CircWEE1/miR-138 axis promotes the malignant progression of glioma by regulating SIRT1

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Background: This study aims to reveal the potential impact of circWEE1 on the malignant progression of gliomas and its mechanism.

Methods: Real-time quantitative polymerase chain reaction (qRT-PCR) were used to detect circWEE1 levels in glioma tissues and cell lines. The relationship between circWEE1 expression and glioma metastasis was analyzed.

Results: After knocking out or over-expressing circWEE1, the effects on glioma cells were examined separately. Subsequently, the regulatory relationship of circWEE1 to miR-138 was detected by a dual luciferase reporter gene. In addition, we evaluated the role of silent information regulator 1 (SIRT1) in the progression of gliomas affected by circWEE1 through a rescue experiment. CircWEE1 was significantly up-regulated in glioma tissues and cell lines. At the same time, its expression level was significantly higher in glioma patients with lymphatic or distant metastasis than in glioma patients with non-metastasis. The down-regulation of circWEE1 reduced the viability, migration, and invasion ability of T98-G cells. The expression of miR-138 is negatively regulated by WEE1, while miR-138 directly targets and regulates the expression of SIRT1.

Conclusions: The rescue experiment confirmed that SIRT1 is a regulator of circWEE1 in the malignant progression of glioma. In summary, the CircWEE1/miR-138 axis may regulate SIRT1 to promote malignant progression in glioma.

Keywords: Glioma; circWEE1; miR-138; silent information regulator 1 (SIRT1); metastasis

Introduction

Glioblastoma is the most common adult central nervous system tumor accounting for 45–50% of CNS tumors (1-4). While gliomas include astrocytoma, oligodendroglioma, and ependymoma, glioblastoma accounts for more than half of these, and is the most malignant. Most gliomas occur slowly, usually in weeks to months from symptom onset to treatment. Since glioma seriously affects the quality of life of patients, its treatment is also the focus of significant clinical research.

Competitive endogenous RNA (ceRNA) is a transcription product that achieves mutual regulation through the competitive binding of miRNA reaction elements to common miRNA, and this mechanism is known as the "ceRNA" hypothesis (5). Since any transcription product with miRNA reaction element structure can theoretically function as ceRNA, ceRNA theory is of great

significance for integrating the pathogenesis of diseases, especially for tumor research. CircRNA is a kind of non-coding RNA produced by special selective shearing. Because it is a closed circular structure, circRNA has no free end and is not affected by RNA exonuclease, so it is more stable than linear RNA (6). While there are few studies on circRNA at present and the mechanism has not been clarified, there are still experiments (7) that confirm that some circRNA are rich in miRNA binding sites and play the role of competitive binding of miRNA in cells, which is a form of efficient ceRNA (8-12).

MicroRNAs (miRNAs) are a class of endogenous, non-coding single-stranded small RNA with a length of about 18–24 nucleotides, which are involved in various stages of tumor development and metastasis. MiR-138 is an important regulatory factor in tumors, and its expression is low in glioma cells and tissues. However, its role in glioma patients is not fully understood. As a member of the Sirtuin family, silent information regulator 1 (SIRT1) is a protein deacetylase that relies on NAD to deacetylate lysine residues of various proteins. Sirtuin is highly conserved in evolution from bacteria to eukaryotes and plays an important role in a variety of cellular functions in mammals, such as gene silencing, control of cell cycle and apoptosis, and energy balance (13). SIRT1 has a wide range of biological functions, such as growth regulation, response to stress response, tumor formation, and life extension. SIRT1 might be a target for the treatment of glioma. Several compounds have been shown to treat gliomas by inhibiting SIRT1. Such as, melatonin modulates the microenvironment of glioblastoma multiforme by targeting sirtuin 1 (14). However, at present, there are few studies on the role of SIRT1 in the pathogenesis of glioma.

In this study, an in vitro overexpression and knockout model of circWEE1 was constructed. At the same time, the target gene and regulation of SIRT1 on glioma progression were further investigated. The objective of this study was to describe the regulatory role of circWEE1/miR-138/SIRT1 axis in glioma and to describe the cellular/molecular mechanism of this axis function. This study is the first to report the biological function of circWEE1 in glioma. Our study reveals new aspects of cell function and pathophysiology of circWEE1 and miR-138. All of them can be considered as potential molecular targets for the treatment of glioma.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/tcr-21-251).

Methods

Patient and glioma samples

A total of 10 matched glioma and adjacent tissues were obtained between September 2017 and October 2019 from The Second Hospital of Shanxi Medical University. The tumor staging of gliomas was evaluated according to criteria proposed by the UICC. The experiment was approved by the ethics committee of Shanxi Medical University in line with the Declaration of Helsinki (as revised in 2013), and informed consent was obtained from all patients.

Cell culture

Human glioma cell lines (U251, U87, T98-G) and human brain normal glial cell lines (HEB) were provided by ATCC (American Type Culture Collection; Manassas, Virginia, USA). All cells were cultured in DMEM medium (DMEM; Thermo Fisher Scientific, Wal-Mart, Massachusetts) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37 ℃ in a humidified incubator with 5% CO_2.

Cell transfection

MiR-138 inhibitor or mimics and NC inhibitor or mimics were purchased from Genechem (Shanghai, China). Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were seeded into 6-well plates, cultured until confluence reached 60% for transfection and then harvested 48 hours after transfection for functional experiments and detection of protein expression.

Cell proliferation assay

Transfected cells were seeded into 96-well plates at a density of 2×10^3 cells per well. On days 1, 2, 3, and 4, a CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) was added to each well and incubated for 2 hours in the dark. Absorbance was recorded at 450 nm with a microplate reader (Japan, Nikon) and after the detection value was obtained, the cell proliferation rate was calculated.

Transwell experiment

Transfected cells were seeded into 24-well plates at 2×10^5 cells per well. 200 μL of the cell suspension was added to the Transwell upper chamber (Millipore, Billerica, MA, USA),

and 600 µL of complete medium with 10% FBS added to the Transwell lower chamber. After 48 hours of incubation, the cells were fixed in methanol for 15 minutes and stained with crystal violet for 10 minutes. Cell migration was then observed under a microscope (15,16).

Scratch test

Cells were seeded into a 24-well plate at 5.0×10^5 cells/well. After the cells adhered, an artificial wound was formed in the fused cell monolayer using a 100 µL pipette tip. Wound closure images were taken at 0 hours (cell adhesion) and 48 hours using an inverted microscope and the percentage of wound closure was calculated.

Real-time quantitative polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA). After total RNA was purified by DNase I treatment, the extracted RNA was reverse transcribed into cDNA using PrimeScript RT reagent (TaKaRa, Otsu, Shiga, Japan). QRTPCR was performed on the obtained cDNA using SYBR Premix Ex Taq™ (TaKaRa, Ostu, Shiga, Japan). The β-actin and U6 were used as internal references and each sample was repeated three times. The relative expression levels of genes were calculated by the $2^{-\Delta\Delta Ct}$ method.

Double-Luciferase reporter assay

After co-transfection of the plasmid for 36–48 h, the medium was discarded, and the cells were washed with 100 µL of 1× PBS. Deionized water diluted the 5× PLB (lysate) to 1× PLB and placed it at normal temperature before use. A 50 µL portion of the diluted 1× PLB was then added to each well and placed on a shaker for 20–30 minutes to ensure that the lysis buffer completely lysed the cells. A white opaque 96-well microtiter plate was then added with 10 µL of supernatant to each well, and 100 µL of premixed Luciferase Assay Reagent II was added to measure the data to measure the luciferase reaction intensity. After the measurement was completed, 100 µL of premixed Stop & Glo Reagent was added to each well and allowed to stand for 2 s, and the data was then measured to determine the intensity of the internal reference Renilla luciferase reaction. The ratio of the two sets of data was then calculated.

In vivo study

Six female nude mice (18–22 g) were purchased from Kay Biological Technology Co. Ltd (Shanghai, China). The BALB/c mice were randomly divided into a sh-NC group and a sh-CircWEE1group. The sh-NC group were injected with 100 µL of phosphate buffered sodium only as control and the sh-CircWEE1group were injected with 100 µL of T98-G cells by subcutaneous injection. After two weeks, mice were dissected and the tumors were obtained and weighed. Experiments were performed under a project license (NO.: IRB-2018-012) granted by ethics board of Shanxi Medical University, in compliance with NIH guidelines for the care and use of animals.

Statistical analysis

SPSS 22.0 (SPSS IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Experimental data are expressed as mean ± standard deviation. Differences between groups were analyzed by t test and multiple groups were compared using ANOVA. $P < 0.05$ was considered statistically significant (17).

Results

CircWEE1 is up-regulated in glioma tissues and cell lines

CircWEE1 expression was significantly up-regulated in glioma tissues compared to adjacent cancer tissues (*Figure 1A*). Similarly, the expression level of circWEE1 in glioma cells was significantly higher than that of normal glioma cells (*Figure 1B*). As T98-G expressed the highest circWEE1 abundance among the three glioma cell lines, it was used in subsequent experiments. CircWEE1 expression is related to metastasis and prognosis in glioma patients. We found that circWEE1 levels were positively correlated with lymphatic and distant metastases and lymphatic or distant metastatic patients with glioma had higher circWEE1 expression (*Figure 1C,D*).

CircWEE1 promotes the progression of gliomas in vivo and in vitro

We transfected T98-G cells with si-circWEE1 to detect the mechanism of circWEE1 in gliomas. *Figure 2A* shows that si-circWEE1 markedly decreased the level of circWEE1, which indicated that the transfection was effective, and
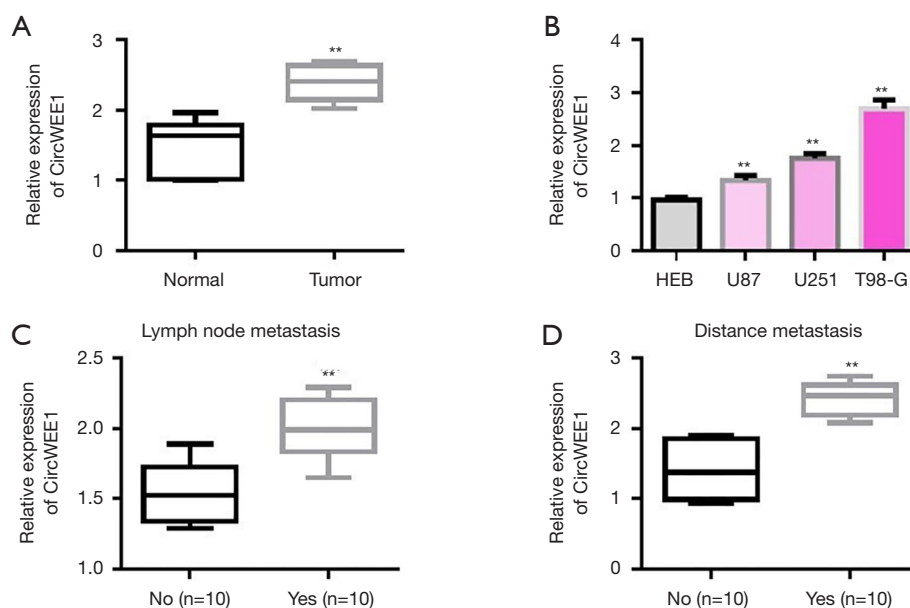
Figure 1 CircWEE1 expression in glioma and adjacent tissues and analysis of clinical information. (A) CircWEE1 level in para-cancerous tissues and glioma tissues; (B) circWEE1 level in normal glial cells and glioma cells; (C) circWEE1 level in glioma patients either with lymphatic metastasis or not; (D) circWEE1 level in glioma patients either with distant metastasis or not. **P<0.01.

Transwell assay and cell scratch test results suggested that si-circWEE1 inhibited the invasion and migration abilities of T98-G cells (*Figure 2B,C*). Further experiment also indicated that sh-circWEE1 inhibited the growth of gliomas in vivo (*Figure 2D*). Collectively, these results show circWEE1 can promote the progression of gliomas in vivo and in vitro.

MiR-138 is the target gene of circWEE1

To further study the mechanism of circWEE1 in gliomas, we first analyzed the miRNAs bound by circWEE1. The results show that circWEE1 binds to miR-138 (*Figure 3A*) and further detection of luciferase reporter gene confirmed the binding site between circWEE1 and miR-138 (*Figure 3B*). To verify the regulatory effect of circWEE1 on miR-138, we overexpressed and knocked down circWEE1, respectively (*Figure 3C*). The expression of miR-138 showed that over-expression of circWEE1 could inhibit the expression of miR-138, while knockdown of circWEE1 could promote the expression of miR-138 (*Figure 3D*). The co-expression test results of circWEE1 and miR-138 showed that they had a negative co-expression correlation (*Figure 3E*).

MiR-138 reduces malignant progression of glioma

Figure 4A suggests that miR-138 mimics upregulated the expression of miR-138 and miR-138 inhibitor down-regulated the expression of miR-138. Cell Transwell experiments showed that miR-138 mimics added to glioma cell lines reduced cell invasion capacity and adding miR-138 inhibitors increased cell invasion (*Figure 4B*). Cell migration experiments showed that miR-138 mimics added to glioma cell lines reduced cell migration capacity, while adding miR-138 inhibitors enhances cell migration capacity (*Figure 4C*). All results indicated that miR-138 could inhibit the malignant progression of glioma.

MiR-138 inhibits SIRT1 expression

To investigate the target genes of miR-138, we used TargetScan to predict the target gene information of miR-138 and *Figure 5A* shows a map of the binding sites of the two. Luciferase reporter assay suggested that miR-138 can bind to SIRT1 (*Figure 5B*). We transfected T98G cells with mimics NC, miR-138 mimics, inhibitor NC, and miR-138 inhibitor and detected the expression of miR-138 by PT-qPCR and the results indicated that the transfection is effective (*Figure 5C*).
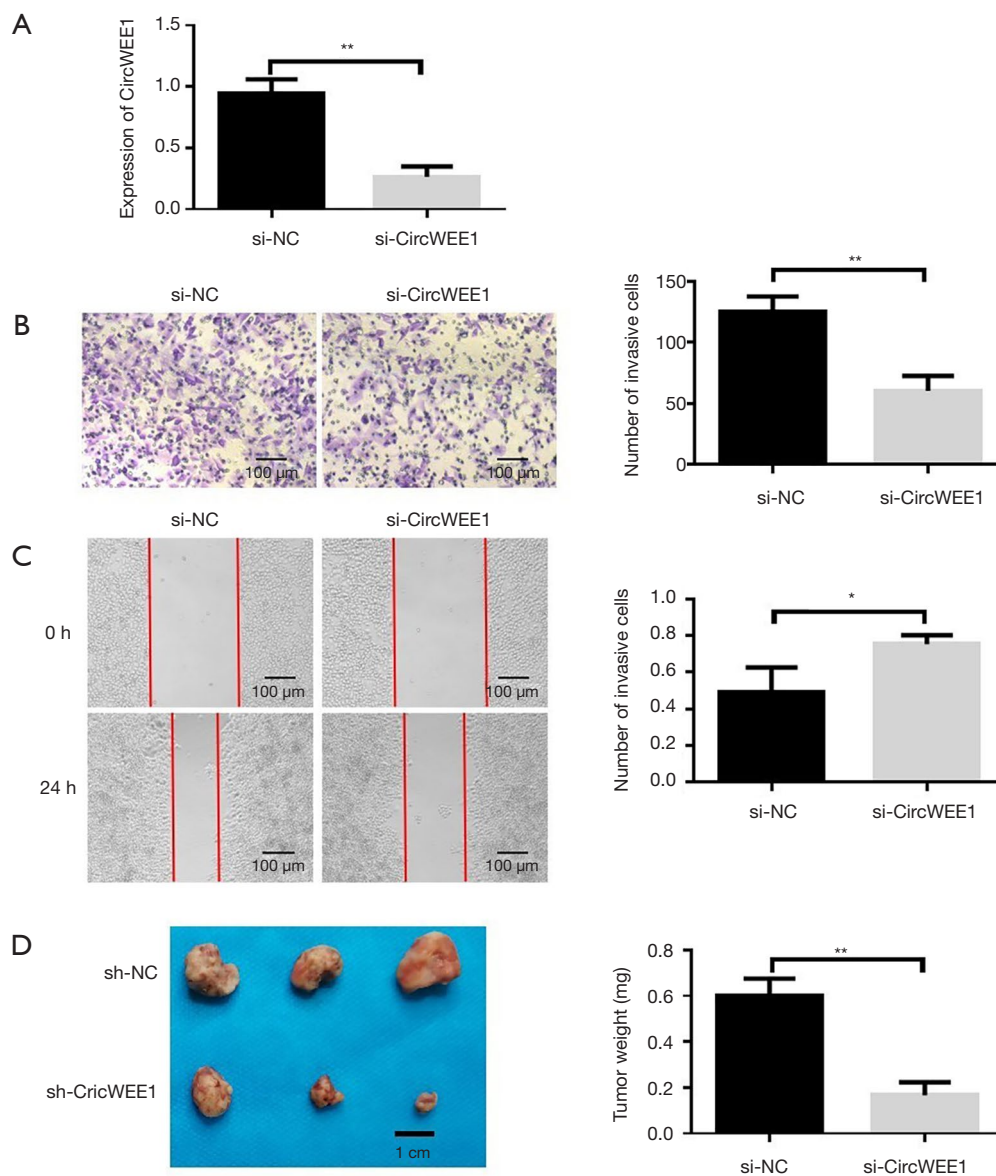
Figure 2 CircWEE1 promotes the progression of gliomas *in vivo* and *in vitro*. (A) RT-qPCR was used to test the level of CircWEE1; (B) si-CircWEE1 decreased the number of invasive cells (Transwell assay, Magnification 100×); (C) si-CircWEE1 inhibited cell migration; (D) Downregulation of CircWEE1 inhibited the growth of tumor *in vivo*. *P<0.05, **P<0.01.

CircWEE1 promotes the malignant evolution of glioma cells by up-regulating the expression of SIRT1 through sponge adsorption of miR-138

To further study the regulatory mechanism of ceRNA between circWEE1 and miR-138, we first tested the expression of SIRT1 and the experimental results showed that si-circWEE1 could inhibit the expression of SIRT1. However, miR-138 inhibitor could reverse the effect of si-circWEE1 on SIRT1 expression (*Figure 6A*). Cell proliferation test results showed that si-circWEE1 could inhibit the proliferation of glioma cells but miR-138 inhibitor could reverse the inhibitory effect of si-circWEE1 (*Figure 6B*). Cell Transwell and scratch detection results showed that si-circWEE1 could inhibit the invasion and migration ability of glioma cells but miR-138 inhibitor could reverse the inhibitory effect of si-circWEE1
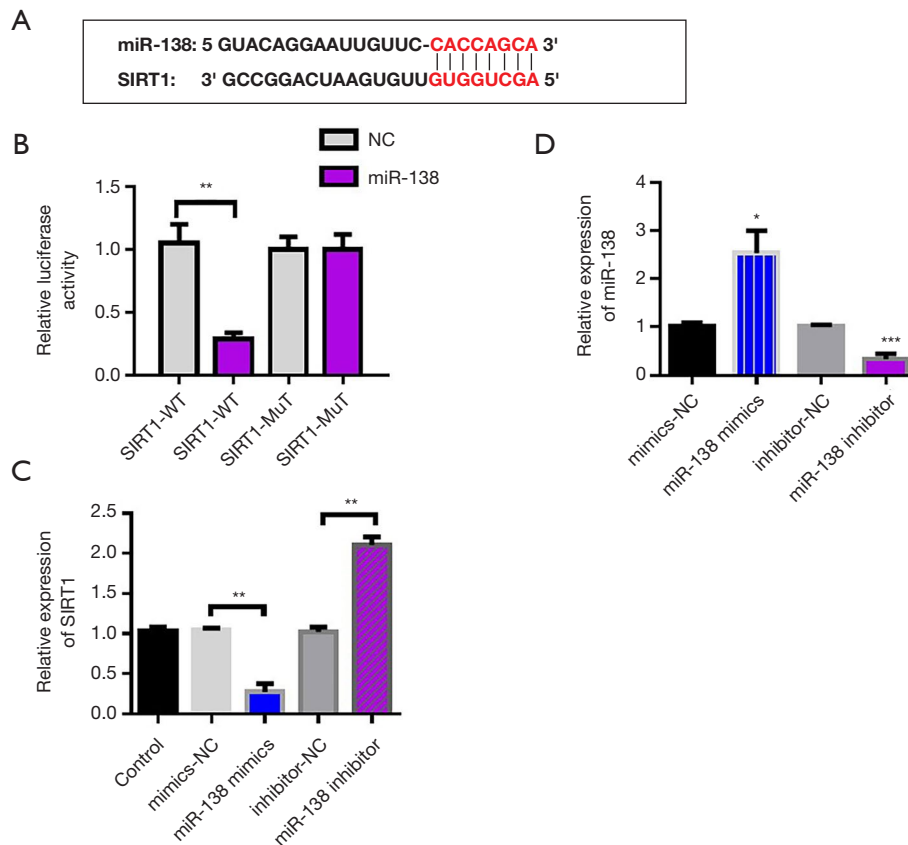
Figure 3 CircWEE1 sponge as miR-138. (A) CircWEE1 and miR-138 binding site information maps; (B) dual luciferase report detects circWEE1 binding to miR-138; (C) circWEE1 overexpression and expression verification after knockdown; (D) circWEE1 inhibits miR-138 expression; (E) correlation analysis between circWEE1 and miR-138 co-expression. *P<0.05, **P<0.01, ***P<0.001.

(*Figure 6C,D*). E-cadherin expression was consistent with SIRT1 expression (*Figure 6E*), while Vimentin expression was correlated with SIRT1 expression (*Figure 6F*). The experimental results showed that circRNA up-regulates the expression of SIRT1 by sponge-adsorbed miR-138 to promote the malignant evolution of glioma cells.

Discussion

Glioma is one of the most common intracranial malignant tumors. The WHO categorizes glioma according to its malignancy degree into four levels. While levels I-II level are considered as low-grade glioma, levels III-IV are high-grade glioma, and the both are categorized by an unclear pathogenesis, high mortality and short survival time (18,19). Therefore, it is particularly important to further understand the occurrence and development of glioma, identify the relevant factors affecting the degree of glioma malignancy,

and find early diagnosis and treatment methods (20-22).

In this study, we found that circWEE1 was significantly up-regulated in gliomas and its level was positively correlated with high metastasis rate and poor prognosis in glioma patients. Therefore, circWEE1 may play a carcinogenic role in the progression of glioma. In vitro experiments showed that knockout of circWEE1 reduced the viability, migration ability, and wound closure rate of glioma cells. To date, several ceRNAs, including PTENP1 and KRAS, have been shown to be related to tumor development (23-26) and the clinical application of ceRNA in tumors is another future research direction. Cerebellar degeneration-related protein 1 antisense (CDR1as) consists of about 1500 nucleotides and has more than 70 miR-7 binding sites. Overexpression of CDR1 will inhibit the activity of miR-7, and this phenomenon is particularly evident in the CNS (6). MiR-7 is abnormally expressed in malignant tumors such as glioblastoma, gastric cancer, and
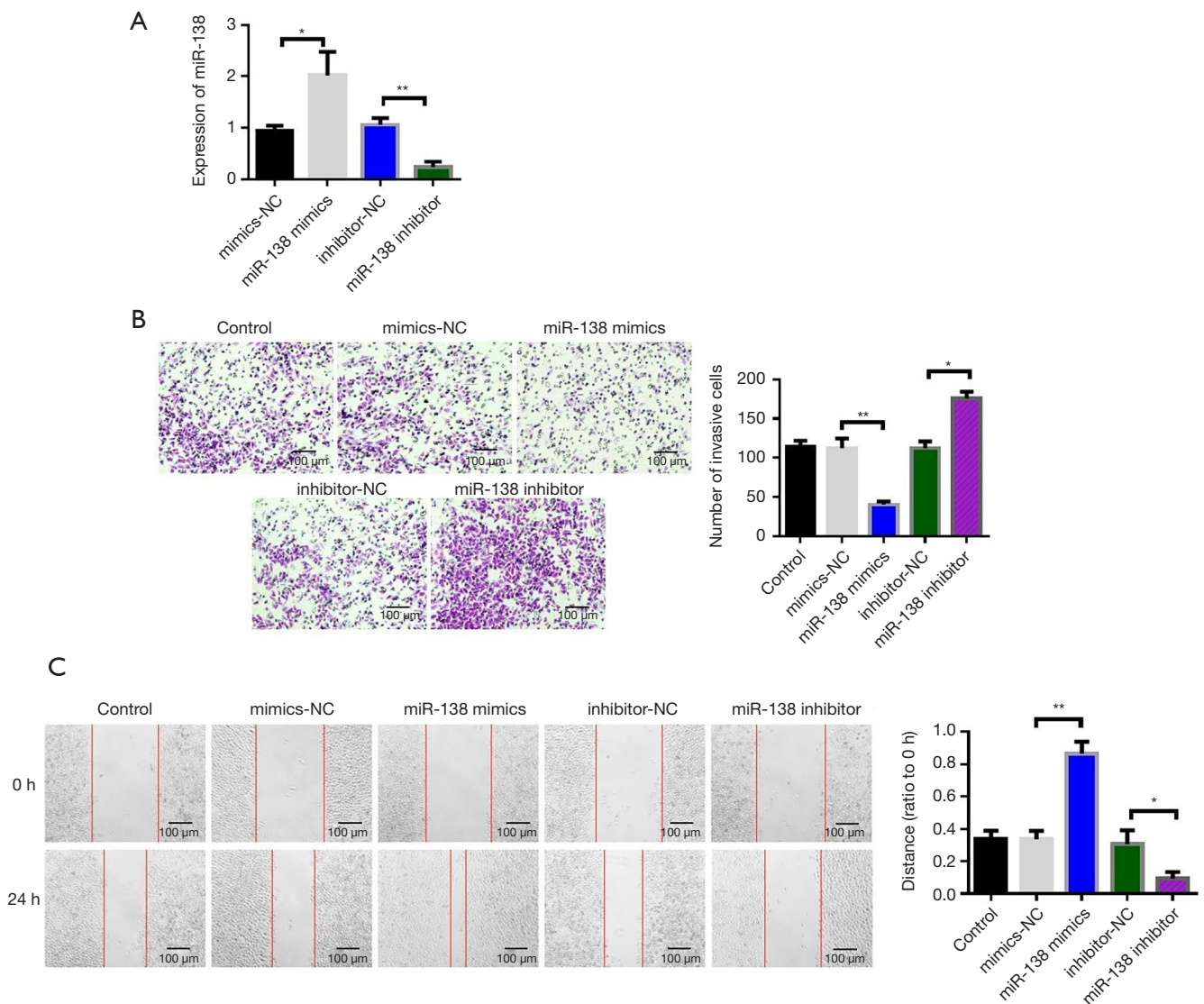
Figure 4 MiR-138 reduces malignant evolution of glioma. (A) RT-qPCR was used to test the level of miR-138; (B) cell invasion experiment. miR-138 overexpression inhibited cell invasion ability and miR-138 knockdown promoted cell invasion ability (Transwell assay, magnification 100×); (C) cell migration experiments. miR-138 overexpression inhibited cell migration ability and miR-138 knockdown promoted cell migration ability, *P<0.05, **P<0.01.

colorectal cancer, and through the competitive binding of miR-7, miR-7 target gene expression is increased. We have reason to suspect that CDR1 may be related to the progress of related tumors and play an important regulatory function (27). In esophageal squamous cell carcinoma, circ-ITCH regulates ITCH levels through competitive binding to miR-7, 17, 214. Barbagallo *et al.* (28) found that compared with normal brain tissue, circ-TTBK2 was significantly up-regulated in gliomas, inhibited tumor

cell apoptosis, and was positively correlated with the pathological grade of gliomas. Using normal astrocytes for comparison, we found that miR-217 was down-regulated in glioma U87 and U251 cell lines, and that returning miR-217 to normal expression could inhibit the proliferation of glioma cells. On this basis, we speculate that miR-217 plays a tumor suppressive role in gliomas. Our results also show that circ-TTBK2 reduced the expression of miR-217 in glioma cells in the manner of RNA-induced silencing
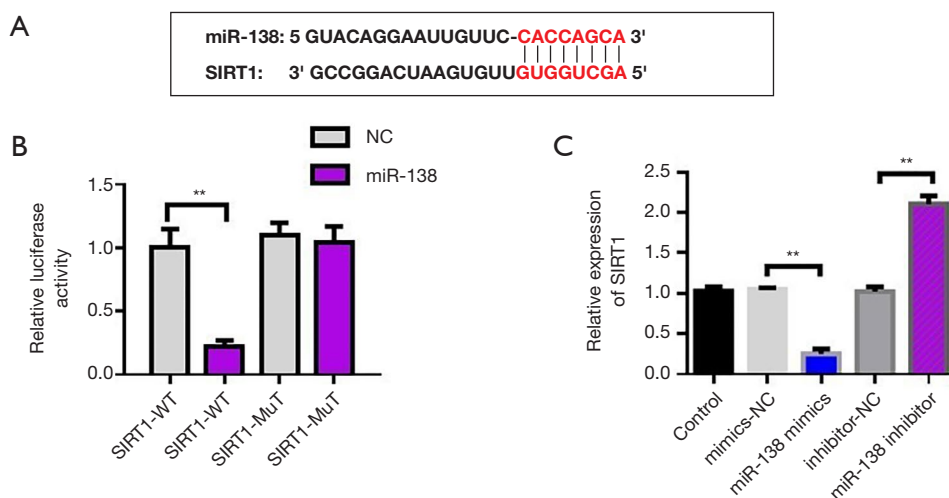
Figure 5 MiR-138 inhibits SIRT1 expression. (A) Information maps of miR-138 and SIRT1 binding sites; (B) dual luciferase report assay detects the relationship between miR-138 and SIRT1; (C) when miR-138 mimics was added to the glioma cell line, SIRT1 content decreased. With the addition of miR-138 inhibitor, SIRT1 content increased. **$P<0.01$.

complex (RISC).

MiRNA is a member of the non-coding RNA family. In recent years, research has found that miRNA plays an important role in the occurrence and development of tumors and has great clinical diagnostic value (29). Lu *et al.* (30) found that down-regulation of the miR-573 expression level promoted the occurrence of gastric cancer and can be used as a tumor marker in its early diagnosis. MiR-138 is a new class of miRNAs with tumor regulation showing low expression in a variety of malignant tumors (31,32). Therefore, miR-138 usually acts as a tumor suppressor gene during tumor formation. Stojcheva *et al.* (33) showed that miR-138 can inhibit autophagy-mediated temozolomide chemotherapy resistance to glioma cells by targeting the inhibitor of apoptosis protein BIM. Studies have also shown that miR-138 down-regulates hTERT protein and its expression inhibits the proliferation and migration of glioma cells. On this basis, miR-138 is considered a new tumor suppressor for gliomas.

The occurrence of tumors is closely related to the overexpression of oncogenes or the low expression of tumor suppressor genes. Type III histone deacetylase SIRT1 gene expression and deacetylase activity are up-regulated in tumor cells and it is speculated that these may cause tumorigenesis. SIRT1 can also cause deacetylation of tumor suppressors and promote tumorigenesis. In the occurrence,

development, and maintenance of various characteristics of tumors, SIRT1 is likely to play an important role because it can promote tumor cell proliferation and inhibit apoptosis and aging. Studies have reported that SIRT1 is significantly overexpressed in human prostate cancer, human primary colon cancer, and acute myeloid leukemia (34-36). In addition, SIRT1 is also highly expressed in human squamous cell carcinoma and basal cell carcinoma (37-38) and can silence growth and apoptosis of human epithelial cancer cells by silencing SIRT1 (39). FOXM1 is an upstream regulator of SIRT1 in glioma cells. FOXM1 is highly expressed in gliomas. The expression of FOXM1 in glioma cells was overexpressed by gene transfection, and the mRNA and protein levels of SIRT1 in glioma cells were significantly increased. Silencing the expression of FOXM1 with siRNA reduced the expression of SIRT1 in glioma cells. At the transcriptional level, FOXM1 was found to bind to the promoter region of SIRT1 gene in glioma cells and activate SIRT1 promoter activity in a dose-dependent manner. The regulation of FOXM1 on SIRT1 in glioma was further confirmed (40).

In summary, circWEE1 is upregulated in glioma tissues and is related to distant metastases and poor prognosis in glioma patients. CircWEE1 up-regulates SIRT1 expression by targeting miR-138, aggravating the malignant progression of glioma.
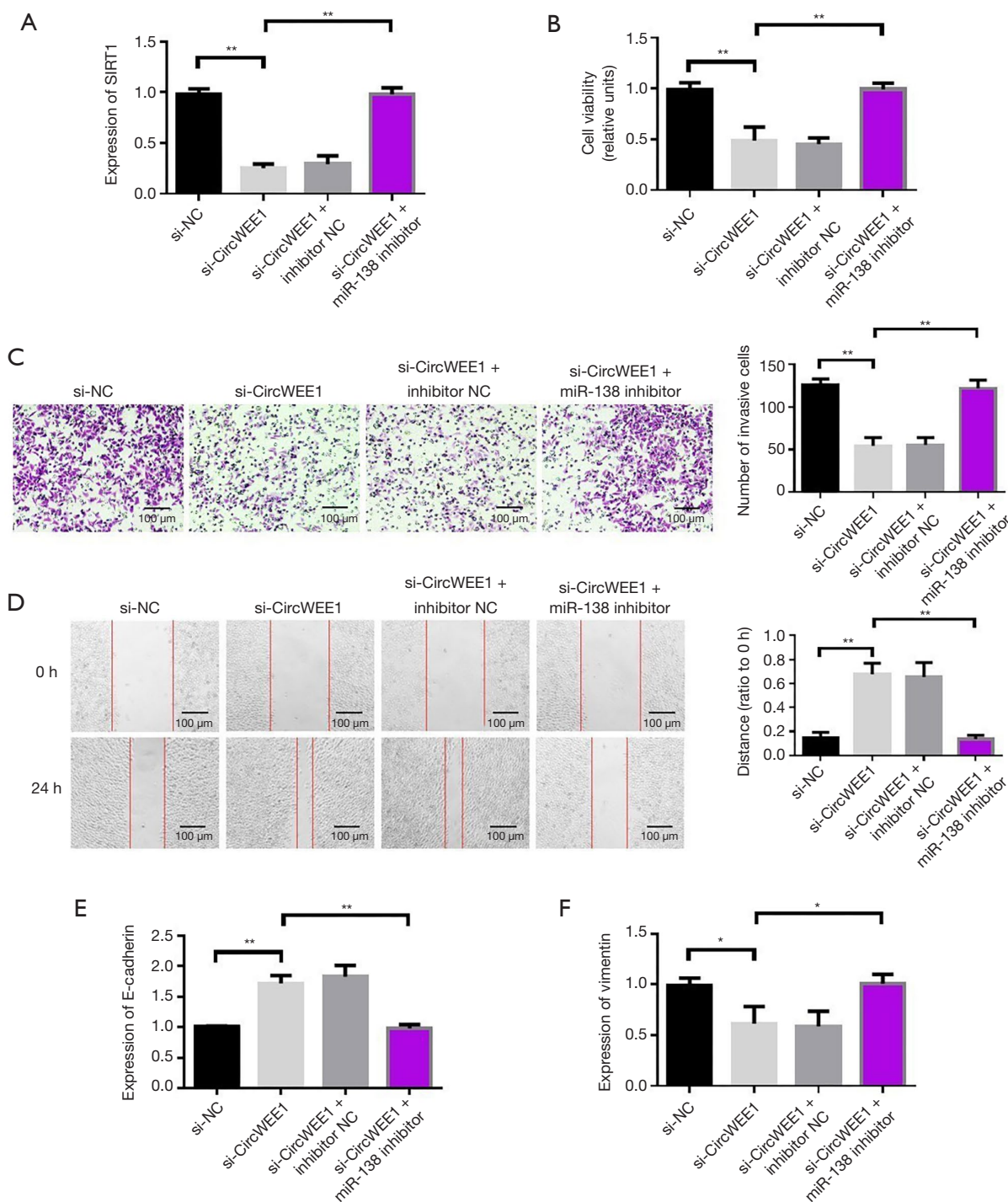
Figure 6 CircWEE1 promotes the malignant evolution of glioma cells by up-regulating the expression of SIRT1 through the adsorption of miR-138 by sponge effect. (A) Detection of SIRT1 expression; (B) cell proliferation detection; (C) detection of cell invasion (Transwell assay, magnification 100×); (D) cell migration detection; (E) detection of E-cadherin expression; (F) detection of Vimentin expression. *P<0.05, **P<0.01.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The experiment involving human was approved by the ethics committee of Shanxi Medical University in line with the Declaration of Helsinki (as revised in 2013), and informed consent was obtained from all patients. Experiments were performed under a project license (NO.: IRB-2018-012) granted by ethics board of Shanxi Medical University, in compliance with NIH guidelines for the care and use of animals.

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