

Original Article

CircKIF4A regulates the progression of gastric carcinoma by sponging miR-135b

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Abstract: Growing evidence shows that circRNA acts as an miRNA sponge to regulate a variety of tumor types. Gastric carcinoma is one of the most malignant cancers. However, the functions of circRNA and its associated regulatory mechanisms remain largely unknown. In this study, we used quantitative real-time PCR (qRT-PCR) to determine that the circKIF4A expression is abnormal in gastric carcinoma tissues and different tumor cell types. Next, we investigated the circKIF4A function in gastric carcinoma using apoptosis and migration assays. Western blot was used to explore the miR-135b sponging function of circKIF4A in gastric carcinoma. A qRT-PCR demonstrated that circKIF4A is up-regulated in gastric carcinoma. The knockdown of circKIF4A promotes apoptosis and the migration of SGC-7901. Moreover, miR-135b regulates the expression of mRNA (including PARP, Pax-4, FOXJ2, and Elf-1) in gastric carcinoma, and circKIF4A inhibits miR-135b's regulatory function on mRNA (including PARP, Pax-4, FOXJ2, and Elf-1). Taken together, our findings show that circKIF4A acts as an miR-135b sponge, which affects the expression of mRNA (including PARP, Pax-4, FOXJ2, and Elf-1) and ultimately acts as a gastric carcinoma promoter.

Keywords: circKIF4A, miR-135b, gastric carcinoma, comparative endogenous RNA

Introduction

As the fifth most frequently-diagnosed cancer and the third leading cause of cancer-related death, gastric carcinoma seriously threatens patients' lives [1]. Molecular targeted therapy is widely used in tumors [2-4] and has brought new light and hope for the treatment of advanced gastric carcinoma. Therefore, investigators urgently need to develop an understanding of the molecular regulatory mechanisms underlying gastric carcinoma.

CircRNA is a new family of non-coding RNAs that affect many mammalian cell processes via the circRNA-miRNA-mRNA network axis [5, 6]. CircRNA, a potential biomarker for cancer, is used to investigate the progression of the different types of cancer. Some circRNAs have been identified as tumor oncogenes. And circAGFG1 has been found to act as a tumor promoter for triple-negative breast cancer [5]. Other circRNAs are known to be tumor suppressors. For example, circMTO1 is reported to suppress hepatocellular carcinoma progres-

sion [7]. These findings suggest that circRNA is closely related to tumor progression. Also, circKIF4A has also been reported to promote triple-negative breast cancer [8]. However, the function of circKIF4A in gastric cancer remains unclear.

One classical microRNA is miR-135b which has key functions in many cellular processes. Significant evidence indicates that miR-135b acts as oncogenes and tumor suppressor for different types of cancer, suggesting its potential as a therapeutic target. MiR-135b has been reported to act as a promoter of the triple-negative breast cancer [9]. Research confirms that miR-135b inhibits the progression of cutaneous squamous cell carcinoma [10]. However, few studies investigate the roles of miR-135b and circKIF4A in the regulation of human gastric carcinoma, and it remains unclear whether circKIF4A acts as an miR-135b sponge to regulate gastric carcinoma.

In this study, we first investigate the effects of circKIF4A on gastric carcinoma. And then we

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Table 1. Primers used for the qRT-PCR and siRNA related sequences

circKIF4A	F: 5'-GAGGTACCCTGCCTGGATCT-3' R: 5'-TGGAATCTCTGTAGGGCACA-3'
KIF4A	F: 5'-TACTGCGGTGGAGCAAGAAG-3' R: 5'-CATCTGCGCTTGACGGAGAG-3'
GAPDH	F: 5'-AGAAGGCTGGGGCTCATTG-3' R: 5'-AGGGGCCATCCACAGTCTTC-3'
U6	F: 5'-AGGGGCCATCCACAGTCTTC-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
circ-KIF4A siRNA-1	CGGACCUAGAUUUGCAUAAU
circ-KIF4A siRNA-2	ACGGACCUAGAUUUGCAUAA
circ-KIF4A siRNA-3	GGACCUAGAUUUGCAUAAU
Relative si-NC	AAUUCUCCGAACGUGUCACGU

explore the roles of circKIF4A and miR-135b in its progression. Finally, we further study the potential mechanism underlying these roles.

Materials and methods

Grouping

To identify the expression of circKIF4A in gastric carcinoma, the tissues were divided into the tumor tissues and the adjacent normal tissues, and we collected the patients' blood serum and the serum of healthy controls, and we collected different tumor cell types.

To clarify the role of circKIF4A in gastric carcinoma, the SGC-7901 cells were divided into four groups: control, si-RNA-1, si-RNA-2, and si-RNA-3.

To explore whether circKIF4A regulates gastric carcinoma by inhibiting miR-135b-3p, the SGC-7901 cells were divided into four groups: miR-135b-3p, control, circKIF4A + miR-135b-3p, and circKIF4A.

Cell lines and culture

The human gastric carcinoma cell lines were provided by the China Center for Type Culture Collection and included MGC-803, MKN-45, SGC-7901, and HGC-27. The normal GES-1 stomach mucosa epithelium cell line was purchased from ATCC. The cell lines were cultured in complete growth medium containing DMEM medium and an additive with 10% fetal bovine serum (Gibco) and 1% streptomycin/penicillin (Hyclone, USA). The trypsin was obtained from

Gibco. The cells were incubated in a 5% CO₂, 37°C environment.

RNA and qRT-PCR assays for the circKIF4A expression

The tissue sample RNAs were extracted with a Trizol reagent (Invitrogen), and the cell sample RNAs were extracted using miRNeasy mini kits (Qiagen). Quantitative real-time PCR (qRT-PCR) was performed with QuantiNova SYBR Green RT-PCR Kits (Qiagen). U6 was used as an internal reference, with GAPDH as the endogenous control. The primer sequences were synthesized with TaKaRa, and the details are shown in **Table 1**. Three technological replicates were used to ensure the reliability of the analysis.

Construction and transfection of the circKIF4A and miR-135b overexpression plasmids

To construct the circKIF4A overexpression plasmids, amplified circKIF4A cDNA was infused into the pLCDH-ciR-vectors (Invitrogen, Shanghai, China), that have a front and back circular frame. We then designed a mock vector control with no circKIF4A cDNA. siRNA-1, 2, and 3 were used to target and knock down the circKIF4A. We also designed siRNA-NC as a control. qRT-PCR was used to examine the knock-down efficiency. The most effective one was siRNA-1, and it as well as siRNA-2 and siRNA-3 were transfected into the plasmid vector. To overexpress the miR-135b, full-length miR-135b was amplified and then transfected into the overexpression plasmid pLCDH-ciR (Invitrogen, Shanghai, China). All the vectors were verified using sequencing (Invitrogen, Shanghai, China).

For the cell transfections, Lipofectamine 2000 (Invitrogen, USA) was used to deliver the vector into the target cell according to the manufacturer instructions

The si-RNA was synthesized (Biosyntech, China), and the related sequence information is shown in **Table 1**.

Cell apoptosis assay

The cells were treated using Hoechst kits (Beyotime Biotechnology, China) and then analyzed to determine their apoptosis-positive cell percentages under a fluorescence microscope

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instrument (Olympus IM2) according to the Hoechst kit's instructions.

Western blot assays

We performed the western blot assays according to the standard protocols. 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to extract and separate the total proteins, and then the separated protein samples were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The PVDF membranes were blocked for 1 h at room temperature using a blocking buffer, and then they were treated for 2 h at room temperature with one of the following primary antibodies as appropriate: anti-Pax-4 (Abcam, #135598), anti-PARP (abcam, #74290), anti-FOXJ2 (Abcam, #22857), anti-Elf-1 (Santa Cruz, #133096). GAPDH was used as the endogenous control and treated with an anti-GAPDH antibody, obtained from Abcam (#181602). After washing the membranes with TBST buffer three times, the PVDF membrane was incubated with an HRP-secondary antibody at room temperature for 2 h, and then the same washing procedure was performed. Finally, the protein bands were analyzed using chemiluminescence. Secondary antibodies induced m-IgG κ BP-HRP (Santa Cruz, #516102) and goat anti-rabbit IgG-HRP (Abcam, #205718).

Transwell invasion assay

A 24-well Boyden chamber with a non-coated filter in the insert chamber (Corning, USA, #pore size 8 μ m) was used for the migration assays. The cells were seeded into the insert chamber in 0.5 mL free-serum DMEM medium. The cells migrated for 24 h into the bottom chamber containing 0.5 mL DMEM medium and 10% FBS in a CO₂ incubator at 37°C. The migrated cells were stained with 4, 6-diamidino-2-phenylindole (DAPI) and then counted using microscopy.

Statistical analysis

We used triplicate data for accuracy and analyzed the data using SPSS statistical software. For two group comparisons, Student t-test was used. For multiple group comparisons, one-way ANOVA was used with Bonferroni post-tests for the comparisons between the two selected

groups and Dunnett post-tests for comparisons among all other treatment groups to the corresponding control. * indicated P<0.05 was considered statistically significant, ** indicated P<0.01 was considered extremely significant.

Results

CircKIF4A expressions in the gastric carcinoma and in the different tumor cell types

According to the reference genome (GRCh37/hg19), circKIF4A is located in chrX69549254-69553539 and derived from the splicing of exons 8, 9, and 10 (Figure S1). We assessed the impact of circKIF4A on gastric carcinoma by performing qRT-PCR to assay the circKIF4A expression in both the tumor tissues and the adjacent normal tissues derived from the blood serum of the patients and the healthy controls. The results showed that the difference in the circKIF4A expressions between the tumor tissues and the adjacent normal tissues was statistically significant, P<0.01 (Figure 1A). The differences in the circKIF4A expressions in the blood serum between the healthy controls and the patients were not significant P>0.05 (Figure 1B and 1C).

We performed an additional qRT-PCR to assay the circKIF4A expression of the different tumor cell types. Our findings indicated that circKIF4A is strongly expressed in MGC-803, SGC-7901, and MDA-MB-231, second only to lung cancer cells relative to the GES-1 cells. Thus, we selected SGC-7901 as our cell model for the regulatory pathway of circKIF4A on gastric carcinoma.

The knockdown of circKIF4A promoted apoptosis and inhibited migration in gastric carcinoma

We performed RNA interference to knock-down the expression of circKIF4A in order to analyze its potential functions in gastric carcinoma. qRT-PCR demonstrated that the expression of circKIF4A was strongly inhibited and circKIF4A with the circle structure resisted the RNase-R digestion (Figure 2A). Next, we found that si-RNA was specific only to circKIF4A, not to KIF4A (Figure 2B). Moreover, we found that KIF4A and free siRNA were sensitive to RNase digestion (Figure 2B). These results showed that the circular structure of cir-

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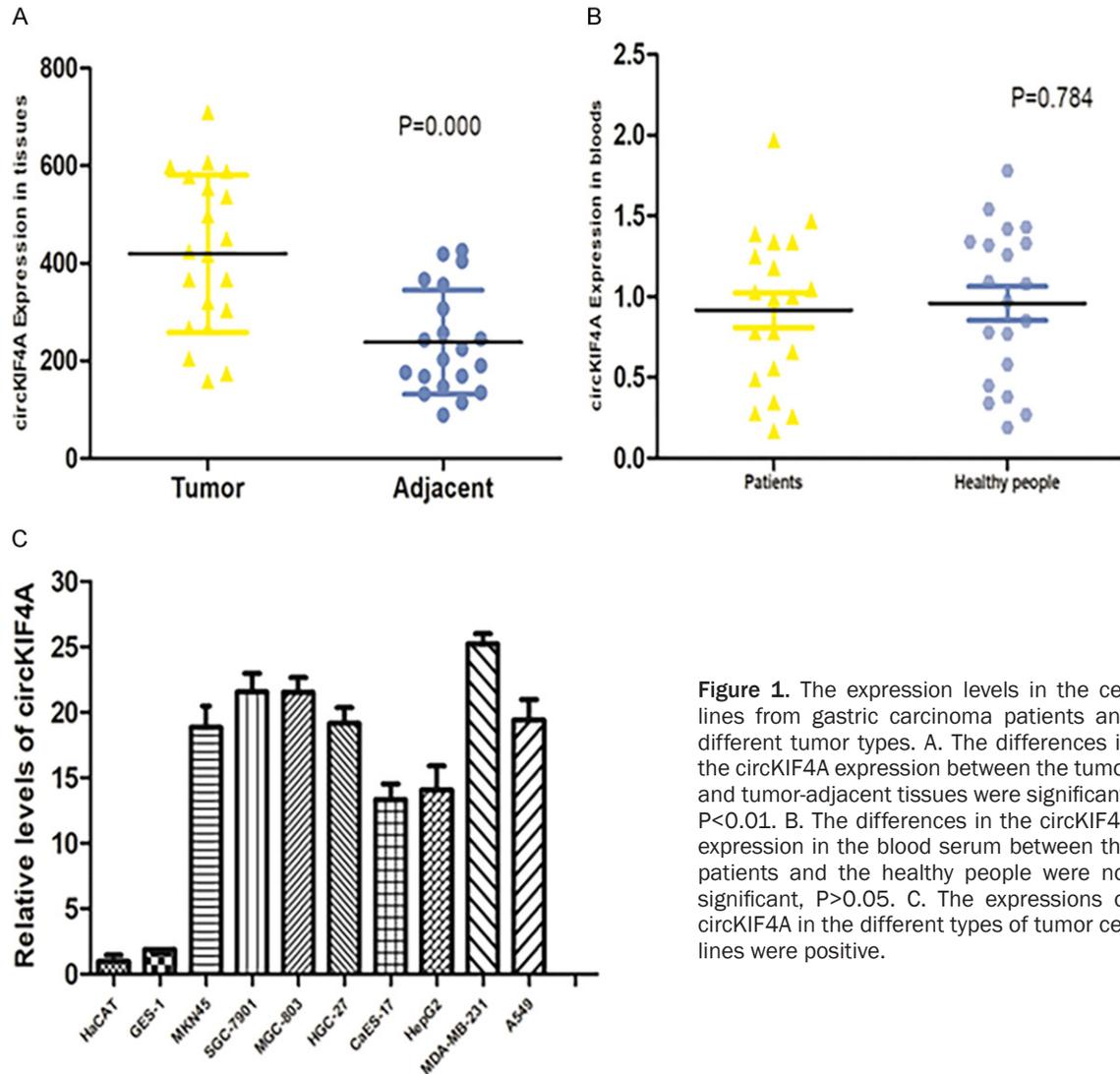


Figure 1. The expression levels in the cell lines from gastric carcinoma patients and different tumor types. A. The differences in the circKIF4A expression between the tumor and tumor-adjacent tissues were significant, $P < 0.01$. B. The differences in the circKIF4A expression in the blood serum between the patients and the healthy people were not significant, $P > 0.05$. C. The expressions of circKIF4A in the different types of tumor cell lines were positive.

crRNA was responsible for its stability and abundance in gastric carcinoma tissues.

The Hoechst-apoptosis assays showed that the inhibition of circKIF4A promotes the apoptosis of SGC-7901 cells (Figure 3). Furthermore, the Transwell assays demonstrated that an over-expression of circKIF4A promoted the migration of SGC-7901 cells. However, the knockdown of circKIF4A inhibited the migration of SGC-7901 cells (Figure 4). These results suggest that circKIF4A plays a role in the promotion of cell growth and in the progression of gastric carcinoma.

CircKIF4A acts as a sponge for miR-135b

The predicted binding sites of miR-135b to circKIF4A according to the RegRNA databases

are shown (Figure S2). We selected miR-135b as a potentially functional miRNA absorbed by circKIF4A in SGC-7901 cells. These genes included Pax-4, PARP, FOXJ2, and Elf-1, which were reported to play roles in cell growth and in the regulation of tumors. Western blots and related analyses were performed. In the next phase, we investigated four cell models: the control, miR-135b transfected only, circKIF4A-transfected only, and miR-135b + circKIF4A co-transfected (Figure 5). Overall, the expression of Pax-4, PARP, and Elf-1 in SGC-7901 was dramatically decreased in the miR-135b-transfected only model, while the PARP was dramatically increased relative to the control. Next, we co-transfected the miR-135b and circKIF4A into SGC-7901 cells and found that the inhibitory effects on PARP, FOXJ2, Pax-4,

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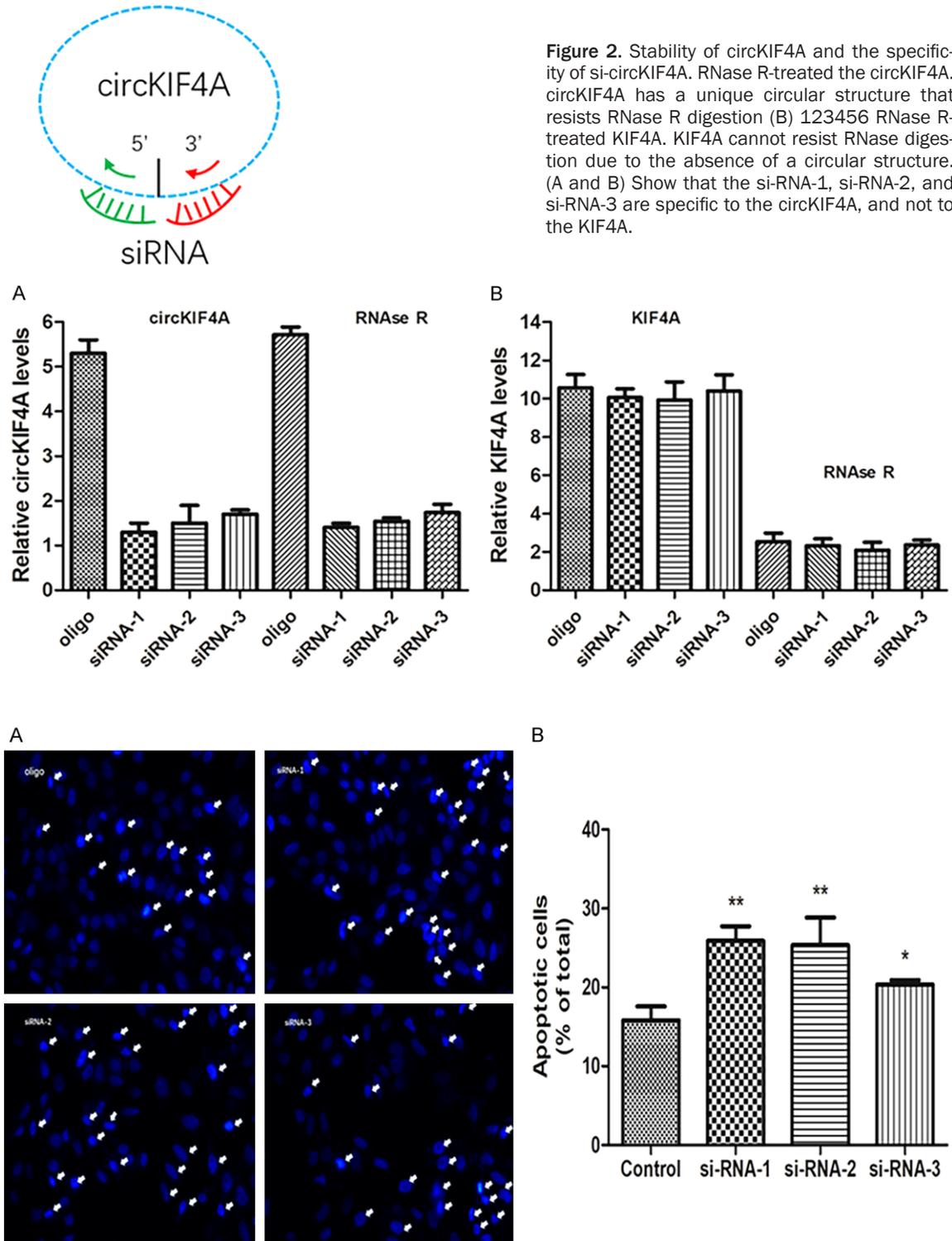


Figure 3. Silencing the circKIF4A induced the SGC-7901 cell line apoptosis. A. A fluorescence micrograph of the SGC-7901 cell line apoptosis via circKIF4A silencing. The fluorescent dye used was Hoechst, excitation max: 350 nm; emission max: 448 nm. B. Statistical analyses of the percentages of the SGC-7901 apoptotic cells. Overall, the statistical differences in the apoptotic percentages between the si-RNA-1/si-RNA-2 treated group and the control were extremely significant, $P < 0.01$. The statistical differences in the apoptotic percentages between the si-RNA-3 and the control were significant, $P < 0.05$.

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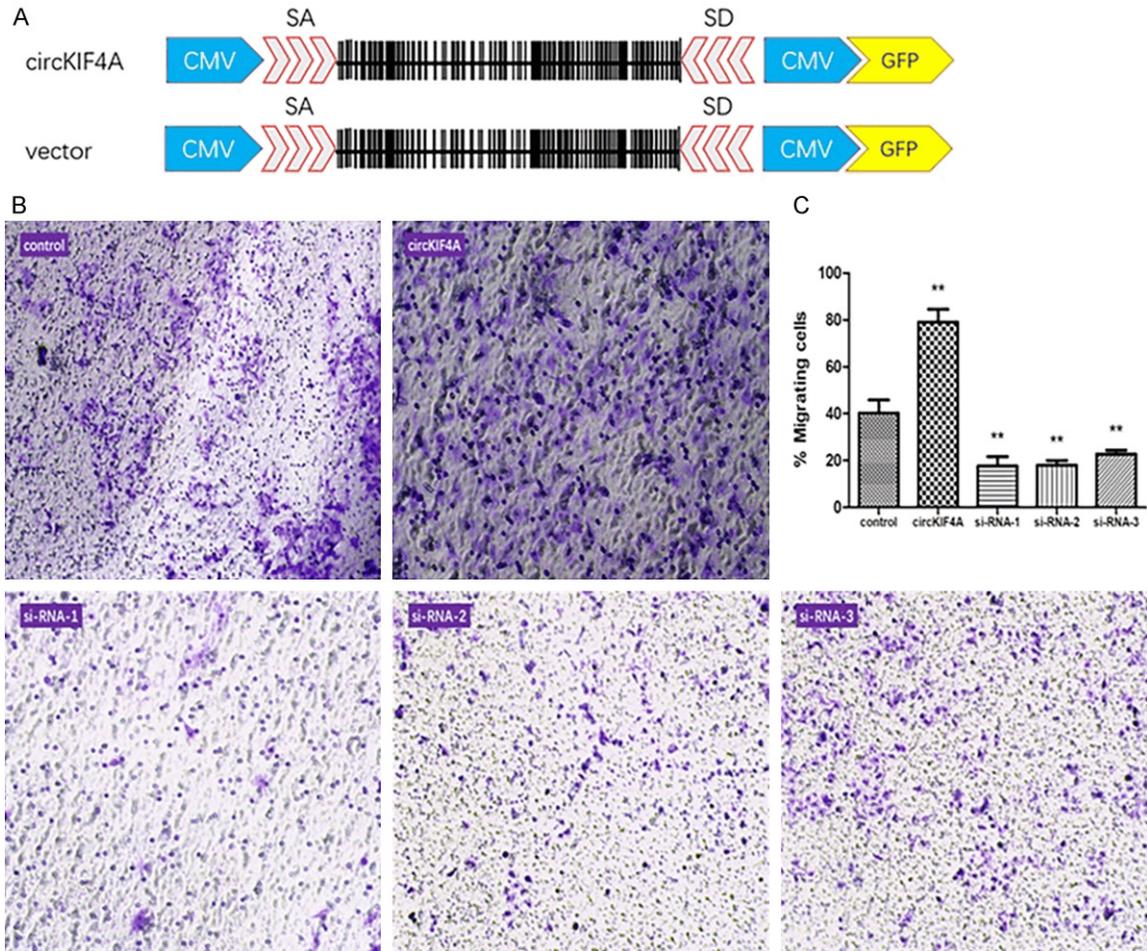


Figure 4. The impact of the overexpression and silencing of circKIF4A on the SGC-7901 cell migration. A. The design strategy for the circKIF4A overexpression in the SGC-7901 cell. B. A micrograph of the impact of the overexpression and silencing of circKIF4A on the migration of SGC-7901 cells. Overall, the overexpression significantly promoted the SGC-7901 cell migration relative to the control. However, the three si-RNA groups significantly inhibited the SGC-7901 cell migration in contrast to the control. The statistical analysis of the percentage is on the positive migrating SGC-7901 cells. Bar = 100 μ m. C. The statistical analysis of the percentages of migration-positive SGC-7901 cells. Overall, differences between the control and overexpression groups were extremely significant, $P < 0.01$. However, the reduction in the percentages of the migration-positive SGC-7901 cells for each of the three si-RNA groups were extremely significant and relative to the control group. $P < 0.01$.

and E1f-1 of miR-135b were reversed by the circKIF4A. In conclusion, circKIF4A acted as a sponge to reverse the function of miR-135b and regulate the progression of gastric carcinoma. The data suggest that the progression of gastric carcinoma is tightly regulated by the circKIF4A/miR-135b/mRNA (PARP, Pax-4, FOXJ2, and E1f-1) axis.

Discussion

Non-coding RNA has been reported to regulate the progression of gastric carcinoma. However, whether circKIF4A acts as a sponge of miR-

135b to regulate this progression remains unknown. CircRNA has a unique circular structure that adds to its stability and makes it a potential tumor biomarker [11, 12]. In our study, we found that the expression of circKIF4A in tumor tissues was greater than it is in normal tissues near tumors. On the other hand, there were no significant differences in the circKIF4A expression in the peripheral blood serum between the healthy controls and the patients. Moreover, the levels in the different cell lines such as MGC-803, SGC-7901, and MDA-MB-231 relative to the controls were particularly high. Significant evidence indicates

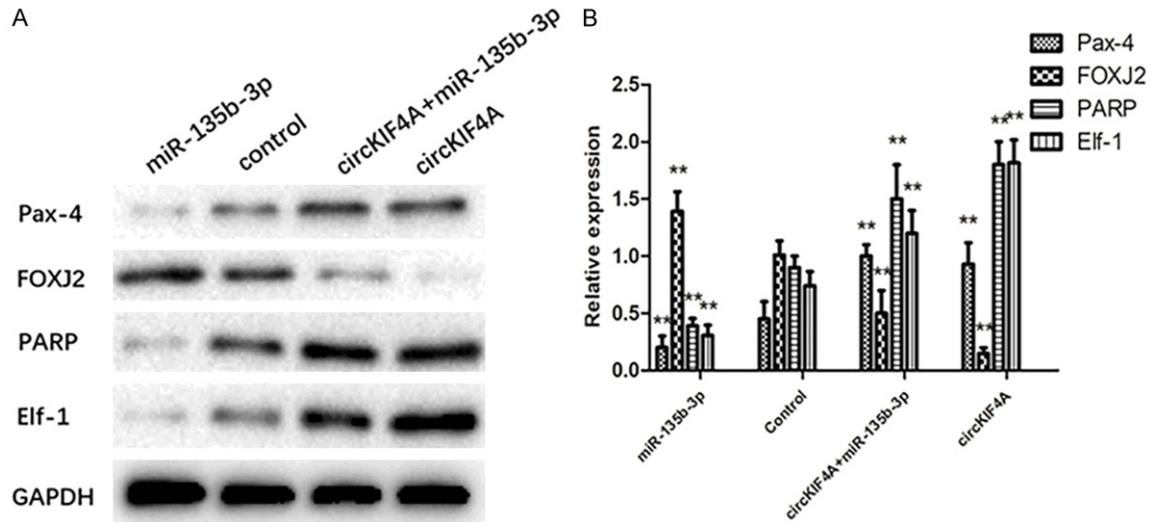


Figure 5. The effects of circKIF4A acting as an miR-135b sponge to regulate the expressions of the targeted genes, including Pax-4, FOXJ2, PARP, and Eif-1 of the SGC-7901 cells. The SGC-7901 cells were divided into 4 groups: control, miR-135b (miR-135b transfection), circKIF4A + miR-135b (cotransfection of circKIF4A and miR-135b), and circKIF4A (circKIF4A transfection). A. The Western blot map of circKIF4A: the data shows that circKIF4A acts as a sponge to reverse the effect of miR-135b regulating the target genes PAX-4, FOXJ2, PARP, and Eif-1. B. A statistical analysis of the effect of circKIF4A as a miR-135b sponge to regulate the expression of the target genes Pax-4, FOXJ2, PARP and Eif-1. Overall, the circKIF4A + miR-135b group reversed the regulation of Pax-4, FOXJ2, and PARP and the Eif-1 expression.

that there is a close relationship between abnormal circRNA expression and tumor progression. We found that the knock-down of circKIF4A in the SGC-7901 cells induced cell apoptosis. This suggests that circKIF4A promotes gastric tumor proliferation.

microRNAs are small (19-to 25-nucleotide) non-coding, single-stranded RNAs that play a role in the tumor process [13]. Studies have found that miR-135b has a key function in the regulation of a variety of cellular process such as cell growth and metastasis in many cancers. In colorectal cancer, miR-135b promotes cancer cell proliferation and inhibits apoptosis through the negative regulation of Transforming Growth Factor Beta Receptor II (TGFB2) [14]. In cervical cancer, miR-135b promotes cell growth by targeting FOXO1 [15]. In cutaneous squamous cell carcinoma, miR-135b promotes cell mobility and invasiveness by down-regulating leucine zipper tumor suppressor 1 (LZTS1) [10]. In prostate cancer, miR-135b acts as a suppressor to inhibit metastasis by targeting the STAT6 [16]. Therefore, it is worth exploring miR-135b-targeted gene therapy for the treatment of different tumors types.

The ceRNA based regulatory network hypothesis described how competing endogenous RNAs (ceRNAs) bind with non-coding RNAs via interaction elements and then inhibit the non-coding RNA regulatory function targeting down-stream gene expression [17, 18]. miR-29b-1 regulates the expression of SPIN1 and suppresses cell growth [19]. Cdr1as acts as a circRNA sponge for miR-671 and regulates brain function [20]. Some studies report construction of a circRNA-associated ceRNA network in the skin and that this network regulates skin chromatinosis [21]. Therefore, circRNA is hypothesized to act as a miRNA sponge for the regulation of cancer progression [22]. One of the key points of our study is that we explored the role of circKIF4A as a miR-135b sponge in the regulation of gastric carcinoma progression.

Elf-1 is reported to regulate tumor cell growth and apoptosis. Elf-1 is up-regulated in cervical cancer and used as a malignant marker for that disease [23, 24]. FOXJ2, a member of the FOX protein family, plays a role in regulating the migration of breast cancer by targeting E-cadherin [25]. PARP plays a role in the process of cell apoptosis, and the inhibition of

PARP reduces the percentage of apoptotic cells [26, 27]. PARP acts as a tumor promoter, and PARP inhibitors are reported to impact the overall survival in ovarian cancer patients [28]. In the present study, we found that miR-135b inhibits the expression of Elf-1, PARP, FOXJ2, and Pax-4 in SGC-7901 cells, and circKIF4A reverses the regulatory role of miR-135b. This evidence supports the theory that circKIF4A acts as a sponge of miR-135b.

Conclusion

In conclusion, our findings show that circKIF4A acts as a miR-135b sponge, which affects the expression of mRNA (including PARP, Pax-4, FOXJ2, and Elf-1) and ultimately acts as a promoter of gastric carcinoma.

Disclosure of conflict of interest

None.

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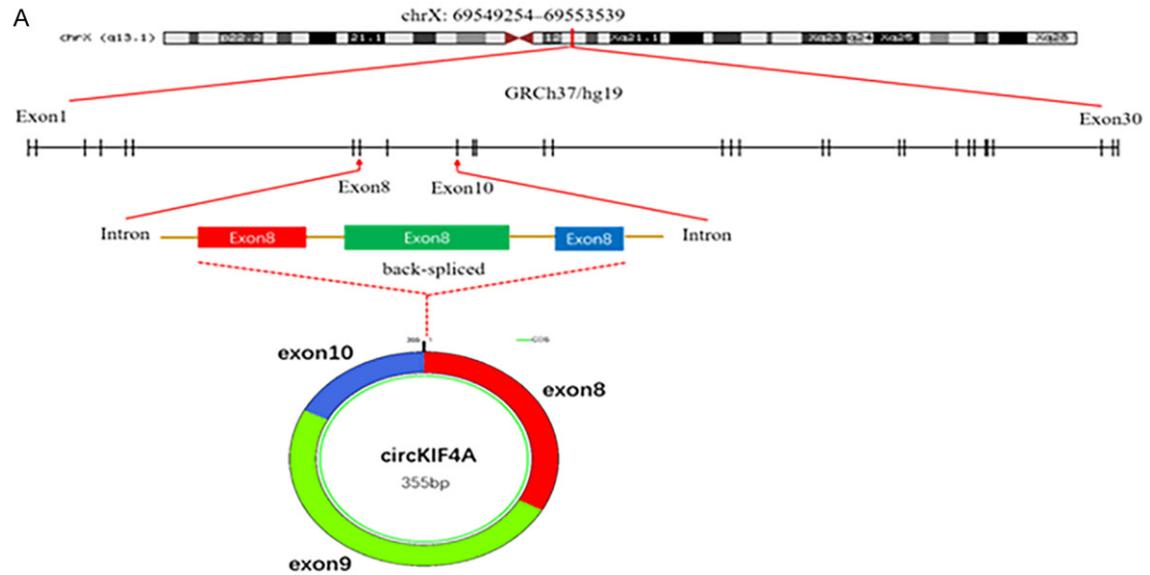
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B

Sequence of circKIF4A

>GRCh37/hg19 located at chrX: 69549255-69553539

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GTATTAATATTAACCGAGGCCTCCTATGCTTGGGAAATGTAATCAGTGCTCTTGGAGATGACAAAAA  
GGGTGGCTTTGTGCCCTACAGAGATTCCAAGTTGACTCGACTGCTTCAAGATTCTCTAGGAGGTAAT  
AGCCATACTCTTATGATAGCCTGTGTGAGTCCTGCTGACTCCAATCTAGAGGAAACATTAATACCC  
TTCGCTATGCTGACAGAGCAAGAAAAATCAAGAACAAACCTATTGTTAATATTGATCCCCAGACAGC  
TGAACCTAATCATCTAAAGCAACAGGTACAACAGCTACAAGTCTTGTTGCTACAGGCCCATGGAGGT  
ACCTGCCTGGATCTATAAC
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Figure S1. Summary depictions of circKIF4A. A. circKIF4A is located at chrX: 69549255-69553539 and is formed by splicing exon 8, exon 9 and exon 10. B. The circKIF4A sequence.

