR-20a overexpression ($P < .05$ or $P < .001$, Figure 8A-F) in both MKN28 and MKN45 cells. Above results implied upregulation of hsa_circ_0001649 restrained the ERK and Wnt/β-catenin pathways via downregulating miR-20a.

**FIGURE 7** Upregulation of hsa_circ_0001649 restrained metastasis by downregulating miR-20a in MKN28 and MKN45 cells. The sequences of miR-20a mimic/NC mimic were, respectively, co-transfected with the recombined overexpression vector hsa_circ_0001649/empty vector. Transwell assay was used to investigate the efficacies of miR-20a overexpression on A, cell migration and B, invasion. (*$P < .05$; **$P < .01$; ***$P < .001$)

**FIGURE 8** Upregulation of hsa_circ_0001649 restrained the ERK and Wnt/β-catenin pathways via downregulating miR-20a in MKN28 and MKN45 cells. The sequences of miR-20a mimic/NC mimic were, respectively, co-transfected with the recombined overexpression vector hsa_circ_0001649/empty vector. Western blot was used to investigate efficacies of hsa_circ_0001649 upregulation and miR-20a overexpression on A-C, Wnt/β-catenin and D-F, ERK pathways in MKN28 and MKN45 cells. (*$P < .05$; **$P < .01$; and ***$P < .001$)

**DISCUSSION**

Gastric carcinoma (GC) is a familiar malignancy of digestive tract.\textsuperscript{21} In this research, we presented hsa_circ_0001649 was downregulated in GC tissues and cells. Upregulation of hsa_circ_0001649 restrained cell viability, proliferation, migration, and invasion, while enhanced
apoptosis in MKN28 and MKN45 cells. In addition, upregulation of hsa_circ_0001649 downregulated the miR-20a expression, and miR-20a overexpression reversed the changes induced by hsa_circ_0001649 upregulation. Finally, we presented hsa_circ_0001649 and miR-20a exerted their efficacies via ERK and Wnt/β-catenin pathways.

Hsa_circ_0001649 was a new discovered anti-carcinoma gene in recent years, which played a restraining efficacy in a variety of carcinomas. For instance, a research displayed that hsa_circ_0001649 downregulated and restrained cell growth in glioma. Another research displayed that upregulation of hsa_circ_0001649 restrained cell growth, migration and invasion in cholangiocarcinoma. On the other hand, there was a research presented that hsa_circ_0001649 was abnormally expressed in GC and the results showed a downward trend. Moreover, it was documented that hsa_circ_0000467 was highly expressed in GC tissues. Because the efficacies and mechanisms of hsa_circ_0001649 in GC were not completely clear until now, we did a further research. In this paper, we presented that hsa_circ_0001649 expression was conspicuously downregulated in GC tissues compared with adjacent non-carcinoma tissues. Moreover, we presented hsa_circ_0001649 is also downregulated in GC cells. This was inconsistent with the expression pattern of hsa_circ_0000467 in GC tissues, while it was in line with other investigations. We think the reason led to this difference might be that the functions of their maternal genes were inconsistent. Further, we found that hsa_circ_0001649 restrained cell viability, proliferation, migration, and invasion and elevated apoptosis in MKN28 and MKN45 cells. This indicated that hsa_circ_0001649 exerted an anti-carcinoma efficacy in GC.

Previous results displayed circRNAs played an efficacy in carcinoma by sponging miRNAs. For instance, a research displayed that circ_0027599 restrained GC by sponging miR-101. Another research displayed that circFAT1 restrained GC by sponging miR-548g. Furthermore, there was a research presented that hsa_circ_0001649 exerted a carcinoma suppressor efficacy in hepatocellular carcinoma by sponging miR-612. Therefore, we speculated that hsa_circ_0001649 may also work in GC by regulating miRNAs. Expectedly, we found that miR-20a was negative regulated by hsa_circ_0001649. miR-20a overexpression could conspicuously reverse the inhibition caused by upregulation of hsa_circ_0001649.

miR-20a is a member of miR-17-92 cluster, which is upregulated in a variety of carcinomas, and it plays a vital efficacy in carcinoma. For instance, a research displayed that miR-20a was upregulated in colorectal carcinoma. Other research displayed that miR-20a promoted multiple myeloma. Meanwhile, researchers demonstrated that miR-20a was highly expressed in GC and it promoted GC cells growth. Otherwise, a research pointed out that overexpression of miR-20a promoted GC cell growth, while inhibition of miR-20a restrained GC cell growth. In this paper, miR-20a overexpression conspicuously abolished hsa_circ_0001649 upregulation-induced changes in cell growth, migration, and invasion in GC cells. This also proved that miR-20a played a carcinogenic effect in GC.

ERK and Wnt/β-catenin pathways are two important pathways in eukaryotic cells. It plays a vital efficacy in the development of GC. On the other side, a research presented that has_circ_0006427 worked in lung carcinoma via Wnt/β-catenin pathway. Hsa_circRNA_0006528 was reported to work in breast carcinoma via ERK pathway. Furthermore, researches presented that miR-20a exerted its efficacies via ERK and Wnt/β-catenin pathways. In this paper, we presented that upregulation of hsa_circ_0001649 declined the levels of Wnt3a and β-catenin and the ratio of p/t-ERK in MKN28 and MKN45 cells. After miR-20a overexpression, hsa_circ_0001649 upregulation-induced decreased expression was elevated. This indicated that hsa_circ_0001649 restrained Wnt/β-catenin and ERK pathways by downregulating miR-20a.

5 | CONCLUSION

In conclusion, we not only demonstrated hsa_circ_0001649 was downregulated in GC tissues and cells. It was also demonstrated that the expression of hsa_circ_0001649 and miR-20a was negatively associated. Upregulation of hsa_circ_0001649 restrained GC cell growth, migration, and invasion, and these effects were noticeably reversed by miR-20a overexpression. These data indicated that hsa_circ_0001649 exerted a carcinoma suppressor efficacy in GC by regulating miR-20a and Wnt/β-catenin and ERK pathways.

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None.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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