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Roles of circ-CSPP1 on the proliferation and metastasis of glioma cancer

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Abstract. – OBJECTIVE: The aim of this study was to explore the association between circ-CSPP1 and the proliferation, invasion, and migration of glioma cancer (GC).

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detected circ-CSPP1 expression in GC tissues and cells. Subsequently, siRNA was transfected to suppress circ-CSPP1 expression *in vitro*. Cell counting kit-8 (CCK-8) assay, colony formation assay, and 5-Ethynyl-2'-deoxyuridine (EdU) staining assay were performed to examine the proliferation of GC cells. Meanwhile, transwell assay was conducted to determine the invasion and migration of GC cells. Furthermore, Western blot assay was conducted to analyze the protein expressions of E-cadherin, N-cadherin, and Vimentin.

RESULTS: Circ-CSPP1 expression was significantly up-regulated both in GC tissues and cells. GC cells with low expression of circ-CSPP1 showed significantly reduced proliferation, invasion, and migration abilities. In addition, up-regulated E-cadherin protein expression, along with down-regulated N-cadherin and Vimentin protein expressions were observed in GC cells with circ-CSPP1 siRNA treatment.

CONCLUSIONS: Circ-CSPP1 promoted the proliferation, invasion, and migration of GC cells.

Key Words:
Glioma cancer (GC), Circ-CSPP1, Proliferation, Metastasis.

Introduction

Glioma cancer (GC), originated from brain neuroglia cells, is the most common malignant tumor of the central nervous system[1]. In China, the annual incidence rate of GC is 5-8/100,000. Meanwhile, its 5-year mortality rate is relatively poor. Although current treatment methods for GC show certain therapeutic effect[2], it is difficult to completely remove the tumor due to the growth characteristics of GC[3]. In addition, the efficacy of radiotherapy and chemotherapy is still far from satisfactory[4,5]. In recent years, gene therapy at the molecular level has been a new direction in the treatment and research on GC[6]. Inhibiting the abnormal activation of signal transduction pathway to regulate the cell cycle has been confirmed to effectively promote apoptosis and block the proliferation of tumor cells[7-9]. Therefore, the exploration of effective therapeutic targets for GC has important significance for prolonging the survival time and improving the life quality of GC patients.

Non-coding ribonucleic acids (RNAs) are a kind of RNA transcripts without protein-coding ability, including miRNAs, lncRNAs, and circRNAs. They constitute competing endogenous RNAs, playing important roles in the occurrence and development of malignant tumors[10]. Among them, circRNAs are highly and stably expressed and widely distributed. Therefore, they have become the front in tumor research[11]. CircRNAs are mainly derived from precursor mRNAs, whose 3'-tail end is connected with 5'-head end. This may help to form a reverse spliced closed covalent ring structure, which makes circRNAs lack 3' poly(A) tail, thereby resisting RNase degradation[12]. CircRNAs are abundantly expressed and conserved in various species, whose expression shows specificity of tissue and developmental timing. They play dual roles of inhibiting or promoting tumor progression by up-regulating tumor suppressor genes or proto-oncogenes. Recently, researchers have found that centrosome/spindle pole-associated protein 1 (CSPP1) acts as a proto-oncogene in human B-cell lymphoma[13] and a candidate oncogene in luminal breast cancer[14], respectively. Li et al[15] have discovered that circ-CSPP1, derived from CSPP1, promotes the proliferation and migration of ovarian cancer cells. However, the exact role of

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circ-CSPP1 in the occurrence and development of GC has not been fully elucidated.

Patients and Methods

Clinical Samples

This investigation was approved by the Ethics Committee of Taizhou First People's Hospital. Informed consent was obtained from patients before the study. GC tissues were collected from patients underwent surgery in our hospital from 2017 to 2019. All histopathology was confirmed by hematoxylin and eosin (HE) staining (Boster, Wuhan, China).

Cell Culture

Cell lines, including NBC, U87, U251 cells, and LN229, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). When the cells in logarithmic growth phase covered about 80% of the culture flask, they were washed with phosphate-buffered saline (PBS) and digested with 0.25% trypsin. After cell contraction, the digestion was terminated. All cells were cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12; Gibco, Rockville, MD, USA) complete medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator, followed by cell passage at a ratio of 1:3 or 1:4.

Cell Transfection

The cells were first seeded into 24-well plates. When cell coverage rate was about 60%, siRNA was mixed with lipofectamine2000 according to the manufacturer's instructions. After incubation for 15 min, the mixture was added to cell culture solution. 6 h later, the medium was removed and replaced with fresh medium. Two groups were established, including NC group (normal GC cells) and siRNA group (GC cells transfected with circ-CSPP1 siRNA). After transfection for 48-72 h, transfection efficiency was examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Extraction and qRT-PCR Analysis

After 48 h of transfection, the cells were harvested. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration, purity, and integrity of extracted RNA were detected by Nano drop instrument. Subsequently, 1 μL of RNA was reversely transcribed into cDNA. Corresponding primers, template, and reagents were added to a 20 μL system, with 3 replicates in each group. Specific reaction conditions were as follows: pre-denaturation for 3 min at 94°C; denaturation at 94°C for 4 s, annealing at 56°C for 5 s, and extension for 6 s at 72°C, for a total of 40 cycles. Using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize the expression of circ-CSPP1, the relative levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: circ-CSPP1, F: 5'-CGAGGTCCAACCCTCTGCTGA-3', R: 5'-GCTAGCCATGTCCACGAAGTGA-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

After 72 h of transfection, the cells were harvested and lysed on ice for 30 min with a protease inhibitor lysate. After centrifugation at 12000 r/min for 4 min at 4°C, protein concentration was determined by the bovine serum albumin (BSA) method. 30 μg protein samples were separated by polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with blocking solution containing 5 g/L skim milk, the PVDF membranes were incubated with primary antibodies overnight. On the next day, the membranes were incubated with the corresponding secondary antibody. After washing with Tris-Buffered Saline and Tween-20 (TBST) for three times, the membranes were added with mixed enhanced chemiluminescence (ECL) reagents A and B at a ratio of 1:1 ratio. Immunoreactive bands were finally exposed under a gel imager.

Cell Counting Kit-8 (CCK-8) Assay

Logarithmic growth phase cells were seeded into 96-well plates at a density of $1x10^4$ cells per well. Then, the cells were cultured overnight in an incubator. 10 μL of CCK8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well, followed by incubation for 2 h in the dark. The plate was taken out after every 24 h, and absorbance at 450 nm was detected by a micro-plate reader. Finally, the viability of cells was calculated.

5-Ethynyl-2'-Deoxyuridine (EdU) Staining Assay

A total of 1×10^4 cells/well were plated into 96-well plates and cultured overnight. 50 μM of EdU medium was prepared, and 100 μL of EdU medium was added into each well for 2 h of incubation. The culture solution was discarded, and the cells were washed with PBS. After fixing with

4% paraformaldehyde, 100 μL of EdU buffer was added into each well for 30 min of incubation at room temperature. After washing with PBS for 3 times, the cells were photographed under a fluorescence microscope. Finally, the percentage of Edu-positive cells was calculated.

Colony Formation Assay

After 48 h of transfection, the cells were harvested and plated into 6-well plates at a density of 1 x 10^3 cells/well. Then, the cells were cultured in a 37°C and 5% CO_2 incubator. Fresh medium was changed every two days. After 7 days, the cells was taken out and washed 3 times with PBS. Next, the cells were fixed in 4% paraformaldehyde solution for 10 min and stained with 1% crystal violet solution. Finally, the number of formed colonies was counted under a light microscope.

Transwell Assay

Matrigel was diluted with serum-free medium at a ratio of 1:8 to make a final concentration of 300 μL/mL. 80 μL of Matrigel was evenly applied on the surface of membrane in the invasion chamber, followed by incubation in the incubator for 30 min. Cells in each group were resuspended with FBS-free medium and counted. Cell concentration was then adjusted appropriately. 150 μL of cell suspension was added into the upper chamber of 24-well plates. Meanwhile, 500 μL of medium containing 10% FBS was added into the lower chamber. After culture for 24 h, transwell chamber were taken out. Cells on the surface of the membrane were gently wiped off using a cotton swab and washed twice with PBS. The lower surface of the chamber was fixed with paraformaldehyde solution at room temperature for 20 min and stained with 0.4% trypan blue dye for 3 min. Next, the cells were washed twice with PBS, followed by observation and photography under an inverted microscope. The number of invading cells on the membrane surface was counted in 5 randomly-selected high-power fields, and the average was calculated.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Differences between two groups were analyzed by using Student's *t*-test. One-way ANOVA was applied to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Circ-CSPP1 Expression Was Upregulated in GC Tissues and Cells

In this study, we first detected the expression levels of circ-CSPP1 at different sites. As depicted in Figure 1A, compared with adjacent normal tissues, the expression of circ-CSPP1 in GC tissues was significantly up-regulated. Highly expressed circ-CSPP1 was observed in GC cell lines when compared with NBC cells, especially in U87 cell lines (Figure 1B). These results indicated an important role of circ-CSPP1 in the occurrence and progression of GC.

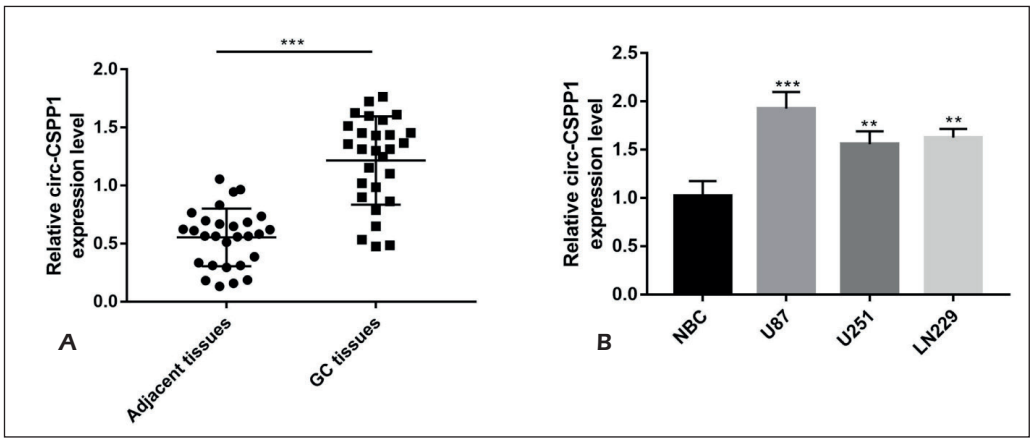

Figure 1. Expression of circ-CSPP1 in GC tissues and cells. **A**, Circ-CSPP1 was up-regulated in GC tissues compared with adjacent tissues detected by qRT-PCR. **B**, Circ-CSPP1 was up-regulated in U87, U251 and LN229 cells when compared with NBC cells detected by qRT-PCR. **$p<0.01$, ***$p<0.001$.

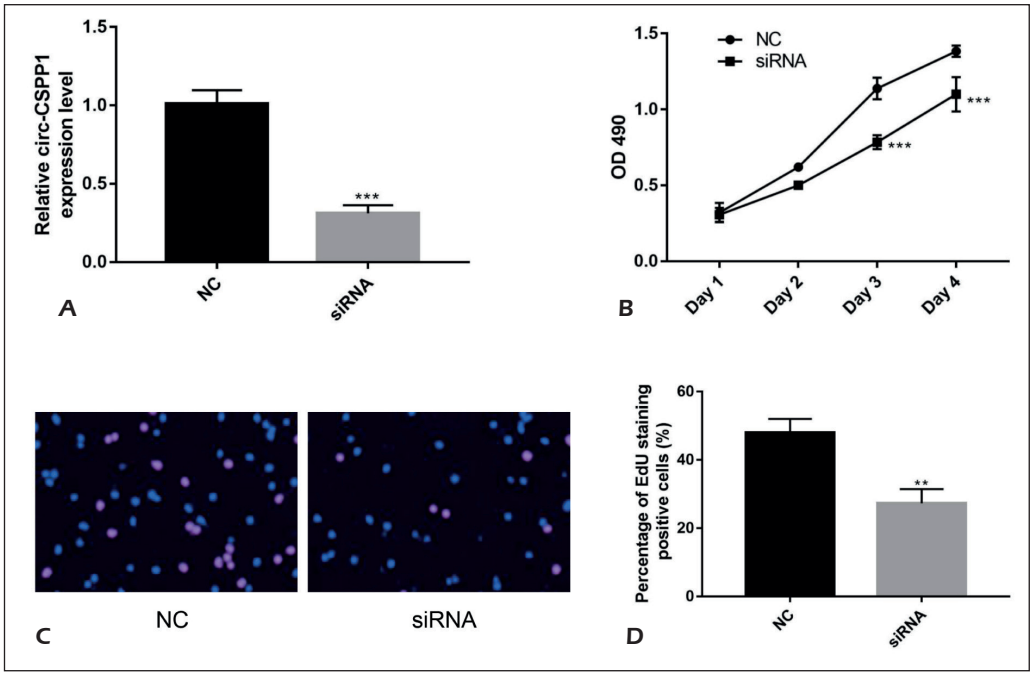

Figure 2. Roles of circ-CSPP1 on the proliferation of GC cells. **A**, Transfection of circ-CSPP1 siRNA in GC cells significantly inhibited circ-CSPP1 expression. **B**, Viability of GC cells decreased significantly in day 3 and day 4 after inhibition of circ-CSPP1. **C**, Representative images of EdU staining in NC group and siRNA group (magnification: 4x). **D**, Percentage of EdU staining positive cells was statistically analyzed. $^{**}p<0.01$, $^{***}p<0.001$.

Roles of Circ-CSPP1 on the Proliferation of GC Cells

Considering high expression of circ-CSPP1 in U87 cell lines, we selected such cell line for further experiments. As shown in Figure 2A, transfection of circ-CSPP1 siRNA significantly down-regulated the expression of circ-CSPP1. CCK8 assay and EdU staining assay (shown in Figure 2B) suggested that inhibition of circ-CSPP1 notably suppressed the viability of U87 cells at day 3 and day 4. In addition, the ratio of EdU staining positive cells was 48 ± 2.309% in normal U87 cells, which was dramatically higher than that of U87 cells transfected with circ-CSPP1 siRNA (27.33 ± 2.404%, Figure 2C, 2D). Moreover, U87 cells transfected with circ-CSPP1 siRNA also showed significantly decreased number of formed colony numbers compared with normal U87 cells (Figure 3A, 3B). Collectively, these findings demonstrated that inhibition of circ-CSPP1 significantly suppressed the proliferation ability of U87 cells.

Inhibition of circ-CSPP1 Impaired the Invasion and Migration of GC Cells

Transwell assay was conducted to examine the effects of circ-CSPP1 on the invasion and migration ability of U87 cells. As shown in Figure 3C, 3D, U87 cells with circ-CSPP1 treatment presented significantly reduced invasion and migration abilities, with 95.33 ± 5.783 invasive cells and 97.67 ± 9.244 migrated cells in NC group compared with 62.33 ± 4.91 invasive cells and 58 ± 5.196 migrated cells in siRNA group, respectively. These results suggested that circ-CSPP1 remarkably affected the invasion and migration abilities of U87 cells.

Effects of Circ-CSPP1 on the Epithelial-Mesenchymal Transition (EMT) of GC Cells

To further explore the underlying mechanism of suppressed invasion and migration abilities of U87 cells, we conducted Western blot analysis. The protein expressions of E-cadherin, N-cadherin, and Vimentin were detected. As shown in Figure 4A, 4B, after down-regulation of circ-CSPP1 in U87 cells, significantly enhanced E-cadherin expression, along with decreased N-cadherin and Vimentin expressions were observed when compared with normal U87 cells. All these data revealed that U87 cells with low expression of circ-CSPP1 were accompanied with impaired EMT biological behavior.

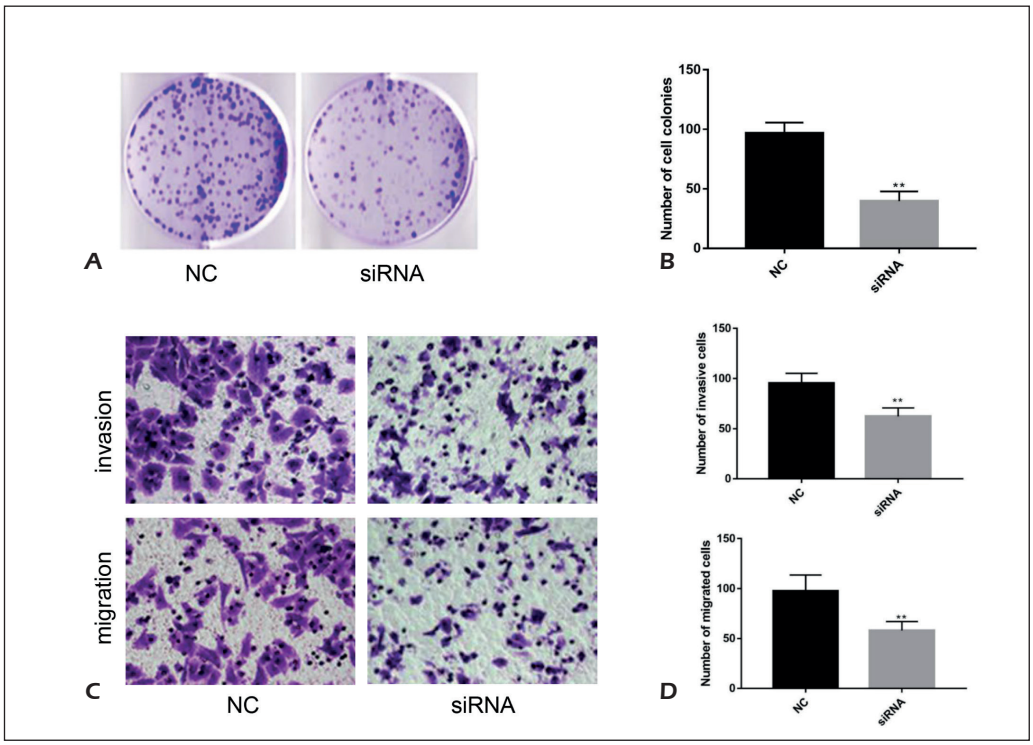

Figure 3. Inhibition of circ-CSPP1 impaired the invasion and migration of GC cells. **A**, Representative images of formed colonies in NC group and siRNA group (magnificaion: 4x). **B**, Number of formed colonies was statistically analyzed. **C**, Representative images of invasion and migration abilities in NC group and siRNA group (magnificaion: 4x). **D**, Number of invasive and migrated cells was statistically analyzed. $^{**}p<0.01$.

Discussion

GC is a kind of malignant tumor in the central nervous system, seriously threatening human health[16]. Currently, certain effects have been achieved in the treatment of GC[17]. However, radical excision is still difficult through surgery due to the invasive growth of GC and its unclear boundary with surrounding brain tissues[18]. In particular, the median survival time of high-grade

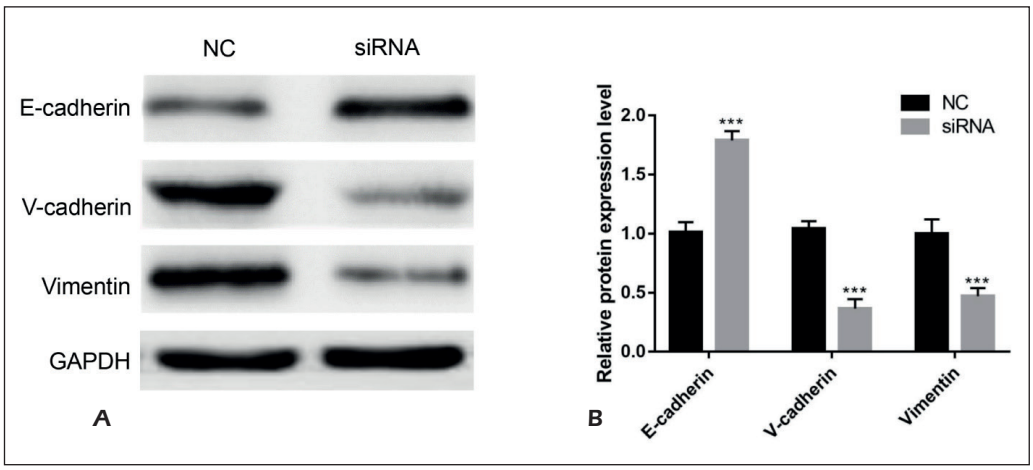

Figure 4. Effects of circ-CSPP1 on the epithelial-mesenchymal transition (EMT) of GC cells. **A**, Related protein expressions of E-cadherin, N-cadherin and Vimentin in NC group and siRNA group detected by Western blot. **B**, Quantification of E-cadherin, N-cadherin and Vimentin expression level. $^{***}p<0.001$.

GC patients is less than one year because of high recurrence rate[19]. Molecular regulation plays an important role in the occurrence and development of GC, including cell apoptosis, proliferation, and invasion[20,21].

With the continuous innovation of molecular research technology, quantities of new circRNAs have been discovered. The mechanism explaining the function of circRNAs is complicated by their origin and location. However, a common one is to combine with miRNAs to exert molecular sponge effects[22]. For example, Luo et al[23] have reported that circRNA-101505 promotes oxidored-nitro domain-containing protein 1 expression by sponging miR-103. Zhang et al[24] have observed that circRNA-TRIM33-12 suppresses hepatocellular carcinoma progression by sponging of miR-191.

At present, increasing circRNAs have been reported to be widely associated with GC. In 2018, a novel circular RNA, circ-0046701, was reported to promote carcinogenesis by up-regulating miR-142-3p expression in glioma[25]. The circ-0007534/miR-761/ZIC5 regulatory loop has been confirmed to be capable of modulating the migration and proliferation of glioma cells[26]. In 2019, circ-U2AF1 was uncovered to promote human glioma *via* down-regulating neuro-oncological ventral antigen 2 by sponging miR-7-5p[27]. Besides, it has been documented that upregulation of circ-0034642 indicates unfavorable prognosis of glioma patients. It can also facilitate cell proliferation, as well as invasion through the miR-1205/BATF3 axis[28]. All of these findings firmly demonstrate that circRNAs play an important role in mediating the occurrence and progress of GC, along with the prognosis of GC patients.

In this paper, circ-CSPP1 was found highly expressed in both GC tissues and cell lines. Therefore, we speculated that circ-CSPP1 played an important role in regulating the development of GC. Subsequently, circ-CSPP1 siRNA was transfected into GC cells. The proliferation and migration abilities of GC cells with low expression circ-CSPP1 were determined. Consistent with our hypothesis, CCK8 assay, colony formation assay, and EdU staining assay showed that low expression of circ-CSPP1 significantly decreased the viability and proliferation abilities of GC cells. Besides, significantly inhibited invasion and migration abilities of GC cells with circ-CSPP1 siRNA treatment were observed by transwell assay.

Since tumor invasion and migration are closely related to EMT[29], we then examined the expressions of E-cadherin, N-cadherin, and Vimentin by Western blot. The results indicated that GC cells with cir-CSPP1 siRNA transfection showed significantly up-regulated E-cadherin protein expression, and down-regulated N-cadherin and Vimentin protein expressions. This was consistent with the results in transwell assay.

Conclusions

Shortly, circ-CSPP1 promoted the proliferation, migration, and invasion of GC cells, thereby providing a better understanding of GC occurrence and progression. All our findings suggested that circ-CSPP1 might serve as a new therapeutic target for the treatment of GC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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