The circ_VCAN with radioresistance contributes to the carcinogenesis of glioma by regulating microRNA-1183

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Abstract
Circular RNAs (circRNAs), a widespread type of noncoding RNA, are produced by reverse splicing with a circular loop structure. Circ_VCAN (hsa_circ_0073237) acts as a novel circRNA, although its roles in the progression and radioresistance of glioma remain unknown.

Expressions of circ_VCAN and microRNA-1183 (miR-1183) were analyzed by quantitative real-time PCR, and the functions of circ_VCAN and irradiate in glioma cell proliferation, apoptosis, migration, and invasion were assessed using cell counting kit-8, flow cytometry, Wound healing, and Transwell assays. The interaction between circ_VCAN and miR-1183 was validated dual-luciferase reporter assay.

Our results revealed that circ_VCAN was significantly upregulated in radioresistant glioma tissues compared with radiosensitive tissues, and that circ_VCAN expression was negatively correlated with miR-1183 expression in glioma tissues. We also determined that circ_VCAN expression was decreased and miR-1183 expression was increased in U87 and U251 cells after irradiation. Both knockdown of circ_VCAN and treatment with miR-1183 mimics inhibited proliferation, migration, and invasion, and accelerated apoptosis of the irradiated U87 and U251 cells. In addition, luciferase reporter assays revealed that circ_VCAN might function as a sponge for miR-1183. Finally, overexpression of circ_VCAN expedited carcinogenesis and reduced glioma radiosensitivity by regulating miR-1183.

Circ_VCAN serves as a potential oncogene of glioma by regulating miR-1183, and plays an essential role in the radioresistance of glioma.

Abbreviations: circRNAs = circular RNAs, DMEM = Dulbecco modified Eagle medium, EMT = epithelial mesenchymal transition, FBS = fetal bovine serum, hsa_circ_0073237 = Circ_VCAN, miR-1183 = microRNA-1183, NC = glioma cells, ncRNAs = noncoding RNAs, Pi = propidium iodide, RT-qPCR = quantitative real-time PCR.

Keywords: circ_VCAN, glioma, miR-1183, radioresistance

1. Introduction
Glioma is a primary intracranial malignant tumor. Its incidence in the population is approximately 3 to 10/100,000, which has increased every year in recent years.[1,2] Glioma is primarily treated by surgical resection and postoperative radiotherapy, which is routinely performed due to aggressive growth of the tumor.[3–5] However, due to the radiobiological characteristics of tumors, most gliomas are not sensitive to radiation, which often leads to treatment failure.[6,7] An increase in radiation dose in the tumor area can improve the local control rate of the tumor; however, an increase in radiation dose is often limited by the tolerance of normal tissues and organs around the tumor.[8] Thus, safe and effective methods to reduce the radiation resistance of malignant glioma are urgently needed; the development of such methods will require an in-depth understanding of the molecular radiobiology mechanism of glioma radiation resistance.[9] Ionizing radiation can cause cell cycle arrest, DNA repair, and apoptosis, which are key factors that determine cell radiation sensitivity.[10,11] Therefore, it will be important to explore novel key regulatory points, which will be pivotal for designing targeted radiotherapy sensitizers and contributing to clinically applicable strategies for the treatment of glioma.

Circular RNAs (circRNAs) are a novel class of noncoding RNAs (ncRNAs) that widely exist in cells.[12] circRNAs are characterized by structural stability and tissue-specific expression because of their closed loop structure.[13,14] Recent studies have indicated that most circRNAs are formed during the human epithelial mesenchymal transition (EMT) through exon and intron circularization, and more than a third are dynamically regulated by the RNA-binding protein Quaking.[15–17] Because the EMT is involved in the development and progression of tumors,[18,19] most circRNAs are closely related to tumor development.[12,20] Circ_VCAN (hsa_circ_0073237) with gene
symbol (VCAN) is located at chr5:82832825–82838087, and its best transcript is NM_004385. Circ_VCAN has been described in previous studies \cite{21-23}; however, the regulatory network and biological function of circ_VCAN in glioma remains unknown.

Numerous studies have shown that circRNAs not only act as a messenger between DNA and coding proteins, but also regulate the expression of target genes and affect the course of disease by competitively binding with corresponding microRNAs (miRNAs) \cite{24,25}. This function of circRNAs is known as competitive endogenous RNA (ceRNA), which is also referred to as molecular "sponging" \cite{26-28}. For example, the sponge effect of CDRI1 on miR-7 has been associated with many diseases, including lung cancer, breast cancer, glioma, and amyotrophic lateral sclerosis \cite{29-36}. In our preliminary experiment, we predicted miRNAs that might bind Circ_VCAN through bioinformatics analysis, and found that microRNA-1183 (miR-1183) may be a regulatory target of Circ_VCAN. Whether circ_VCAN participates in the glioma process and radiation resistance by miR-1183 has not been reported.

In the present study, we investigated the correlation between circ_VCAN and miR-1183 expression in glioma, and circ_VCAN expression in radioresistant and radiosensitive glioma tissues. In addition, we explored the effect of circ_VCAN knockdown combined with irradiation on proliferation, apoptosis, migration, and invasion of glioma cells. We revealed that circ_VCAN acts as a miRNA sponge of miR-1183, and overexpression of circ_VCAN increased proliferation, migration, and invasion, and inhibited apoptosis, of the irradiated glioma cells by regulating miR-1183.

2. Materials and methods

2.1. Tissue specimens

A total of 57 radiosensitive glioma tissues and 57 radioresistant glioma tissues were obtained from patients who were diagnosed with glioma from 2015 to 2019 from the Xinjiang Uygur Autonomous Region People’s Hospital. Written informed consent was obtained from all participants. The study was approved by the Ethics Committee of the Xinjiang Uygur Autonomous Region People’s Hospital. Collected tissues were frozen at –80°C until use.

2.2. Cell culture

The 293T, U87, and U251 cells were purchased from American Type Culture Collection and grown in Dulbecco modified Eagle medium (DMEM; Hyclone, UT, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Massachusetts, USA). All cells were maintained at 37°C in a humidified environment with 5% CO2.

2.3. Cell transfection

The circ_VCAN overexpression plasmid, empty vector, miR-1183 mimics, and negative control (NC) mimics were purchased from GenePharma (Shanghai, China). For transfection, glioma cells were cultured at 37°C for 8 hours followed by transient transfection with the corresponding oligonucleotides with Lipofectamine 3000 (Invitrogen, CA, USA) according to the manufacturer’s instructions.

2.4. Radiation treatment

A high energy X-ray accelerator (2 Gy/min) with a dose of 6.4 Gy was used to irradiate U87 and U251 cells in exponential proliferation phase. The irradiation exposure was obtained using an X-ray irradiator (Rad Source Technologies, RS2000-PRO, FL, USA).

2.5. RNA extraction and quantitative real-time PCR (RT-qPCR) assay

Total RNA was extracted using TRIZol reagent (Takara, Japan) from glioma tissues and U87 and U251 cells according to the manufacturer’s instructions. Complementary DNA synthesis was performed using the Bestar qPCR RT kit (DBI Bioscience, China) according to the manufacturer’s instructions. The expression levels of circ_VCAN and miR-1183 were quantified using the Bestar qPCR MasterMix (DBI Bioscience) on the ABI PRISM 7500 Sequence Detection System (Life Technologies, NY, USA). The sequences of primers were exhibited in Table 1.

2.6. Cell counting kit-8 assay

Cell proliferation of U87 and U251 cells was assessed using gliomaK-8 (Beiyotime Institute of Biotechnology, China). U87 and U251 cells were seeded in 96-well plates at a concentration of 3000 cells/well for 8 hours followed by transfection with circ_VCAN and its vector control, as well as miR-1183 mimics and its NC (miR-NC) before irradiation. At 0, 12, 24, 36, and 48 hours after transfection, 10 μL of gliomaK-8 (5 mg/mL) was added to each well and the cells were maintained at 37°C for 3 hours. The absorbance of each well was determined using a microtiter plate reader (SpectraMax, Molecular Devices, CA, USA) at 450 nm.

2.7. Flow cytometry detection

Treated U87 and U251 cells were washed with cold phosphate-buffered saline. After centrifugation, the cells were resuspended in 1 × Annexin V binding buffer. The suspension liquid with cells in each group was transferred to centrifuge tubes and 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) solution were added. The mixture was incubated for 10 minutes at room temperature in the dark. Apoptotic cells were evaluated using flow cytometry.

2.8. Wound healing assay

Treated U87 and U251 cells (2 × 104 cells/well) were seeded in 6-well plates. After 24 hours, the cells were scratched using a
200-μL pipette tip followed by removal of the supernatant and addition of culture medium. Wound healing was examined under an inverted microscope (Olympus, Tokyo, Japan).

2.9. Invasion analysis
Matrigel matrix (BD Biosciences, CA, USA) was added into the superstratum of Transwell chambers (8-μm pores, Corning Incorporated, NY, USA) and incubated at 37°C for 30 minutes. Treated U87 and U251 cells were harvested and resuspended in serum-free DMEM medium, and the cells were counted and diluted at a final concentration of 1 × 10⁵ cells/ml. Then, 200 µL of cell suspension was added into the upper chamber, and 600 µL of DMEM medium with 10% FBS was added into the lower chamber. After incubation for 24 hours, the cells were fixed with paraformaldehyde and stained with 0.5% Crystal Violet. After washing, the invaded cells were observed under an inverted microscope (Olympus).

2.10. Dual luciferase reporter assay
The interaction between circ_VCAN and miR-1183 was verified by dual-luciferase reporter assay. circ_VCAN containing wild-type or mutant miR-1183 binding sites was amplified and inserted into the psi-CHECK2 vector (Promega, Madison, WI) to generate the following plasmids: psiCHECK2-circ_VCAN-WT (circ_VCAN-WT) and psiCHECK2-circ_VCAN-WT (circ_VCAN-Mut). 293T cells (1 × 10⁵ cells/well) were seeded in 96-well plates and transfected with circ_VCAN-WT or circ_VCAN-Mut and miR-1183 or miR-NC. Luciferase activity was determined using the Dual-Luciferase Assay System (Promega).

2.11. Biotin pull-down assay
Biotin-miR-NC, biotin-miR-1183, and biotin-miR-1183 mut were purchased from Integrated Biotech Solutions (Shanghai, China) and transfected into U87 and U2512 cells for 48 hours. U87 and U2512 cell lysates were obtained using lysis buffer, and added to 50 µL of beads solution (Dynabeads MyOne Streptavidin C1, Life Technologies). RNA was extracted from the mixtures using TRizol reagent followed by qRT-PCR detection.

2.12. Statistical analysis
Data are expressed as mean ± standard deviation, and the results were analyzed using GraphPad Prism Software (Ver. Prism 7). The expression levels of circ_VCAN and miR-1183 in radioresistant tissues and radiosensitive tissues were determined using normal distribution test and then analyzed using paired t test; the correlation between circ_VCAN and miR-1183 was counted using Pearson correlation analysis; other data were analyzed Student t test or 1-way analysis of variance. P < .05 was considered statistically significant.

3. Results
3.1. Circ_VCAN was negatively correlated with miR-1183 following glioma irradiation
To determine the relationship between circ_VCAN and radioresistance in glioma, we collected 57 radiosensitive glioma tissues and 57 radioresistant glioma tissues. Circ_VCAN expression was examined by RT-qPCR, and the results revealed that circ_VCAN expression was higher in radioresistant tissues than radiosensitive tissues (P < .001, Fig. 1A). We then analyzed miR-1183 expression in glioma, and confirmed its correlation with circ_VCAN. Pearson correlation analysis revealed that circ_VCAN expression was inversely related to that of miR-1183 (R² = 0.1045, P = .03, Fig. 1B). We further investigated the changes in expression of circ_VCAN and miR-1183 in response to ionizing radiation in U87 and U2511 cells. As shown in Figure 1C, circ_VCAN expression was significantly downregulated in U87 and U2511 cells while miR-1183 expression was significantly upregulated in U87 and U2511 cells following irradiation (Fig. 1D). These data indicate that irradiation decreased circ_VCAN expression and increased miR-1183 expression in glioma.

3.2. Knockdown of circ_VCAN inhibited proliferation and increased apoptosis in the irradiated glioma cells
To further identify the effects of circ_VCAN on glioma cell proliferation and apoptosis following irradiation, U87 and U2511 cells were transfected with small interfering RNAs targeting circ_VCAN before irradiation. circ_VCAN expression was significantly reduced following circ_VCAN knockdown in the irradiated and untreated cells (P < .05, P < .01, P < .001, Fig. 2A). The gliomaK-8 assay revealed that cell proliferation was significantly decreased in the circ_VCAN and irradiation groups relative to the NC group; cell proliferation was also dramatically attenuated in the irradiation + circ_VCAN group compared with the irradiation + NC group (P < .05, P < .01, Fig. 2B). Annexin V-FITC/PI double staining indicated that the rate of apoptosis was markedly increased in the circ_VCAN and irradiation groups compared with the NC group, and apoptosis was also increased in the irradiation + circ_VCAN group compared with the irradiation + NC group (P < .05, P < .01, Fig. 2C). Therefore, we can conclude that circ_VCAN knockdown suppressed proliferation and induced apoptosis in the irradiated glioma cells.

3.3. Knockdown of circ_VCAN suppressed migration and invasion in the irradiated glioma cells
The effects of circ_VCAN knockdown on irradiation-mediated migration and invasion were also determined by wound healing and Transwell assays in circ_VCAN-silenced U87 and U2511 cells following radiation exposure. Knockdown of circ_VCAN inhibited the migration and invasion of U87 and U2511 cells; irradiation exposure also suppressed cell migration and invasion. circ_VCAN-silenced U87 and U2511 cells treated with X-ray irradiation exhibited distinct decreases in migration and invasion capacities relative to cells treated with irradiation alone (Fig. 3). Thus, we propose that circ_VCAN knockdown suppressed glioma migration and invasion following irradiation.

3.4. Circ_VCAN might be a miRNA sponge of miR-1183
Next, we predicted the potential target miRNAs of circ_VCAN using bioinformatics tools, and identified a possible binding site between circ_VCAN and miR-1183. As shown in the dual luciferase reporter assay, the luciferase activity of 293T cells driven by circ_VCAN-WT could be significantly weakened by miR-1183, but not miR-NC, while the luciferase activity of 293T
cells driven by circ_VCAN-Mut was not affected ($P < .001$, Fig. 4A). A pulldown assay was also performed to further confirm the interaction between circ_VCAN and miR-1183 in U87 and U251 cells. We found that the relative enrichment of circ_VCAN was significantly enhanced in the bio-miR-1183 group compared with the bio-NC group, while there was no change in the bio-miR-1183 mutation group compared with the bio-NC group ($P < .001$, Fig. 4B and C). Therefore, our results indicated that circ_VCAN might serve as a sponge for the miR-1183.

3.5. Circ_VCAN accelerated proliferation and inhibited apoptosis in the irradiated glioma cells by regulating miR-1183

As circ_VCAN cloud regulate miR-1183, we performed a recovery experiment to explore the effects of circ_VCAN overexpression in glioma progression and radiation resistance. Before irradiation, the circ_VCAN-overexpression plasmid and miR-1183 mimics were co-transfected into U87 and U251 cells,

Figure 1. Circ_VCAN is negatively correlated with miR-1183 expression following irradiation of glioma tissue. (A) Circ_VCAN expression was assessed by RT-qPCR in radiosensitive ($n = 57$) and radioresistant ($n = 57$) glioma tissues. (B) Expression correlation between circ_VCAN and miR-1183 was analyzed using Pearson correlation analysis ($R^2 = 0.1045$, $P = .0284$). (C) RT-qPCR analysis of circ_VCAN in U87 and U251 cells after 2 Gy exposure. (D) RT-qPCR analysis of miR-1183 in U87 and U251 cells after 2 Gy exposure. RT-qPCR = quantitative real-time PCR.
Figure 2. Knockdown of circ_VCAN inhibited proliferation and increased apoptosis of the irradiated glioma cells. U87 and U251 cells were transfected with NC and circ_VCAN siRNAs and irradiated. (A) The level of circ_VCAN was examined by RT-qPCR in treated U87 and U251 cells (*P < .01, **P < .001 vs NC group; #P < .05 vs IR + si-NC group). (B) The effect of circ_VCAN knockdown on irradiated cell viability was determined by gliomaK-8 assay (*P < .05 vs NC group; ##P < .01 vs IR + si-NC group). (C) Radiation-induced apoptosis was assessed using Annexin V-FITC/PI double staining in circ_VCAN-silenced U87 and U251 cells. NC = negative control, siRNAs = small interfering RNAs.
Figure 3. Knockdown of circ_VCAN suppressed migration and invasion of the irradiated glioma cells. Circ_VCAN was silenced in U87 and U251 cells before 6 Gy X-ray irradiation. (A) The change in cell migration capacity was assessed by wound healing assay. Magnification, ×100; scale bar = 100 μm. (B) The effect of circ_VCAN knockdown on invasion of the irradiated glioma cells was determined by Transwell assay. Magnification, ×200; scale bar = 50 μm.
irradiated glioma cells could be reversed by miR-1183 mimics. In addition, the increased migration and invasion of the irradiated glioma cells were promoted migration and invasion, while overexpression of circ_VCAN showed that irradiation dramatically suppressed glioma cell healing, and Transwell assays were performed. Our results indicated that irradiation inhibited glioma cell proliferation, while overexpression of circ_VCAN accelerated proliferation of the irradiated glioma cells, and miR-1183 further attenuated the increased proliferation of glioma cells induced by circ_VCAN overexpression in the irradiated U87 and U251 cells (P < .05, P < .01, P < .001, Fig. 5A). Our results indicated that irradiation inhibited glioma cell proliferation, while overexpression of circ_VCAN accelerated proliferation of the irradiated glioma cells, and miR-1183 further attenuated the increased proliferation of glioma cells induced by circ_VCAN overexpression in the irradiated U87 and U251 cells (P < .05, P < .01, P < .001, Fig. 5B). We also demonstrated that irradiation facilitated glioma cell apoptosis, while overexpression of circ_VCAN inhibited apoptosis of the irradiated glioma cells; the miR-1183 further weakened the inhibition of apoptosis mediated by circ_VCAN overexpression in the irradiated U87 and U251 cells (Fig. 5C).

3.6. Circ_VCAN enhanced migration and invasion of the irradiated glioma cells via miR-1183

To assess the effects of circ_VCAN-mediated miR-1183 on glioma cell migration and invasion following irradiation, wound healing, and Transwell assays were performed. Our results showed that irradiation dramatically suppressed glioma cell migration and invasion, while overexpression of circ_VCAN promoted migration and invasion of the irradiated glioma cells. In addition, the increased migration and invasion of the irradiated glioma cells could be reversed by miR-1183 mimics (Fig. 6).

4. Discussion

At present, the treatment of glioma is still a major clinical problem. The primary treatment is surgery, supplemented by radiotherapy and chemotherapy; however, the prognosis is still very poor. With the development of molecular biology technologies, targeted therapy has received increasing attention, although postoperative radiotherapy with 2.0 Gy X-ray is still the most effective treatment. However, a high dose of X-ray radiation can cause irreversible damage to normal glial cells, which can cause abnormalities in the central nervous system. Therefore, finding new effective targets will be important in improving the sensitivity of glioma radiotherapy and reducing the damage to normal brain tissue.

As an important subtype of ncRNAs, circRNAs have been shown to function as both oncogenes and tumor suppressors of glioma through various mechanisms. For instance, circCFH and circSMARCA5 might be potential markers for glioma; circSHPRH and circFBXW7 might exert tumor inhibitory effects in glioma; circNT5E and circNFIx may contribute to the development of glioma. Therefore, circRNAs could be used as potential therapeutic targets of glioma. Although several circRNAs have been identified as meaningful molecules in the clinical diagnosis of glioma, the regulatory mechanisms of most circRNAs, such as circ_VCAN, in glioma remain largely unclear. In the present study, we showed that knockdown of circ_VCAN inhibited proliferation, migration, and invasion, and enhanced apoptosis, of glioma cells.

Numerous studies have demonstrated that a large number of biomolecules can improve or reduce the sensitivity of glioma radiotherapy. For example, radiation-induced miR-30e accelerates the metastasis of glioma via EGFR stabilization; miR-212 is thought to facilitate the radioresistance of glioma through BRCA1; miR-630 suppresses the radioresistance of glioma cells by targeting CDC14A; and long ncRNA HMMR-AS1 could affect the radiosensitivity of glioma by reducing DNA repair. While the effects of circRNAs on the radiosensitivity of glioma have not been fully elucidated, in the present study, we showed that circ_VCAN was highly expressed in radioresistant tissues relative to radiosensitive tissues, and knockdown of circ_VCAN significantly prevented the progression of the irradiated glioma cells.

The circRNAs, with many miRNAs binding sites, act as ceRNAs. Previous studies have shown that circRNAs can participate in the development of glioma by targeting miRNAs. For example, the hsa_circ_0046701/miR-142-3p/ITGB8 regulatory loop facilitates the carcinogenesis of glioma; circ-TTBK2 accelerates the progression of malignant glioma by targeting miR-217 to upregulate the HNFiB/Derlin-1 pathway; and a novel circRNAs, hsa_circ_0007534, has been shown to promote the carcinogenesis of glioma by targeting miR-761 to increase...
ZIC5 expression.\textsuperscript{[55]} The present study indicated that miR-1183 was negatively correlated with circ_VCAN in irradiation-treated glioma, and overexpression of circ_VCAN enhanced proliferation, migration, and invasion, and inhibited apoptosis, of the irradiated glioma cells by regulating miR-1183. Prior research has also suggested that hsa_circ_0004015 can enhance the development and tyrosine kinase inhibitor drug resistance of non-small cell lung cancer by targeting miR-1183.\textsuperscript{[56]}

Figure 5. Circ_VCAN accelerated proliferation and inhibited apoptosis of the irradiated glioma cells by regulating miR-1183. U87 and U251 cells were transfected with circ_VCAN-overexpression plasmid and/or miR-1183 mimics before irradiation. (A) RT-qPCR analysis of miR-1183. (B) Detection of cell viability using the gliomaK-8 assay. (C) Flow cytometry analysis of apoptosis. $P < .05$, $^\# P < .01$, $^{**} P < .001$ versus control group; $^{*} P < .05$, $^{*\#} P < .01$, $^{**\#} P < .001$ versus IR + Vector group; $^{*} P < .05$, $^{*\#} P < .01$, $^{**\#} P < .001$ versus IR + circ_VCAN + NC group. IR = irradiate, NC = negative control, RT-qPCR = quantitative real-time PCR.
5. Conclusions

We have shown that circ_VCAN was inversely correlated with miR-1183 expression in response to irradiation in glioma, and that circ_VCAN negatively regulated miR-1183 by direct binding. In addition, circ_VCAN accelerated the proliferation, migration, and invasion, and inhibited apoptosis, of the irradiated glioma cells by regulating miR-1183. To our knowledge, this is the first report of the role of the circ_VCAN/miR-1183 regulatory loop in glioma following irradiation.

Acknowledgments

The authors would like to acknowledge all the people for their review to this article.

Author contributions

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