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Circular RNA circ-PRKCI promotes cell proliferation and invasion by binding to microRNA-545 in gastric cancer

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Abstract. – OBJECTIVE: The carcinogenic effects of circular RNA circ-PRKCI have been recognized in a variety of malignancies. However, the exact biological function of circ-PRKCI in gastric cancer has not been fully elucidated. Therefore, the aim of this study was to explore the expression of circ-PRKCI in gastric cancer (GC) and to investigate its potential regulation mechanism in the pathogenesis and progression of GC.

PATIENTS AND METHODS: The expression of circ-PRKCI in 50 GC tissues and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Statistical methods were used to analyze the relation between circ-PRKCI expression and overall survival rate of patients. The effect of circ-PRKCI on GC cell proliferation was examined by cell counting kit-8 (CCK-8) and cell colony formation assays. Meanwhile, the effect of circ-PRKCI on the invasion ability of GC cells was determined by transwell invasion assay. Flow cytometry was used to detect the apoptosis of GC cells. Bioinformatics was used to search for miRNAs that might have direct effects with circ-PRKCI. In addition, the binding of circ-PRKCI to microRNA-545 was validated using Dual-Luciferase reporter gene assay.

RESULTS: Circ-PRKCI was significantly highly expressed in GC tissues, as well as cell lines. High expression of circ-PRKCI was positively associated with a poor prognosis of GC patients. Overexpression of circ-PRKCI significantly promoted the proliferation and invasion of GC cells, whereas reduced the proportion of apoptotic GC cells. Subsequent Dual-Luciferase reporter gene assay revealed that circ-PRKCI could bind to microRNA-545 and inhibit its expression in GC cells. These results indicated that circ-PRKCI might promote the development of GC by adsorbing microRNA-545 in a sponge manner.

CONCLUSIONS: Circ-PRKCI can be used as a potential prognostic indicator of GC, providing a new perspective for the potential bio-molecular mechanism in GC.

Key Words:
Gastric cancer (GC), Circ-PRKCI, Cell proliferation, Cell invasion.

Introduction

Gastric cancer (GC) is one of the most common gastrointestinal malignancies in the world. The morbidity and mortality of GC rank fourth and second among all cancers, respectively. Currently, the five-year survival rate of GC is only about 20%, leading to great harm to human life and health[1]. Previous studies have demonstrated that the onset of gastric cancer is insidious. Most GC patients have already been in the middle or late stage when first diagnosed, thus losing the opportunity of surgical treatment. Current treatment and prognosis of GC are not ideal. Meanwhile, the specific mechanism of the occurrence and development of GC has not been fully elucidated[2]. Numerous studies [3]have shown that several oncogenes and tumor suppressor genes are related to GC. Therefore, it is of great significance to clarify the exact mechanism of the occurrence and development of GC and to find early diagnostic markers and therapeutic targets for GC patients.

Cyclic RNA (circ-RNA) is a kind of closed-loop, single-stranded RNA that was previously considered as a "waste" produced during RNA cleavage without any function. In recent years, high-throughput sequencing has revealed the presence of large amounts of circular RNAs in eukaryotic cells, including humans[4]. For example, in all tested cells or tissues, more than 10% of genes are capable of producing circular RNA. Many circular RNAs are highly abundant, with

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cell or tissue specificity. During the epithelial to mesenchymal transition of human cells, the expression of hundreds of circular RNAs has changed[5]. The above findings indicate that these highly expressed circular RNAs are not waste products generated during the splicing process. Indeed, many studies have shown that circular RNA can adsorb microRNAs or bind proteins. Changes in circular RNA level can cause changes in the expression of tumor-related genes, eventually affecting the occurrence and development of tumors[6]. A few studies have explored the role of circular RNA in GC. For example, circPSMC3 can act as a competitive endogenous RNA to promote the expression of phosphate and tension homology deleted on chromosome ten (PTEN) *via* sponge-binding miR-296-5p. This can ultimately inhibit GC cell proliferation and metastasis[7]. Besides, circNRIP1 can act as a microRNA-149-5p sponge *via* AKT1 / mammalian target of rapamycin (mTOR) pathway to promote the progression of GC[8]. Circular RNA YAP1 can inhibit GC cell proliferation and invasion by regulating the miR-367-5p/p27 Kip1 axis[9]. In addition, hsa_circ_0000673 has been found significantly down-regulated in GC and can inhibit its proliferation and invasion by targeting microRNA-532-5p[10]. However, there are few works on the role of circRNAs in GC, which requires further study.

Previous researches[11-13] have shown that circ-PRKCI (hsa_circ_0067934) can promote malignant growth and metastasis of tumor cells. However, its expression and exact function in GC have not been fully elucidated. Therefore, the aim of this study was to explore the role of circ-PRKCI in GC and its possible underlying mechanism.

Patients and Methods

Sample Collection

50 paired GC tissues and adjacent normal tissues were obtained from patients undergoing radical resection at Renmin Hospital of Wuhan University from March 2016 to October 2018. This investigation was approved by the Ethics Committee of Renmin Hospital of Wuhan University. Signed written informed consents were obtained from all participants before the study.

Cell Culture and Transfection

Four GC cell lines (including SGC-7901, MKN-45, BGC-823, and MGC-803) and one normal gastric mucosal cell line (GES-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). SGC-7901 , and MGC-803 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco; Rockville, MD, USA), while GES-1 was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 100 μg/mL streptomycin and 100 UI/mL penicillin in a humidified incubator at 37°C with 5% CO_2.

Circ-PRKCI stable overexpression vector was constructed by cloning the circ-PRKCI cDNA into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). Cells in log phase were first plated to 6-well plates and cultured to a density of approximately $1x10^6$ cells/well. Negative controls (NC), si-circ-PRKCI, microRNA-545 mimics, and microRNA-545 inhibitors supplied by GeneChem Co, Ltd (Shanghai, China) were transfected into cells according to the instructions of Lipofectemine™ 3000 (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, cells were collected for subsequent experiments.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cells or tissues. Next, extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed with the SYBR Green Master Mix (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references for mRNA and miRNA, respectively. Specific qRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences were as follows: circ-PRKCI, F: 5'-CGGAGGTTCCAGCTCGTTAGTC-3', R: 5'-GCAACCGGAATGTGGAATTGA-3'; miR-545, F: 5'-GCTGTACACACCACACGTTCTG-3', R: 5'-GATTACCGCTCGTAGCAGTCAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded into 96-well plates at a concentration of 1×10^4/well. After incubation for 0. 24, 48, and 72 h, respectively, CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well, followed by incubation at 37°C for 2 h in the dark. Absorbance at 450 nm was detected using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Colony Formation Assay

Transfected GC cells were trypsinized, counted and plated into 3.5 cm wells. After 14 days of incubation, visible colonies were stained with crystal violet and photographed. Finally, the number of colonies was counted.

Cell Apoptosis

Cell apoptosis was quantified by flow cytometry using annexin V-FITC (fluorescein isothiocyanate)/ Propidium Iodide (PI) kit (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, GC cells were transfected with corresponding transfection reagents for indicated time points. Subsequently, the cells were collected and incubated with annexin V-FITC/PI for 15 min in the dark. Finally, flow cytometry was applied for cell apoptosis detection.

Cell Invasion Assay

Cell invasion assay was performed using Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA). 1×10^5 GC cells in serum-free medium were seeded into the upper chamber. Meanwhile, the lower chamber was added with complete medium containing 10% fetal calf serum (FCS). After 24 h of incubation, the cells adhering to the membrane were fixed and stained with 0.5% crystal violet. Five fields of view were randomly selected and the number of invading cells was counted under a microscope.

Nuclear Separation Experiment

Cells were first collected and lysed with Lysis Buffer J. After centrifugation, cytoplasmic RNA was obtained in the supernatant, while nuclear RNA was in the remaining liquid. Next, buffer SK and ethanol were added to cytoplasmic and nuclear RNA, respectively. Finally, the separation was performed by centrifugation through a separation column.

Luciferase Reporting Gene Assay

5×10^4 GC cells were seeded into 24-well plates. Then, pGL3-circ-PRKCI WT, pGL3-circ-PRKCI-MUT, microRNA-545 mimics, and microRNA-545 NC were co-transfected into cells. After 24 h, the level of luciferase activity was determined using a dual luciferase reporter kit (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (SPSS IBM, Armonk, NY USA) or GraphPad Prism 7.0 (La Jolla, CA, USA) was used for all statistical analysis. Experimental data were represented as mean ± standard deviation (SD). Spearman rank correlation was used to analyze the correlation between the expression of circ-PRKCI and clinical features of patients. Survival analysis was calculated using the Kaplan-Meier method. $p < 0.05$ was considered statistically significant.

Results

Circ-PRKCI is Highly Expressed in GC Tissues and Cells

First, we detected circ-PRKCI expression in 50 pairs of GC tissues and adjacent normal tissues by qRT-PCR. As shown in Figure 1A, circ-PRKCI was significantly up-regulated in GC tissues when compared with normal tissues. Similarly, circ-PRKCI expression was significantly higher in GC cells than that of normal cells (Figure 1D). To further explore the association between circ-PRKCI expression and clinical features of patients, the correlation of circ-PRKCI expression with TNM staging and tumor metastasis in GC patients was analyzed. As shown in Figure 1B and 1C, high expression of circ-PRKCI was significantly correlated with TNM staging and tumor metastasis. In addition, the Kaplan-Meier analysis showed that high expression of circ-PRKCI predicted poor prognosis of GC patients (Figure 1E). These results suggested that circ-PRKCI might play an important role in the progression of GC.

Highly Expressed Circ-PRKCI Promotes the Proliferation and Inhibits the Apoptosis of GC Cells

To clarify the specific function of circ-PRKCI in GC, we first established a circ-PRKCI stable expression cell line by transfection of LV-circ-PRK-

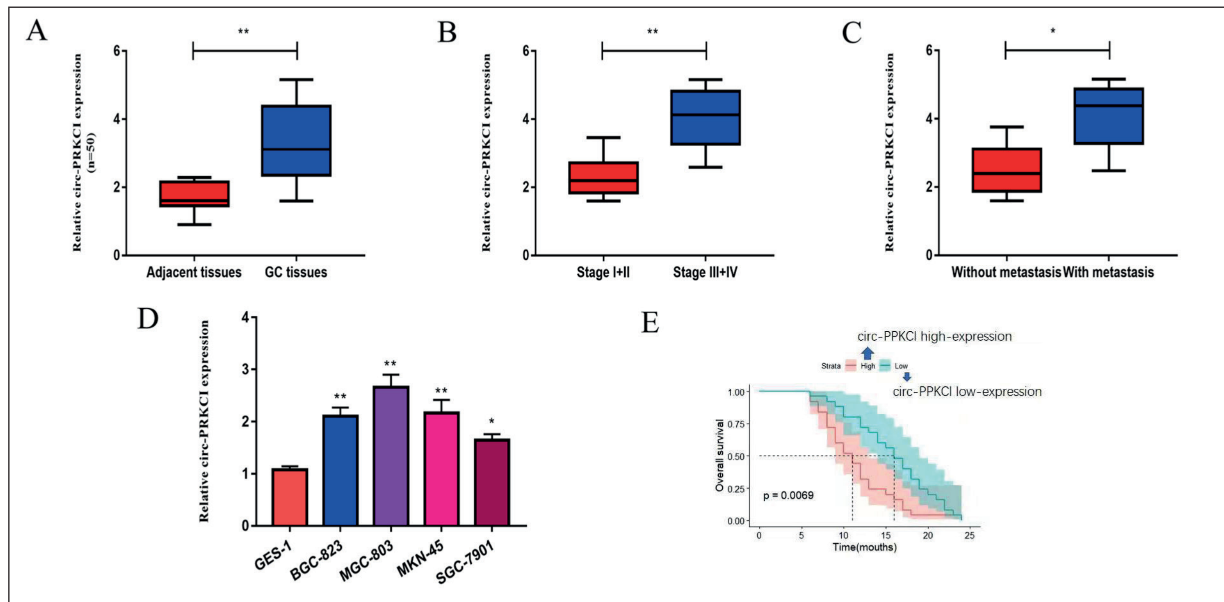

Figure 1. Circ-PRKCI is highly expressed in GC tissues and cells. **A,** Expression of circ-PRKCI in GC tissues and adjacent normal tissues was detected by qRT-PCR. **B,** Expression levels of circ-PRKCI in GC patients with different stages were analyzed by qRT-PCR. **C,** Expression of circ-PRKCI in GC patients with and without distant metastasis was analyzed by qRT-PCR. **D,** Expression of circ-PRKCI in ges-1 normal gastric epithelial cells and GC cell lines (bgc-823, mgc-803, mkn-45, and sgc-7901) was detected by qRT-PCR. **E,** Kaplan-Meier was used to plot the survival curves of GC patients in circ-PRKCI high-expression group and circ-PRKCI low-expression group. *$p<0.05$; **$p<0.01$.

CI *in vitro*. Transfection efficiency was measured by qRT-PCR (Figure 2A). Subsequently, the effect of circ-PRKCI on the proliferation of GC cells was detected by CCK8 assay. Results revealed that circ-PRKCI overexpression significantly enhanced the proliferation of GC cells (Figure 2B). Furthermore, we confirmed the promotion of cell proliferation by colony formation assay (Figure 2C). To explore whether circ-PRKCI could affect cell proliferation by inhibiting apoptosis, we examined the effect of circ-PRKCI on the apoptosis of GC cells by flow cytometry. As shown in Figure 2D, cells transfected with LV-circ-PRKCI exerted a significantly lower percentage of apoptotic cells than blank control group. In addition, to observe the effect of circ-PRKCI on cell metastasis, we performed transwell invasion assay. Results demonstrated that overexpressed circ-PRKCI significantly promoted the invasive ability of GC cells (Figure 2E). Therefore, we could conclude that highly expressed circ-PRKCI promotes the proliferation and inhibits the apoptosis of GC cells

Circ-PRKCI Exerts its Biological Function in GC by Adsorbing MicroRNA-545

It is well known that the distribution of non-coding RNAs in cells determines their potential molecular mechanism to some extent. Therefore, in this work, we first tested the distribution of circ-PRKCI. As shown in Figure 3A, most circ-PRKCI were distributed in the cytosol, with only a small part in the nucleus. This suggested that circ-PRKCI might play a role at the post-transcriptional level. Subsequent bioinformatics was used to predict miRNAs that could bind to circ-PRKCI. Finally, microRNA-545 was screened out and selected (Figure 3B) by literature searching and functional analysis. The binding relation was confirmed by dual luciferase reporter gene assay. As shown in Figure 3C, microRNA-545 mimics effectively reduced the luciferase activity of circ-PRKCI-wt, while circ-PRKCI-mut showed no significant changes. These findings suggested that microRNA- 545 could directly target circ-PRKCI in GC. At the same time, qRT-PCR results showed that microRNA-545 was markedly reduced in GC tissues (Figure 3D). The Spearman rank correlation analysis demonstrated that the expression of circ-PRKCI was negatively correlated with microRNA-545 in GC ($R=-0.8536$, $p<0.001$) (Figure 3E). Subsequently, we transfected GC cells with LV-circ-PRKCI and detected the expression of microRNA-545. The results displayed that circ-PRKCI overexpression

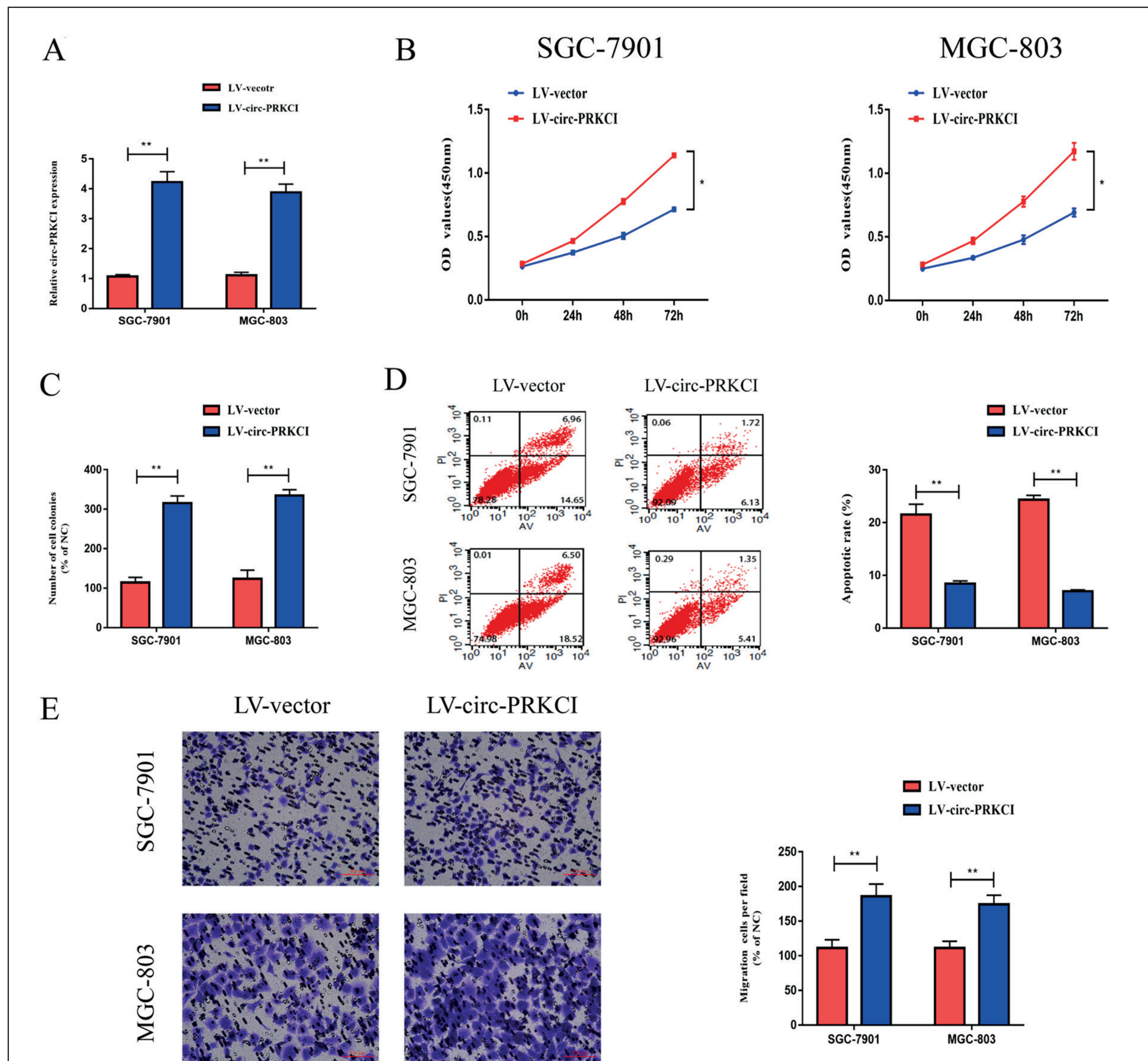

Figure 2. Overexpression of circ-PRKCI can promote the proliferation and invasion and inhibits the apoptosis of GC cells. **A,** Expression of circ-PRKCI in lv-vector and lv-circ-PRKCI cells of sgc-7901 and mgc-803 was detected by qRT-PCR. **B,** Effect of circ-PRKCI on the proliferation of sgc-7901 and mgc-803 cells was detected by CCK-8 assay. **C,** Colony formation assay was used to further explore the effect of circ-PRKCI on the proliferation of sgc-7901 and mgc-803 cells. **D,** Effect of circ-PRKCI on the apoptosis of sgc-7901 and mgc-803 cells was detected by flow cytometry. **E,** Transwell invasion assay was used to detect the effect of circ-PRKCI on the invasion of sgc-7901 and mgc-803 cells. (Magnification 20×) *$p<0.05$; **$p<0.01$.

markedly inhibited microRNA- 545 expressions in GC cells (Figure 3F). All these findings indicated that circ-PRKCI could exert its biological function by adsorbing microRNA-545.

MicroRNA-545 Reverses the Function of Circ-PRKCI in GC Cells

To further confirm whether circ-PRKCI could promote GC progression by adsorbing microRNA-545, a rescue experiment was conducted. First, we examined the transfection efficiency of circ-PRKCI inhibitor and microRNA-545 mimics by qRT-PCR (Figures 4A, 4B). Subsequently, circ-PRKCI inhibitor, microRNA-545 mimics or negative controls were co-transfected into GC cells. The proliferation and metastasis abilities of GC cells were then detected. The results of *in vitro* experiments showed that the proliferation and metastasis abilities of GC cells decreased significantly, whereas the proportion of apop-

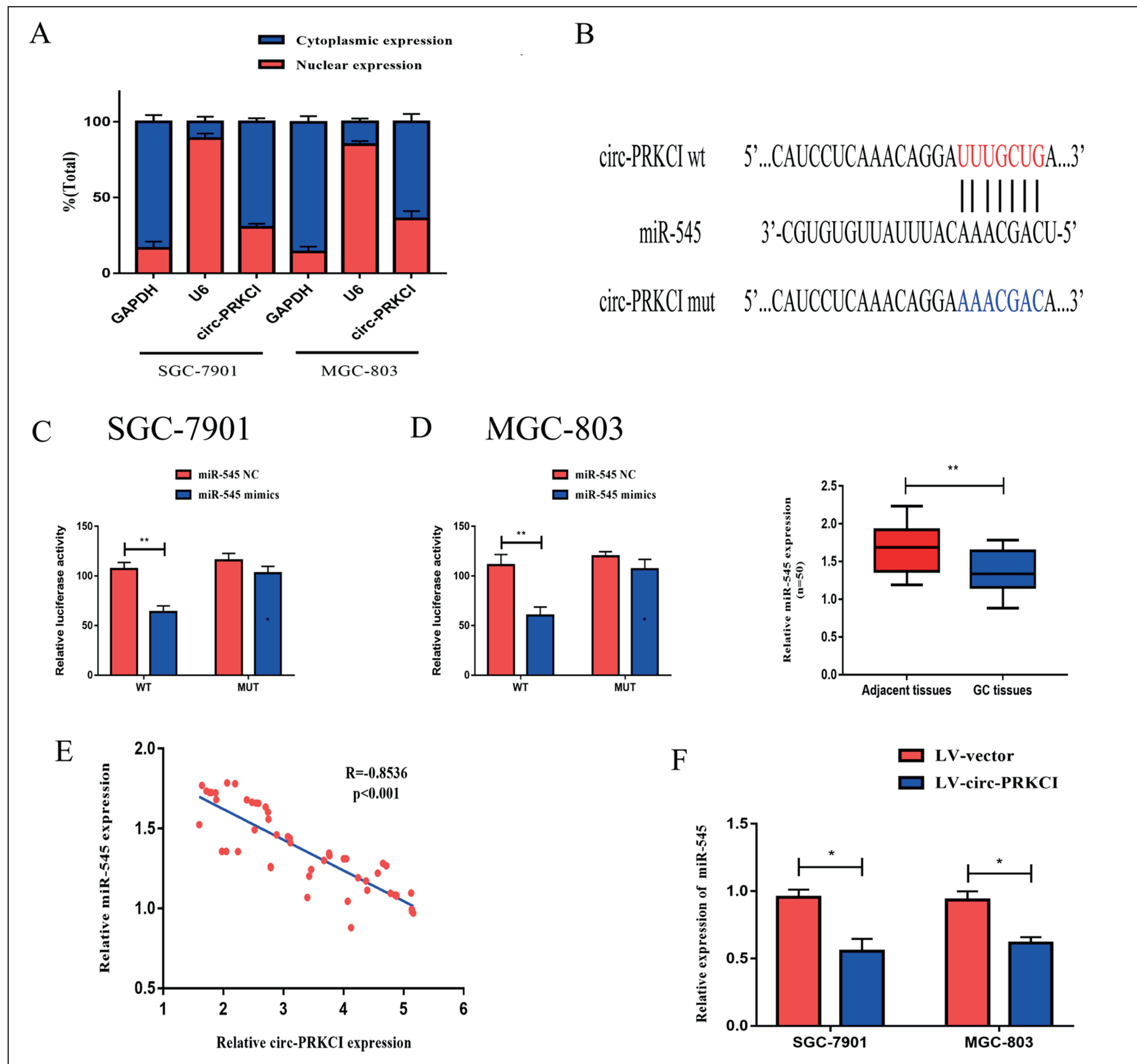

Figure 3. Circ-PRKCI promotes the growth and metastasis of GC cells by adsorbing miR-545. **A,** Distribution of circ-PRKCI in the cytoplasm and cytoplasm of sgc-7901 and mgc-803 was detected by nucleoplasmic separation experiment. **B,** Predicted circ-PRKCI and miR-545 binding site sequence. **C,** Luciferase activity of 3'UTR reporter gene was determined after co-transfection of miR-545 mimics and luciferase reporter gene containing circ-PRKCI-wt or circ-PRKCI-mut transcripts in sgc-7901 and mgc-803 cells. **D,** Expression of miR-545 in GC tissues and adjacent normal tissues was detected by qRT-PCR. **E,** Spearman's rank correlation analysis was used to analyze the correlation between circ-PRKCI and miR-545 expression in GC tissues. **F,** Expression of miR-545 was detected by qRT-PCR after transfection of lv-vector and lv-circ- PRKCI with sgc-7901 and mgc-803. *p<0.05; **p<0.01.

totic cells was up-regulated. At the same time, microRNA-545 overexpression remarkably enhanced cell proliferation and decreased the proportion of apoptotic cells (Figures 4C-4F). These results indicated that circ-PRKCI could promote GC cell proliferation and metastasis by adsorbing microRNA-545, which might be a potential molecular mechanism to promote the treatment of GC.

Discussion

GC is one of the most common malignant tumors in human beings, whose mortality rate is still on the rise at present. The main reason lies in the low detection rate of GC at an early stage, meanwhile, the effect of early and late treatment of GC ranks differently[14]. Currently, the 5-year survival rate for early GC can be as high as 90 to 95%. However, once

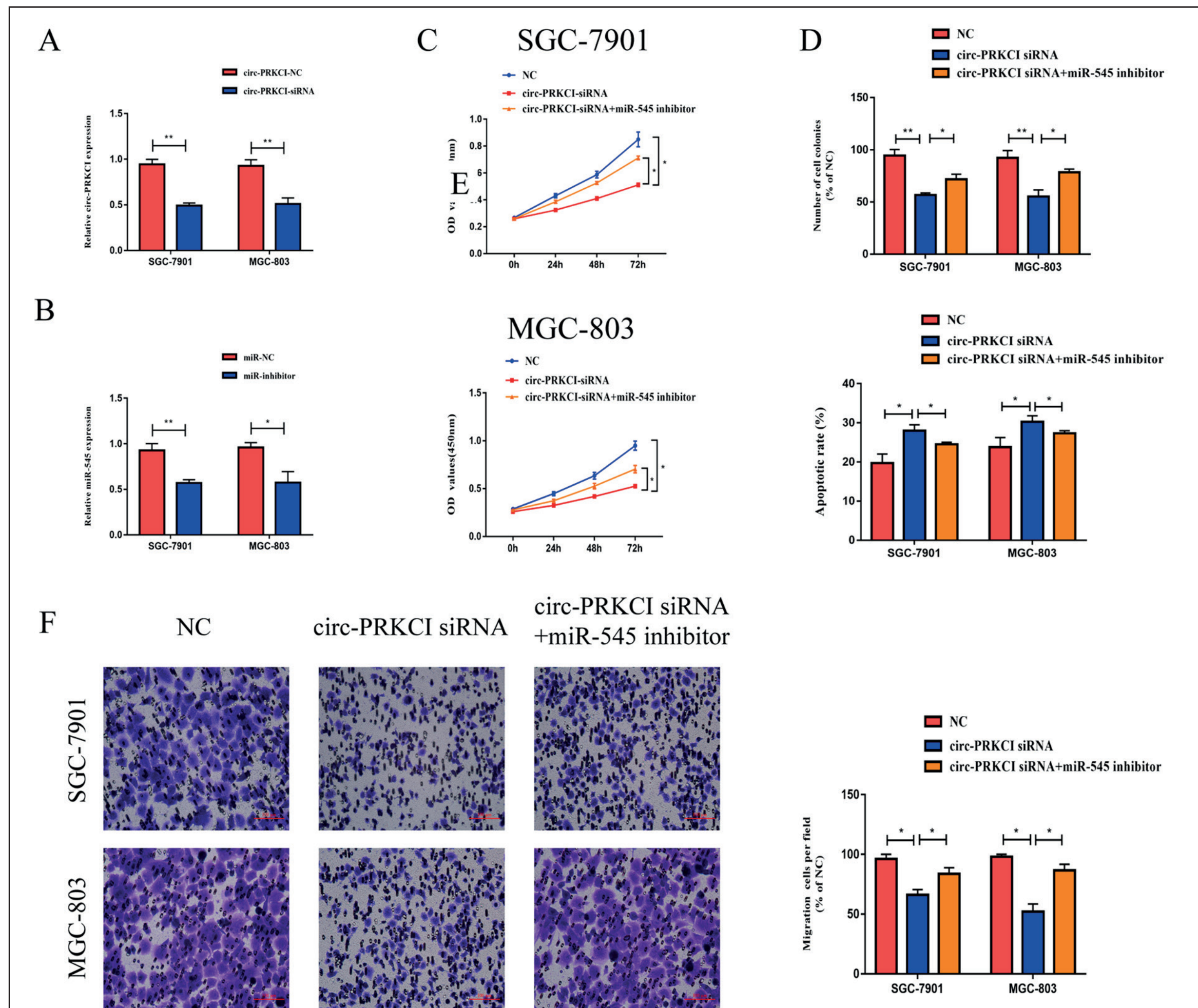

Figure 4. MiR-545 inhibitor was able to reverse the effect of low expressed-PRKCI on the proliferation, apoptosis, and invasion of GC cells. **A,** After transfection of circ-PRKCI-NC and circ-PRKCI-siRNA in sgc-7901 and mgc-803 cells, the expression of circ-PRKCI was detected by qRT-PCR. **B,** Expression of miR-545 was detected by qRT-PCR after transfection of miR-545-nc and miR-545-inhibitor in sgc-7901 and mgc-803 cells. **C-D,** Cell growth and proliferation detected by CCK-8 assay and colony formation assay. **E,** Cell apoptosis ratio detected by flow cytometry. **F,** Transwell invasion assay was used to detect cell invasion ability. (Magnification 20×) *$p<0.05$; **$p<0.01$.

diagnosed at an advanced stage, the mortality rate will be very high. Therefore, the search for stable and effective molecular diagnostic markers in the early stage of GC is of vital importance for its early diagnosis. Furthermore, this may also effectively reduce the mortality of GC patients[15].

CircRNAs act as a novel class of non-coding RNAs. Compared with lncRNA and miRNA, circRNA has the remarkable feature of being more stable in the body. This makes it an ideal molecular diagnostic marker of early GC[16,17]. However, the research on circRNA is still in its infancy. At present, there are few studies on the biological functions of circRNA in human diseases, especially in malignant tumors. Circ-PRKCI has previously been confirmed to have cancer-promoting properties in a variety of tumors. For example, circ_0067934 promotes the development of thyroid cancer by promoting EMT[13]. Meanwhile, it is able to promote tumor growth and metastasis in hepatocellular carcinoma[12]. In addition, circ_0067934 has also been found highly expressed in NSCLC tissues and can enhance NSCLC cell proliferation[18].

MicroRNAs (miRNAs) are a class of non-coding small RNAs that are widely involved in the

regulation of gene expression. They are relatively conservative in biological evolution and can be found in the genomes of lower organisms such as bacteria, viruses, etc.[19]. However, a single miRNA possesses a large number of target genes. It is estimated that about 60% of mammalian genes are regulated by miRNAs. Therefore, miRNAs are involved in important cell life activities, such as cell growth, differentiation, apoptosis, etc.[20]. Previous studies[21,22] have demonstrated that microRNA-545 can act as a tumor suppressor in the occurrence of tumors. Moreover, it can inhibit the malignant growth and metastasis of tumor cells by targeting the degradation of downstream genes.

In this work, we showed that circ- PRKCI expression increased significantly in GC tissues and cell lines. High expression of circ-PRKCI indicated poor prognosis of GC patients. Subsequently, we found that the proliferation and migration of GC cells were significantly enhanced after circ-PRKCI overexpression *in vitro*. Researches have found that circ-RNAs can exert their biological functions by regulating the expression of miRNAs. From this, we speculated that circ-PRKCI might promote GC progression by regulating certain miRNAs. Using bioinformatics prediction tools, microRNA-545 was selected as a possible binding target of circ-PRKCI. Then, we found that circ-PRKCI could bind to microRNA-545. Meanwhile, the overexpression of circ-PRKCI in cells inhibited the expression of microRNA-545. Furthermore, rescue experiments indicated that the proliferation and invasion abilities of GC cells were significantly weakened after knocking down circ-PRKCI. However, knocking down microRNA-545 at the same time could restore this inhibitory effect. This suggested that circ-PRKCI might exert its biological effects by binding to microRNA-545. However, this study has not yet explored the downstream target genes of microRNA-545. Furthermore, we have not verified the cancer-promoting properties of Circ-PRKCI in animals, which need to be further investigated.

Conclusions

Our findings indicate that Circ-PRKCI is aberrantly highly expressed in GC tissues and cell lines. Highly expressed circ-PRKCI can promote GC cell proliferation and invasion by binding to microRNA-545. Our findings may provide a solid pathophysiological basis for the potential mechanism of circ-PRKCI/microRNA-545 in GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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