ORIGINAL ARTICLE

Circ-ZNF609 promotes migration of colorectal cancer by inhibiting Gli1 expression via microRNA-150

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Summary

Purpose: To investigate the effect of circular (circ)-ZNF609 on the pathogenesis of colorectal cancer and its underlying mechanism.

Methods: 24 cases of postoperative colorectal cancer tissues and 36 cases of mucosa tissues were selected as experimental group and control group, respectively. Circ-ZNF609 expression in colorectal cancer tissues and mucosa tissues were detected by quantitative real-time PCR (qRT-PCR). For in vitro experiments, subcellular localization of Circ-ZNF609 in nuclear and cytoplasmic HCT116 cells was assessed. MicroRNA-150 was found to bind to Circ-ZNF609 by dual luciferase reporter assay. Furthermore, migration ability of transfected HCT116 cells was assessed by Transwell assay. Additionally, mRNA and protein levels of glioma-associated oncogene 1 (Gli1) in HCT116 cells were detected by qRT-PCR and Western blot, respectively.

Results: Higher expressions of Circ-ZNF609 and Gli1 were found in colorectal cancer tissues compared to paracancerous tissues. MicroRNA-150 was downregulated in colorectal cancer tissues. Pearson correlation analysis showed that Circ-ZNF609 was positively correlated with Gli1, and micro-RNA-150 was negatively correlated with Circ-ZNF609 and Gli1. Dual luciferase reporter assay confirmed that micro-RNA-150 was bound with cytoplasmic Circ-ZNF609. Furthermore, downregulated Circ-ZNF609 inhibited migration of HCT116 cells. In addition, knockdown of Circ-ZNF609 or overexpression of microRNA-150 inhibited cell migration, which was reversed by co-transfection with microRNA-150 inhibitor and Circ-ZNF609 siRNA.

Conclusions: Circ-ZNF609 regulates Gli1 expression via microRNA-150, thus affecting the migration of colorectal cancer.

Key words: CircRNA, colorectal cancer, migration, proliferation

Introduction

Colorectal cancer is one of the most common malignant tumors. In 2012, there were 1.4 million new cases and 693,900 death cases of colorectal cancer worldwide [1]. Since sporadic colorectal cancer without genetic or family history is typically represented, variations of molecular signaling pathways related to the occurrence and progression of colorectal cancer are of great significance. Although a large number of studies have been carried out, the specific pathogenesis of colorectal cancer has not yet been fully elucidated. Non-coding RNAs are well recognized in recent years, and demonstrate close relationship with regulatory genes and tumors. In addition to a number of long non-coding (lnc)RNAs that were associated with colorectal cancer [2-4], accumulating evidence demonstrated that circRNAs are directly involved in colorectal cancer via regulating proliferation, metastasis and invasion of tumor cells.

CircRNAs participate in precursor RNA cleavage, regulation of RNA expression, protein translation and protein interaction at post-transcriptional

level. Yang et al. showed that circRNAs which are inserted with internal ribosome entry site (IRES) and enriched with m6A can be translated in cells [5]. Bachmayra-Heyda et al. found that circRNAs are downregulated in colorectal cancer tissues by RNA-seq technology [6]. Other researchers have identified 39 differentially expressed circRNAs in colorectal mucosa and colorectal cancer tissues [7]. Among them, overexpressed ciRS-7 in colorectal cancer cells can inhibit miR-7 and activate oncogenes, EGFR and RAR1, which is a promising prognostic biomarker for colorectal cancer patients [8]. In addition, hsa_circ_001988 was found to serve as a potential biomarker for colorectal cancer [9]. Circ-ZNF609 has been shown to be involved in myogenesis and vascular endothelial dysfunction [10,11], but its possible role in colorectal cancer still remains unclear.

It has been reported that Gli1 is downregulated in colorectal cancer tissues. Inactivation of Gli1 decreases the negative regulation of PIP3/Akt signaling pathway. The accumulation of PIP3 further leads to enhanced activation of Akt, thus resulting in uncontrolled cell proliferation, cancer deterioration, tumor development and increased possibility of metastasis [12-14]. Prognostic analysis of colorectal cancer found that Gli1 deficiency significantly affects the efficacy of Cetuximab in patients with wild-type KRAS metastatic colorectal cancer. It is reported that 19.9% of colorectal cancer patients with Gli1 deficiency have poorer overall survival, suggesting that Gli1 might serve as an independent prognostic indicator of colorectal cancer [15]. Recent studies have confirmed that downregulated microRNA-150 in colorectal cancer increases Gli1 expression [16]. Our study mainly explored the possible roles of Circ-ZNF609, microRNA-150 and Gli1 in the pathogenesis of colorectal cancer and their underlying mechanism.

Methods

Subjects

Twenty-four paraffin-embedded specimens of colorectal cancer tissues from patients surgically resected and diagnosed with colorectal cancer in Ningbo No.9 Hospital from July 2012 to July 2017 composed the experimental group. All the included patients did not receive any preoperative radiotherapy and chemotherapy. There were 14 males and 10 females, aged from 36 to 86 years (median 52). Among them, 2 cases were well-differentiated, 18 cases were moderately differentiated and 4 cases were poorly differentiated. There were 9 cases with lymph node metastasis and 15 cases without. Three cases were in Dukes stage A, 15 cases in Dukes B and 6 cases in Dukes C. In addition, 36 normal mucosa tissues 5 cm away from the tumor edge composed

the control group. All subjects signed informed consent and the study was approved by the ethics committee of Ningbo No.9 Hospital.

QRT-PCR

Total RNAs in cells and tissues were extracted by TRIzol reagent and reverse transcribed into cDNAs according to the instructions of PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan). GAPDH was selected as the loading control. The relative concentration was calculated by the $2^{\Delta\Delta CT}$ method. Primer sequences used in this study were as follows: Circ-ZNF609 Forward primer: 5'-CAGCGCTCAATCCTTTGGGA-3', Reverse primer: 5'-GACCTGCCACATTGGTCAGTA-3'; Gli1 Forward primer: 5'-GGGTGCCGGAAGTCATACTC-3', Reverse primer: 5'-GCTAGGATCTGTATAGCGTTTGG-3'; GAPDH Forward primer: 5'-GCACCGTCAAGGCTGAGAAC-3', Reverse primer: 5'-GGATCTCGCTCCTGGAAGATG-3'.

Cell culture and transfection

HCT116 colorectal cancer cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin. Cells were maintained in a 5% CO_2 incubator at 37°C. Circ-ZNF609 siRNA and overexpression plasmid, microRNA-150 mimics and inhibitor, as well as negative control (NC) were constructed for cell transfection based on the instructions of Lipofectmaine 2000. Briefly, HCT116 cells were seeded in 6-well plates at a density of 4×10^5/mL and then transfected with the abovementioned plasmids when cell confluence was up to 80-90%. Subsequent *in vitro* experiments were performed after 24-48 hrs of transfection. Transfection reagents were the following: Circ-ZNF609 siRNA Sense 5'-GUCAAGUCUGAAAAGCAAUGATT-3', Antisense 5'-UCAUUGCUUUUCAGACUUGACTT-3'; microRNA-150 mimics Sense 5'-UCUCCCAACCCUUGUACCAGUG-3'.

Western blot

The total protein was extracted by radioimmuno-precipitation assay (RIPA) reagent (Sigma, St. Louis, MO, USA). Protein samples were then separated by 10% sodium dodecyl sulphate (SDS) protein electrophoresis after the concentration of each sample was adjusted to the same level. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane and routinely immunostained at 4°C overnight (anti-Gli1, Abcam, ab49314, Cambridge, MA, USA). After washing with tris buffered saline-tween (TBST) for 3 times, membranes were incubated with the secondary antibody (l:1000) at room temperature for 1 hr. All membranes were exposed by enhanced chemiluminescence method.

Transwell assay

Forty-eight hrs post-transfection, the cells were digested with trypsin and resuspended in serum-free medium. The cell density was adjusted to 2.0×10^5/mL. Transwell chambers with or without Matrigel were placed in the 24-well plates, respectively. Two hundred μL of cell suspension were added to the upper chamber, and 500 μL of DMEM medium containing 10% FBS were

added to the chamber. After 48-hr inoculation, cells were fixed with 4% polyoxymethylene for 30 min at room temperature. After fixation, colonies were stained with crystal violet for 15 min and washed with PBS twice. The inner surface of the basement membrane was carefully cleaned. Finally, cells were counted from 5 randomly chosen fields per well.

Dual luciferase reporter assay

HCT116 cells were seeded in a 24-well plate and co-transfected with microRNA-150 mimics and wild-type pGL3-Circ-ZNF609, microRNA-150 mimics and mutant-type pGL3-Circ-ZNF609, NC and wild-type pGL3-Circ-ZNF609, NC and mutant-type pGL3-Circ-ZNF609, respectively. After incubation for 48 hrs, relative luciferase activity was detected based on the recommendations of Dual-Glo®Luciferase Assay System (Promega, Madison, WI, USA).

Cytoplasmic and nuclear extraction

HCT116 cells were collected, resuspended in 100 μL Buffer A and placed on ice for 15 min (Buffer A: 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.15% NP-40), followed by centrifugation at 12,000 rpm at 4°C for 1 min. The supernatant was discarded and the pellet was washed with 1 mL of Buffer A 3 times and resuspended in 150 μL of Buffer B (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40), followed by centrifugation at 12,000 rpm at 4°C for 30 min. The supernatant nuclear extraction was collected.

Statistics

Stata 9.2 software was used for statistical analyses. Continuous variables were shown as mean ± standard deviation. The *t*-test was used to compare the data between two groups, and chi-square test was used to compare categorical variables. P<0.05 indicated the difference was statistically significant (*p<0.05,**p<0.01,***p<0.001).

Results

Expressions of Circ-ZNF609, Gli1 and microRNA-150 in colorectal cancer tissues

Higher mRNA expressions of Circ-ZNF609 and Gli1 were observed in colorectal cancer tissues compared to paracancer tissues (p<0.001, Figure 1A, 1B). MicroRNA-150 was downregulated in colorectal cancer tissues (p<0.001, Figure 1C). In addition, Circ-ZNF609 was positively correlated with Gli1 (R^2=0.6091, p<0.001, Figure 1D), and microRNA-150 was negatively correlated with Circ-ZNF609 (R^2=0.3884, p=0.0012) and Gli1 (R^2=0.4059, p=0.008, Figure 1E, 1F), respectively.

Subcellular localization of Circ-ZNF609

Subcellular localization of lnc RNA was directly related to its biological function. To determine the subcellular localization of Circ-ZNF609 in HCT116
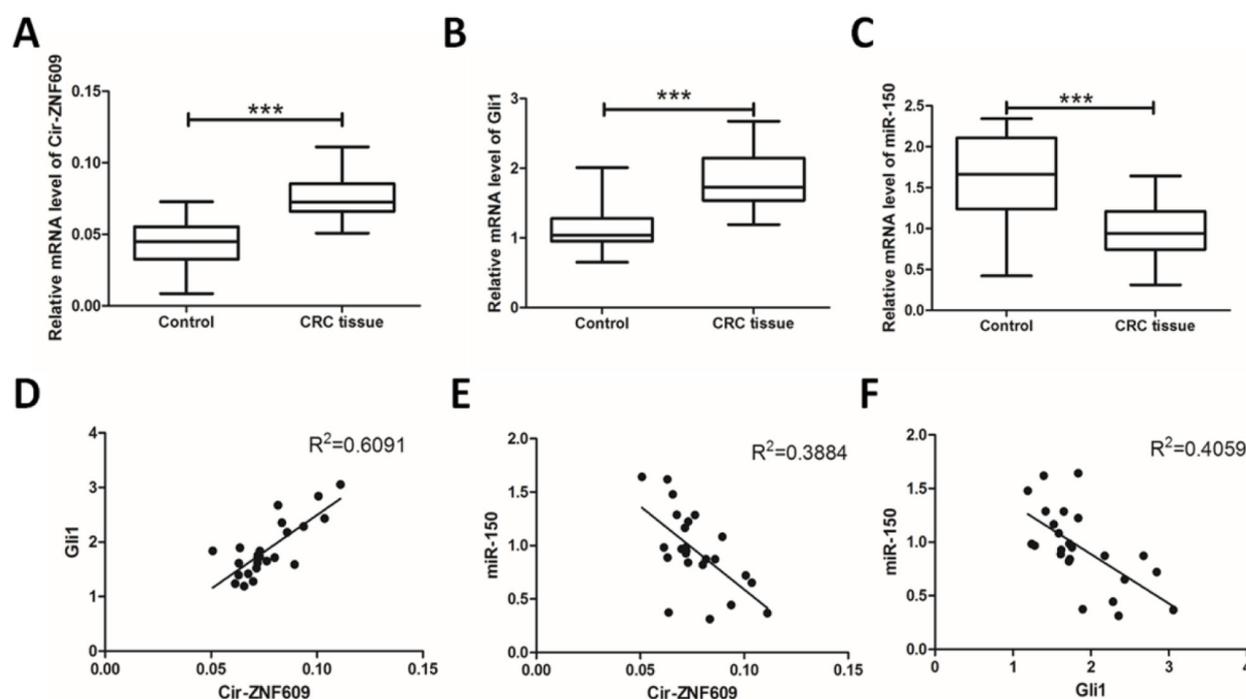

Figure 1. Expressions of Circ-ZNF609, microRNA-150 and Gli1 in colorectal cancer tissues. **A-C:** Higher mRNA expressions of Circ-ZNF609 and Gli1 in colorectal cancer tissues were found compared with those of paracancer tissues (***p<0.001). MicroRNA-150 was downregulated in colorectal cancer tissues (***p<0.001). **D:** Circ-ZNF609 was positively correlated with Gli1 in colorectal cancer tissues (R^2=0.6091, p<0.001). **E:** MicroRNA-150 was negatively correlated with Circ-ZNF609 in colorectal cancer tissues (R^2=0.3884, p=0.0012). **F:** MicroRNA-150 was negatively correlated with Gli1 in colorectal cancer tissues (R^2=0.4059, p=0.008).

cells, the cytoplasmic and nuclear extraction assay were performed. QRT-PCR results found that U6 was mainly present in the nucleus, while GAPDH was mainly present in the cytoplasm, indicating that Circ-ZNF609 was predominantly presented in the cytoplasm of HCT116 cells (Figure 2A). The study indicated that Circ-ZNF609 participated in the development and progression of colorectal cancer at the post-transcriptional level.

Circ-ZNF609 was bound to microRNA-150

MicroRNA-150 was verified to bind to Circ-ZNF609 by starBase prediction [17] (Table 1). To determine the interaction between microRNA-150 and Circ-ZNF609, HCT116 cells were co-transfected with microRNA-150 mimics and wild-type pGL3-Circ-ZNF609, microRNA-150 mimics and mutant-type pGL3-Circ-ZNF609, NC and wild-
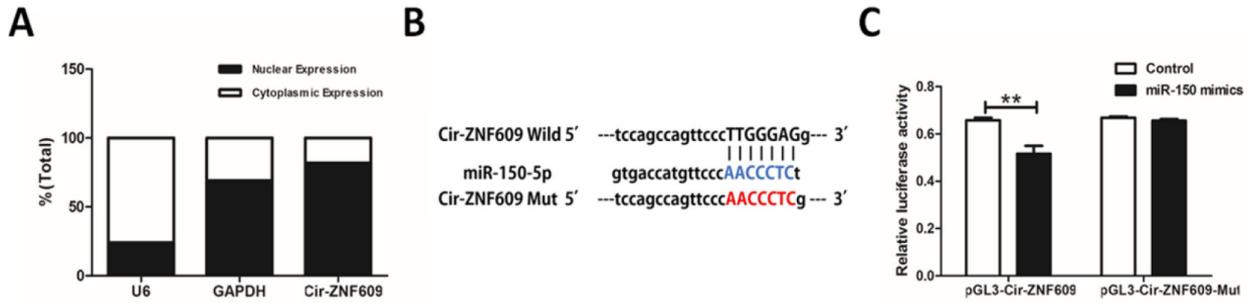

Figure 2. Interaction between Circ-ZNF609 and microRNA-150. **A:** Circ-ZNF609 was mainly present in the cytoplasm. **B:** Binding sites of microRNA-150 mimics and wild-type and mutant-type Circ-ZNF609, respectively. **C:** Fluorescence values in cells transfected with microRNA-150 mimics and wild-type Circ-ZNF609 were remarkably decreased (**p=0.009). No significant differences in fluorescence values were found in other groups.
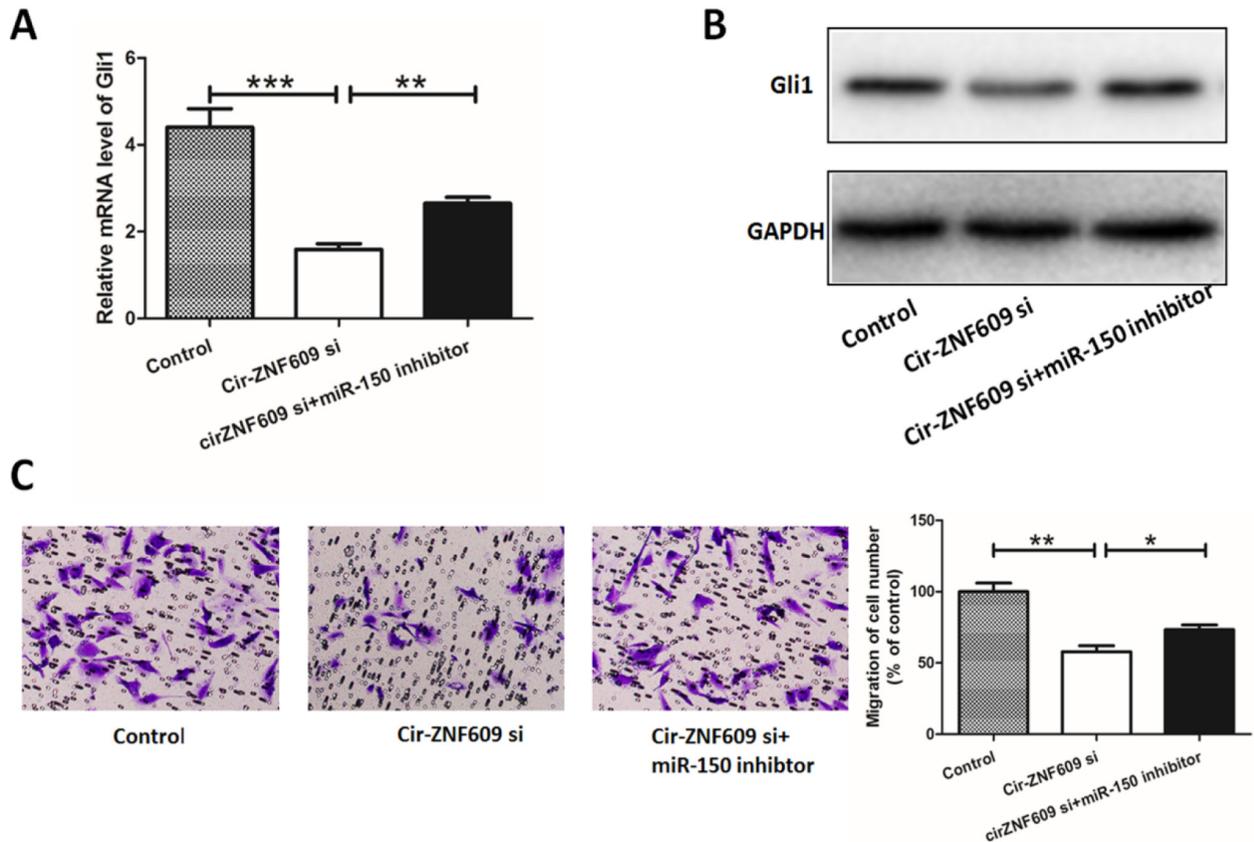

Figure 3. Circ-ZNF609 promoted cell migration and regulated Gli1 expression. **A, B:** The mRNA and protein expressions of Gli1 were decreased after transfection of Circ-ZNF609 siRNA in HCT116 cells, compared with the control group; meanwhile, the mRNA and protein expressions of Gli1 were increased after co-transfection of Circ-ZNF609 siRNA and microRNA-150 inhibitor in HCT116 cells, compared with transfection of Circ-ZNF609 siRNA. **C:** decreased cell migration was observed after transfection of Circ-ZNF609 siRNA in HCT116 cells (p<0.001), which was all reversed by co-transfection of Circ-ZNF609 siRNA and microRNA-150 inhibitor. *p<0.05, **p<0.01, ***p<0.001.
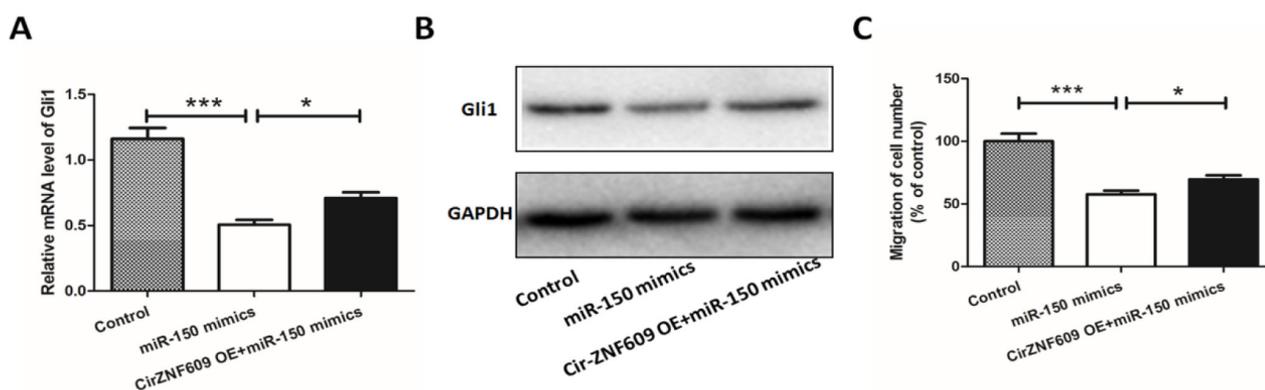
Figure 4. Circ-ZNF609 regulated cellular function via microRNA-150 and Gli1. **A, B:** Lower mRNA and protein levels of Gli1 were found in cells transfected with microRNA-150 mimics (p<0.01), which were both reversed by co-transfection of microRNA-150 mimics and Circ-ZNF609 overexpression plasmid (p=0.038). **C:** After co-transfected with microRNA-150 mimics and Circ-ZNF609 overexpression plasmid, cell migration was significantly higher than those transfected with microRNA-150 mimics. *p<0.03, ***p<0.001.

Table 1. MiRNAs that bind to cir-ZNF609 in starBase

Name	mir Accession	clip ReadNum
Has-miR-150-5p	MIMAT0000451	40
Has-miR-16-5p	MIMAT0000069	14
Has-miR-15a-5p	MIMAT0000068	14
Has-miR-195-5p	MIMAT0000461	14
Has-miR-497-5p	MIMAT0002820	14
Has-miR-15b-5p	MIMAT0000417	14
Has-miR-424-5p	MIMAT0001341	14
Has-miR-338-3p	MIMAT0000763	8
Has-miR-1271-5p	MIMAT0005796	8
Has-miR-96-5p	MIMAT0000095	8
Has-miR-142-3p	MIMAT0000434	1

type pGL3-Circ-ZNF609, NC and mutant-type of pGL3-Circ-ZNF609, respectively (Figure 2B). Our data demonstrated that fluorescence values in cells transfected with microRNA-150 mimics and wild-type Circ-ZNF609 were remarkably decreased (p=0.009, Figure 2C). However, no significant differences in fluorescence values were found in other groups, indicating that microRNA-150 is bound to wild-type pGL3-Circ-ZNF609.

Circ-ZNF609 regulated cellular function via microRNA-150 and Gli1

To determine whether Circ-ZNF609 regulates Gli1 expression by binding to microRNA-150, we determined mRNA and protein expressions in Gli1 after changing expressions of Circ-ZNF609 and microRNA-150. Reduced mRNA and protein expressions of Gli1, as well as decreased cell migration were observed after transfection of Circ-ZNF609 siRNA in HCT116 cells (p<0.001), which were all

reversed by co-transfection of Circ-ZNF609 siRNA and microRNA-150 inhibitor (Figure 3A-3C).

Furthermore, lower mRNA and protein levels of Gli1 were found in cells transfected with microRNA-150 mimics (p<0.01), which were both reversed by co-transfection of microRNA-150 mimics and Circ-ZNF609 overexpression plasmid (p=0.038, Figure 4A, 4B). The migration ability of cells transfected with microRNA-150 mimics was inhibited (p=0.0004). After co-transfected with microRNA-150 mimics and Circ-ZNF609 overexpression plasmid, cell migration was remarkably increased compared to those transfected with microRNA-150 mimics (p=0.03, Figure 4C).

Discussion

Colorectal cancer is one of the most common malignant tumors of the digestive system. CircRNA, as a new endogenous RNA, is involved in various diseases including cancer. Effects of circRNAs in colorectal cancer have been well explored [18]. In order to verify whether circRNA expression is reduced in normal colorectal mucosa compared with colorectal cancer tissue, 21635 reverse cleavage sites have been found by RNase R digestion followed by deep sequencing and the results suggested the feasible detection of all circRNAs [6]. CircRNA plays a crucial role in the development, invasion and metastasis of colorectal cancer by binding to miRNA. Studies have shown that circ_001569 upregulates E2F5, BAG4 and FMNL2 by directly inhibiting miR-145 [19]. In addition, upregulated hsa_circ_0000069 in colorectal cancer tissues is correlated with TNM staging. Hsa_circ_0000069 knockdown significantly inhibits the proliferative, migratory and invasive abilities and arrests cell cy-

cle in G0/G1 phase [20]. Recently, circRNA has been confirmed to be involved in multiple pathological processes of colorectal cancer, and is expected to become a novel prognostic marker of colorectal cancer, such as Circ-BANP, hsa_circ_001988 and Circ CCDC66 [20-22]. Further in-depth explorations are urgently needed to identify other circRNAs that are involved in colorectal cancer.

Gli1 is a transcriptional factor in the Hedgehog pathway that affects cell proliferation, apoptosis, migration and invasion. Overexpressed Gli1 can activate the Hedgehog pathway and participate in the development and progression of cancer [23,24]. It has been shown that Gli1 is upregulated in colorectal cancer. In addition, downregulated miR-150-5p in colorectal cancer is correlated with progression, treatment and prognosis of colorectal cancer [25,26]. Moreover, microRNA-150 alleviates epithelial-mesenhymal transition (EMT) and cell invasion of colorectal cancer by targeting Gli1 [16]. Currently, studies have elucidated that circ-ZNF609 promotes AKT3 expression by binding to microRNA-150, thereby promoting cell proliferation and migration [11]. Therefore, we speculated that circ-ZNF609 may be involved in the pathogenesis and progression of colorectal cancer by regulating Gli1 expression via microRNA-150.

In this study, we detected circ-ZNF609 expressions in 24 colorectal cancer tissues and 36 paracancer tissues. Higher circ-ZNF609 expression in colorectal cancer tissues was observed than in paracancer tissues. In addition, downregulated circ-ZNF609 led to decreased migration in HCT116 cells. These results indicated that abnormally expressed circ-ZNF609 may be related to the development of colorectal cancer. We further verified that circ-ZNF609 regulated microRNA-150 by positively regulating Gli1 expression, thus promoting cell migration. Our study provides a new basis for investigating the pathogenesis of colorectal cancer.

Conclusion

Circ-ZNF609 regulates Gli1 expression via microRNA-150, thus affecting the migration of colorectal cancer.

Conflict of interests

The authors declare no conflict of interests.

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