

Original Research Article

CircRTN4 inhibits the progression of gastric cancer by sponging miR-424-5p and regulating LATS2 expression

Xiaoli Li^{1,2}, Tianming Wang¹, Qian Chen¹, Qi Fu¹, Yan Cui¹, Haibang Pan^{1*}

¹First Clinical Medical School, Gansu University of Chinese Medicine, Lanzhou 730000, ²Gansu Provincial Maternity and Child-care Hospital, Lanzhou 730050, China

*For correspondence: **Email:** phbwbb@gszy.edu.cn; **Tel:** +86-0931-5162887

Sent for review: 20 October 2022

Revised accepted: 30 April 2023

Abstract

Purpose: To determine the expression of circRTN4 in gastric cancer (GC) and its role in tumor progression.

Methods: GEO dataset GSE93541 was analyzed using GEO2R. Starbase website was used to predict the combination of miRNA and circRTN4. The relationship between circRTN4 and prognosis was analyzed using Kaplan-Meier Plotter database, while expression levels of circRTN4, miR-424-5p, and LATS2 were assessed by quantitative real time-polymerase chain reaction (qRT-PCR). CCK8, EDU, Transwell, and Western blot were used to assess GC proliferation, migration, invasion, and stemness. Lastly, co-transfection of miR-424-5p or si-LAST2 was reversely used to demonstrate the regulatory effect of circRTN4 on the progression of gastric cancer cells. Significantly downregulated circRTN4 in GC was screened, and the combined miR-424-5p and downstream gene LATS2 were predicted by Starbase.

Results: The average relative expression of circRTN4 mRNA in GC tissues was significantly lower than in adjacent tissues. miR-424-5p was highly expressed in tumor tissues, while LATS2 decreased ($p < 0.05$). Low expression of circRTN4 was associated with a low survival rate in patients. pLCDH-circRTN4 significantly inhibited the proliferation of gastric cancer cells ($p < 0.05$). Overexpression of circRTN4 inhibited the migration and invasion of tumor cells, while pLCDH-circRTN4 reduced the ability of GC stem cells and expressions of MMP2 and OCT4.

Conclusion: Expression of circRTN4 decreases in GC, and contributes to the development and progression of this disease by increasing the levels of LATS2 and binding with miR-424-5p. This suggests that circRTN4 may serve as a promising prognostic marker as well as a potential therapeutic target for gastric cancer.

Keywords: Gastric cancer, Circular RNA, miRNA

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Gastric cancer (GC) is a prevalent malignancy affecting the digestive tract. Despite significant advancements in treatment, the primary cause of

high mortality rates is the metastasis and recurrence of advanced gastric cancer [1]. Therefore, it is of great significance to explore new diagnostic markers and therapeutic targets for the treatment and prognosis of gastric cancer.

Circular RNA (circRNA) is a type of non-coding RNA that is circular in shape and found in mammals, which mainly regulates the transcription and post-transcription of genes [2]. More studies have found that some circRNAs bind to miRNAs, regulate gene transcription, and interact with RNA-binding proteins involved in tumorigenesis [3]. In this study, the GEO database was utilized to identify the differentially expressed circRNAs in gastric cancer. Further screening was conducted on the microRNAs (miRNAs) that bind to these circRNAs and their downstream target genes. Furthermore, this study investigates the correlation between the expression levels of circRTN4 in gastric cancer and incidence and progression of tumors.

EXPERIMENTAL

Screening of circRNA

The GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) was used to search and screen for gastric cancer gene expression profile GSE93541. Three pairs of gastric cancer tissues and three pairs of paracancerous tissues were obtained from the above database, and the data were analyzed using GEO2R online analysis tool (<HTTPS://www.ncbi.nlm.nih.gov/geo/geo2r/>), with circRTN4 as research target from differentially expressed circRNAs.

Prediction of circRNA-binding miRNA and target genes

circRTN4-binding miRNA was predicted using Starbase website (<https://starbase.sysu.edu.cn/>), and then downstream target genes of miR-424-5p were predicted using Starbase.

Survival analysis

Kaplan-Meier Plotter online database (<HTTPS://kmplot.com/analysis/>) was used to analyze the relationship between circRTN4 or miR-424-5p and prognosis of gastric cancer patients.

Quantitative real-time-polymerase chain reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cells, PrimerScript RT Reagent Kit kit was used for reverse transcription, and SYbr® premix Ex Taq II was used for PCR amplification. The PCR primer sequences used are as follows (Table 1).

CCK8 cell activity assay

The HGC-27 or AGS cells of different treatments (pLCDH and pLCDH-circRTN4) were seeded in 96-well plates (5000 cells/well) and cultured at 37 °C in an incubator. After 24, 48, and 72 h, 10 µL of Cell Counting Kit-8 (CCK-8) reaction reagent (Biotech Institute, Haimen, China) was added to each well for 4 h. Cell activity was measured at a wavelength of 450 nm under a microplate reader.

EdU test

Gastric cancer cells were incubated with 50 µM EdU reagent for 22 h. Then, nuclei were stained with 4,6-diamino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA). Fluorescence images were taken under a fluorescence microscope (Olympus, Tokyo, Japan) and the analysis was repeated three times.

Transwell assay

A 24-well Transwell chamber with a pore size of 8 µm was used for migration and invasion assays. For the migration assay, transfected gastric cancer cells (2×10^4) were inoculated using the upper cavity of a Transwell without fetal bovine culture medium. The lower chamber was filled with 1640 medium containing 10 % fetal calf serum. After 36 hours, the cells were fixed with 4 % paraformaldehyde, stained with 0.1 % crystal violet, and observed under an inverted microscope. The invasion assay was similar to the migration assay except that 100 µL of fresh Matrigel medium was dispensed in the upper Transwell chamber before cell seeding.

Balling study

Gastric cancer cells were seeded on transparent round-bottom ultra-low attachment microplates (Corning) and cultured for 7 days. The diameter of the sphere was evaluated under a microscope. The sphere formation test was performed three times.

Western blot assay

Cells were lysed on ice with RIPA lysate and centrifuged at 10000 × g for 13 min at 4 °C to aggregate the precipitates, and then the upper layer of clarified protein lysate was packed and the precipitates were discarded. Proteins were denatured by heating at 100 °C for 10 min and then subjected to SDS-PAGE gel electrophoresis.

Table 1: Primer sequences

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
circRTN4	AGATCCCTGACAGCTGTATTGT	CTTGACCAGACGAAACAGTGT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
U6	GCTTCGGCAGCACATATACTAAAT	CGCTTCACGAATTTGCGTCTCAT
miR-424-5p	GAGGCGTTGTTATATTTTTTCGT	GAAGTTCCTTCTACTCCTAAACCG
LATS2	AAGAGCTACTCGCCATACGCCTT	AGCTTTGGCCATTTCTTGCTCCAG

After transferring, the substance was blocked with 5 % skim milk for 1 hour, and primary antibodies (OCT4, MMP2, LATS2, GAPDH) were incubated overnight at 4 °C, followed by secondary antibody incubation for 1 h. The ECL method development Image J analysis processing was done.

RNA knock-down studies

Biotin-labeled probes, bio-miR-424-5p-wt, and bio-miR-424-5p-mut, were utilized to analyze the enrichment of circRTN4 in cell lysates via RT-qPCR. Microbeads were incubated with the probes and cell lysates. The experiment was conducted in triplicate.

RNA immunoprecipitation (RIP) assay

The RIP analysis was performed using an RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). Cell extracts were mixed with beads coated with Argonaute2 (Ago2; microwell) or immunoglobulin G (IgG; microwell) antibodies. The enrichment of circRTN4 and miR-424-5p was analyzed by qPCR. The RIP analysis was performed in triplicate.

Immunohistochemistry (IHC)

The expression of LAST2 in tumor tissues was determined by immunohistochemistry. The tissue slides were microwaved in 10 mM sodium citrate buffer at pH 6.0 for 10 min for antigen and tissue repair. The cells were incubated with blocking solution containing 0.1 % Triton-X and 10 % normal goat serum at room temperature for 1 h. Then, the primary antibody LAST2 (1: 100) was diluted in blocking solution and incubated overnight at 4 °C, the secondary antibody was incubated for 30 min at room temperature, and HRP-labeled SP working solution was then added and incubated for 30 minutes at room temperature.

Actinomycin D treatment

The stability of circRTN4 was analyzed using the transcription inhibitor actinomycin D (Sigma, St. Louis, MO, USA). Gastric cancer cells were co-cultured with 22 mg/mL actinomycin D for 6, 12, 18, and 24 h. The RNA was collected with TRIzol

reagent and the expression level of circRTN4 was analyzed by RT-qPCR.

Dual-luciferase reporter assay

The cells were co-transfected with miR-424-5p or miR-NC and the reporter plasmid. Luciferase activity was determined using the Dual-Luciferase Reporter Kit (Promega, Madison, WI, USA). The dual luciferase reporter assay was performed in triplicate.

Statistical analysis

SPSS statistical analysis software (version 26.0) was used for data analysis. Relative expression of circRTN4 mRNA is expressed as mean \pm standard deviation (SD), and *t*-test was used to compare the two groups. Kaplan-Meier method was used to draw survival curves, and log-rank test was used for survival analysis. The difference was statistically significant when $p < 0.05$.

RESULTS

Low expression of CircRTN4 in gastric cancer tissues and cell

The GEO database analysis found low expression of circRTN4 in gastric cancer tissues (Figure 1 A). The expression levels of circRTN4 in 70 pairs of GC cancer tissues and corresponding paracancerous tissues were further determined by qRT-PCR, and the results showed that the expression of circRTN4 in GC was significantly reduced, and the difference was statistically significant ($p < 0.001$; Figure 1 B). Taking circRTN4 median-expression value of GC tissue in Figure 1 B as the cut-off, seventy GC patients were divided into circRTN4 low-expression group ($n = 35$) and circRTN4 high-expression group ($n = 35$). Survival analysis showed that the overall survival rate of circRTN4 low-expression group was significantly worse ($p < 0.01$; Figure 1 C). The expression of circRTN4 in gastric cancer cells HGC-27 and AGS was significantly reduced compared to human normal gastric epithelial cells GES-1 ($p < 0.01$; Figure 1 D). At the same time, RNA stability experiments report that after actinomycin D and RNase R treatment, there was no significant effect on

circRTN4 (Figure 1 E and F). Chi-square test was used to analyze the relationship between the expression of circRTN4 and clinicopathological data of GC in the low-expression group (n = 35) and the high-expression group (n = 35) of circRTN4. It was found that circRTN4 expression was closely related to the tumor, node, metastases (TNM) stages, and distal node metastasis ($p < 0.05$). However, it is not related to the patient's gender, age, tumor differentiation, and tumor size (Table 2).

Overexpression of circRTN4

In order to further verify the effect of circRTN4 on the occurrence and development of gastric cancer, first, qRT-PCR was used to confirm that pLCDH-circRTN4 could effectively overexpress circRTN4 in cells compared with pLCDH (Figure 2A). The CCK8 results showed that overexpression of circRTN4 could effectively reduce the light absorption value of HGC-27 and AGS gastric cancer cells at 450 nm wavelength, and the difference was statistically significant compared with the pLCDH group ($p < 0.01$). EUD results indicate that overexpression of circRTN4 could reduce the positive staining rate of EDU in cells, indicating that overexpression of circRTN4 could significantly inhibit the proliferation of gastric cancer cells (Figure 2 B and C). Next, the inhibitory effect of overexpression of circRTN4 on cell migration and invasion was determined by chamber experiment. The results are shown in Figure 2 D and E. Compared with control group, migration, and invasion of cells in pLCDH-circRTN4 group were significantly reduced ($p < 0.01$). Overexpression of circRTN4 also limited the ability to inhibit stemness of gastric cancer cells (Figure 2 F), and Western blot also showed that overexpression of circRTN4 down-regulated MMP2 and OCT4 protein levels (Figure 2 G). These results indicate that overexpression of circRTN4 inhibits the migration, invasion, proliferation, and stem cell capacity of gastric cancer cells.

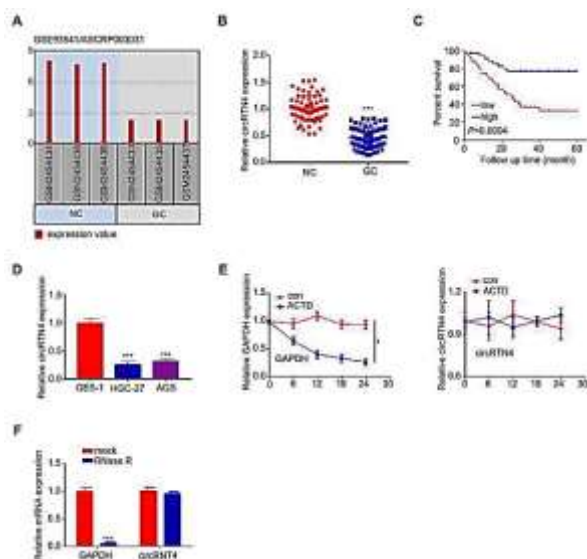


Figure 1: Expression levels of CircRTN4 in GC tissues and cells

Table 2: Relationship between circRTN4 and GC clinicopathological data (n = 35)

Factor	circRTN4 expression		P-value
	Low	High	
Gender			0.809
	Male	20	19
	Female	15	16
Age			0.3372
	≤ 60	21	17
	> 60	14	18
Tumor size			0.3372
	≤ 5 cm	14	18
	> 5 cm	21	17
Pathological differentiation			0.0937
	Well/moderate	13	20
	Poor	22	15
TNM			0.0303
	I/II	15	24
	III/IV	20	11
Lymph node metastasis			0.0312
	Absence	12	21
	Presence	23	14
Distant metastasis			0.0157
	Absence	10	20
	Presence	25	15

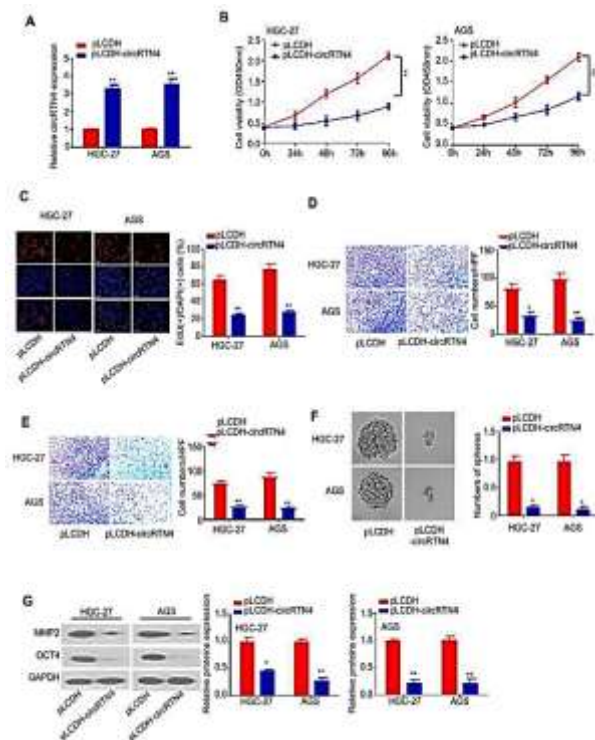


Figure 2: Overexpression of circRTN4 inhibited proliferation, migration, invasion, and stemness of gastric cancer cells

CircRTN4 targeted miR-424-5p

Results showed that expression level of circRTN4 in cytoplasm was significantly higher than that in nucleus (Figure 3 A). It was found that circRTN4 could target miR-424-5 p by bioinformatics prediction and that overexpression of miR-424-5 p could inhibit the activity of luciferase in cells compared with miR-NC by luciferase reporter gene experiment. After the predicted miR-424-5p was mutated at circRTN4 binding site, its inhibitory effect disappeared (Figures 3 B and C). The results of RNA pull-down experiments showed that miR-424-5p probe enrich more circRTN4 than NC probe (Figure 3 D). The results of RIP-qrt-PCR experiments showed that Ago2 group was enriched in more circRTN4 and miR-424-5p than IgG group (Figure 3 E).

Meanwhile, the expression of miR-424-5p was significantly elevated in 70 pairs of gastric cancer (GC) tissues compared to their corresponding paracancerous tissues. Kaplan-Meier survival curve evaluation found that the prognosis of miR-424-5p high-expression group was significantly worse than that of miR-424-5p low-expression group, ($p < 0.01$) (Figures 3 F and G). MiR-424-5p and circRTN4 negatively correlated with the expression of GC (Figure 3 H).

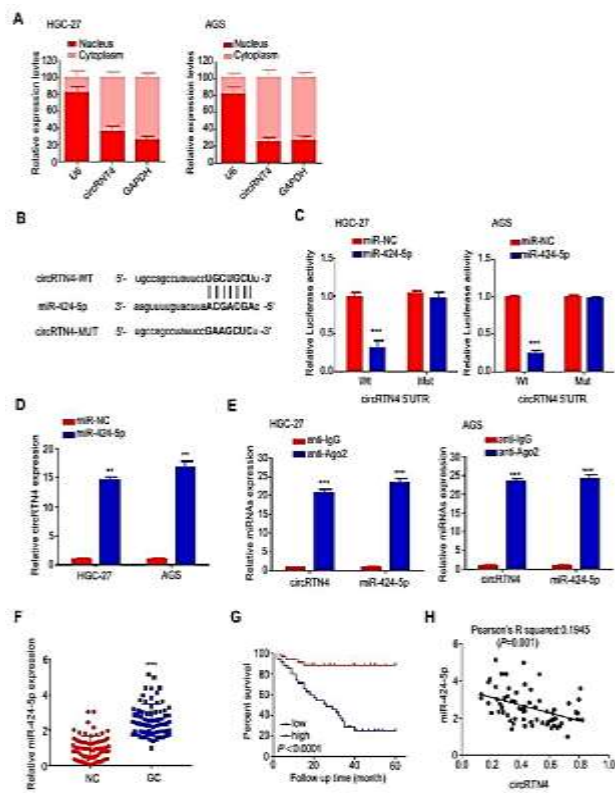


Figure 3: CircRTN4 targeted miR-424-5p

MiR-424-5p targeted LATS2

A miR-424-5p binding site was predicted to exist in a LATS2 3' non-coding region through Starbase, and a luciferase report assay was adopted, and it was discovered that compared with miR-NC, the overexpression of miR-424-5p inhibits the activity of luciferase in cells, Mutation of the predicted LATS2 3'-UTR binding site abolished its inhibitory effect, as shown in Figure 4 A. Western blot results showed that the overexpression of miR-424-5p down-regulated the protein expression of LATS2 (Figure 4 B). QRT-PCR was used to compare the expression of LATS2 in 70 pairs of GC and paracancerous tissues, and the expression of LATS2 in GC tissues was significantly reduced, and the difference was statistically significant, ($p < 0.001$; Figure 4 C).

The prognosis of LATS2 low-expression group was significantly worse than that of LATS2 high-expression group ($p < 0.01$). The rate of positive staining for LATS2 in gastric cancer tissues was low (Figure 4 E). Spearman correlation coefficient analysis found a positive correlation between circRTN4 and LATS2, and a significant negative correlation trend between miR-424-5p and LATS2 expression, ($p < 0.001$; Figure 4 F and G).

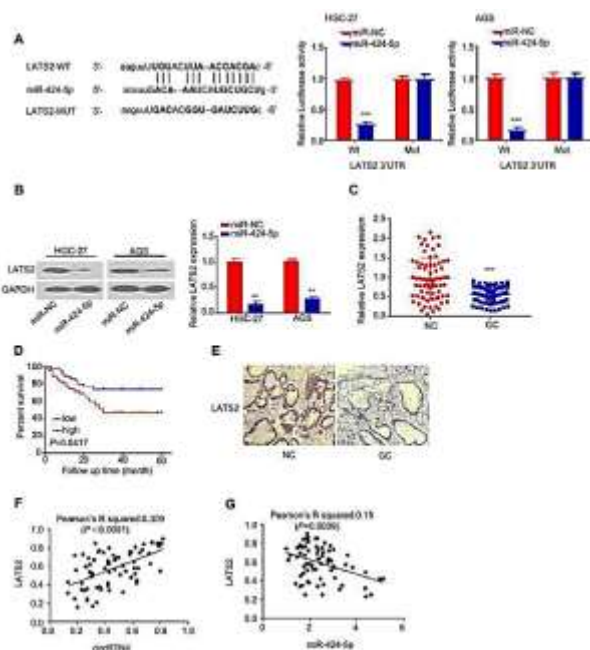


Figure 4: MiR-424-5p Targeted LATS2. (A) Mutation of the predicted LATS2 3'-UTR binding site abolished the inhibitory effect. (B-C) Overexpression of miR-424-5p down-regulated the protein expression of LATS2, and the expression of LATS2 was significantly reduced in GC cancer tissues compared to paracancerous tissues, (D) with a worse prognosis in the LATS2 low expression group. (E) Immunohistochemistry showed low positive staining rate of LATS2 in GC tissues. (F-G) Spearman correlation coefficient analysis found a positive

correlation between circRTN4 and LATS2 and a significant negative correlation trend between miR-424-5p and LATS2 expression

CircRTN4 regulated gastric cancer cell progression

Overexpression of circRTN4 up-regulated the expression level of LATS2 protein, which was partially reduced when co-transfected with miR-424-5p or si-LATS2 ($p < 0.01$) (Figure 5 A). The results of CCK8 and EDU experiments showed that overexpression of circRTN4 reduced cell proliferation, while co-transfection of miR-424-5p or si-LATS2 partially increased cell proliferation (Figure 5 B and C). Transwell results showed that over-expression of circRTN4 reduce the migration and invasion ability of cells; co-transfection of miR-424-5p or si-LATS2 partially increased the migration and invasion ability of cells ($p < 0.01$; Figure 5 D and E). The results of the balling assay showed that overexpression of circRTN4 inhibited the protein levels of MMP2 and OCT4, while co-transfection of miR-424-5p or si-LATS2 partially increased the protein levels of MMP2 and OCT4 ($p < 0.01$; Figure 5 G).

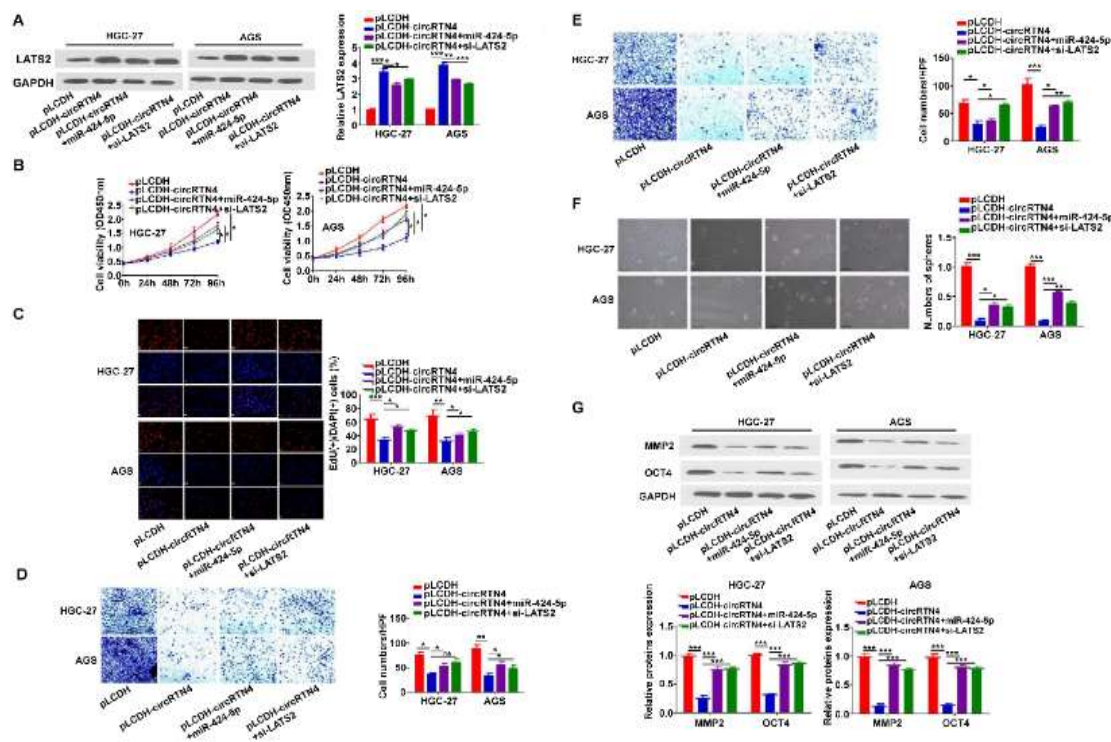


Figure 5: CircRTN4 inhibited GC cell progression by sponging miR-424-5p and regulating LATS2 expression

DISCUSSION

There are new pieces of evidence indicating that circRNA has tissue-specific expression characteristics and plays an important role in the occurrence and development of many human malignant tumors, including gastric cancer [4]. Studies have found that circRTN4 is less expressed in the peripheral blood of gastric cancer patients [5]. The expression and role of circRTN4 in gastric cancer have not been reported.

Here, circRTN4 is down-regulated in gastric cancer by GEO analysis. Based on the Starbase prediction results, miRNAs bound by circRTN4 and downstream target genes were analyzed in depth. This study found that circRTN4 target the expression of miR-424-5p and downstream LATS2. It has been reported that miR-424-5p is highly expressed in gastric cancer tissues [6], but its role in gastric cancer has not been reported, while LATS2 is lowly expressed in gastric cancer and plays a role as a tumor suppressor gene [7,8].

In order to explore the downstream target genes of circRTN4/miR-424-5p, miR-424-5p, and si-LATS2 were transfected at the same time, and the results showed that circRTN4 affected the proliferation, migration, and invasion of gastric cancer cells by regulating the downstream LATS2 gene expression through binding to miR-424-5. Stem cells possess the ability to form spheres, and their expression of MMP2 and OCT4 is notable.

CONCLUSION

Low expression of circRTN4 in gastric cancer tissues and cells regulates the occurrence and development of gastric cancer by up-regulating miR-424-5p and inhibiting the expression of downstream gene LATS2, which is a potential prognostic marker and therapeutic target.

DECLARATIONS

Acknowledgements

This work was supported by University Research Projects in Gansu Province (no. BH2012-021) and Gansu Province outstanding graduate students' "Innovation Star" project.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES

1. Wang H, Wang J. *SLC6A14 promotes cell migration and inhibits autophagy in gastric cancer by regulating JAK2/STAT3 signaling.* *Trop J Pharm Res* 2022; 21(6):1177-1182 doi: 10.4314/tjpr.v21i6.6
2. Qu S, Zhong Y, Shang R, Zhang X, Song W, Kjems J, Li H. *The emerging landscape of circular RNA in life processes.* *Rna Biol* 2017; 14(8): 992-999.
3. Qu S, Yang X, Li X, Wang J, Gao Y, Shang R, Sun W, Dou K, Li H. *Circular RNA: A new star of noncoding RNAs.* *Cancer Lett* 2015; 365(2): 141-148.
4. Ye X, Liu H, Zeng Z. *Long non-coding RNA KCNQ1 opposite transcript 1 (KCNQ1OT1) regulates cell proliferation and invasion via targeting miR-124-3p in gastric carcinoma.* *Trop J Pharm Res* 2021; 20(1):1-9 doi: 10.4314/tjpr.v20i1.1
5. Li T, Shao Y, Fu L, Xie Y, Zhu L, Sun W, Yu R, Xiao B, Guo J. *Plasma circular RNA profiling of patients with gastric cancer and their droplet digital RT-PCR detection.* *J Mol Med (Berl)* 2018; 96(1): 85-96.
6. Zhang J, Liu H, Hou L, Wang G, Zhang R, Huang Y, Chen X, Zhu J. *Circular RNA_LARP4 inhibits cell proliferation and invasion of gastric cancer by sponging*

- miR-424-5p and regulating LATS1 expression. Mol Cancer* 2017; 16(1): 151.
7. Hum M, Tan HJ, Yang Y, Srivastava S, Teh M, Lim YP. WBP2 promotes gastric cancer cell migration via novel targeting of LATS2 kinase in the Hippo tumor suppressor pathway. *Faseb J* 2021; 35(2): e21290.
 8. Sun D, Wang Y, Wang H, Xin Y. The novel long non-coding RNA LATS2-AS1-001 inhibits gastric cancer progression by regulating the LATS2/YAP1 signaling pathway via binding to EZH2. *Cancer Cell Int* 2020; 20: 204.