

MiR-21 enhanced glioma cells resistance to carmustine via decreasing Spry2 expression

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Abstract. – **OBJECTIVE:** Gliomas are accompanied with high mortality owing to their invasive peculiarity and vulnerability to drug resistance. miR-21 is a vital oncogenic miRNA that regulates drug resistance of tumor cells. This study aims to elucidate the function of miR-21 in human glioma cells resistant to carmustine (BCNU) and to demonstrate the underlying molecular mechanism.

MATERIALS AND METHODS: BCNU-sensitive cells (SWOZ2 cells) were transfected with miR-21 agomir and negative control, and BCNU-resistance cells (SWOZ2-BCNU cells) were transfected with miR-21 antagonist and negative control. The Real-time fluorescence quantitative PCR was used to detect and compare the levels of miR-21 expression between SWOZ2-BCNU and SWOZ2 cells. The drug sensitivity of these cells to BCNU was determined by Cell Counting Kit-8 (CCK-8) assay. The protein expression of Spry2 was detected by Western blotting.

RESULTS: The expression level of miR-21 was remarkably higher in SWOZ2-BCNU cells than that in SWOZ2 cells. The half-maximal inhibitory concentration (IC50) of BCNU was obviously higher for SWOZ2-BCNU cells than that for SWOZ2 cells. Besides, we found that aberrant expression of miR-21 in SWOZ2-BCNU cells is responsible for glioma BCNU-resistance. Consistently, Spry2 protein levels were significantly reduced in SWOZ2-BCNU as well as in miR-21 agomir-transfected cells, inversely correlated to miR-21 expression. The results of si-Spry2 co-transfection suggested that the effect of miR-21 on glioma BCNU-resistance is mediated through Spry2.

CONCLUSIONS: miR-21 enhances the resistance of human glioma cells to BCNU by decreasing the expression of Spry2 protein. Thus, Spry2 may be a novel therapeutic target for treating glioma BCNU-resistance.

Key Words:

Spry2, Glioma cells, Drug resistance.

Introduction

Glioma is one of the most common primary brain tumors with high mortality and morbidity

worldwide. Nowadays, strategies to treat these tumors include surgery, radiation therapy and chemotherapy. Despite advances in these strategies, the prognosis for glioma has not significantly improved recently¹, and the ineffectiveness of current treatments can be ascribed to chemo-resistance partially. Recent studies have shown that glioma is resistant to the cytotoxic effects of chemotherapeutics^{2,3}. Therefore, here is a need to develop novel therapeutic targets to optimize the effectiveness of chemotherapeutic.

MicroRNAs (miRNAs) are small non-coding RNAs that can bind to target mRNA partially or fully, leading to mRNA degradation or translational inhibition. Besides, miRNAs are reported to be involved in cell differentiation, apoptosis, proliferation and cancer^{4,5}. Particularly, studies have found multiple miRNAs that are associated with neural diseases and brain tumors^{6,7}. Among these miRNAs, miR-21 is an important oncogenic miRNA that can regulate drug resistance of tumor cells⁸. Previous research demonstrated that miR-21 could regulate multiple genes, including PTEN, RECK and MARCKS in some types of cancers⁹⁻¹². Besides, mounting evidence has suggested that miR-21 was over-expressed in glioma tissues and cell lines as well. A study⁸ reported that miR-21 inhibitor therapy can sensitize glioblastomas resistant cells to anthracyclines by enhancing apoptosis. Thus, miR-21 level can be useful to determine the chemotherapy response of glioma. However, the underlying mechanism of miRNA-21 function in glioma chemotherapy-resistance is not fully discovered. Carmustine (1,3-bis (2-chloroethyl)-1-nitrosourea, BCNU) is currently one of the standard adjuvant chemotherapy for glioma owing to its ability easy through the blood brain barrier. Thus, this study aims to elucidate the function of miR-21 in human glioma cells resistant to carmustine (BCNU) and to demonstrate the underlying molecular mechanism.

Materials and Methods

Cell Line and Reagents

Human SWOZ2 and SWOZ2-BCNU glioma cells were obtained from School of Medicine Pathology Department of Jinan University (Guangzhou, China). Rabbit polyclonal anti-Spry2 was purchased from Upstate Biotechnology (Lake Placid, NY, USA). miR-21 agomir and antagomir as well as siRNA against Spry2 were obtained from Ribobio Biological Technology Company (Guangzhou, Guangdong, China).

Cell Culture and Transfection

SWOZ2 and SWOZ2-BCNU glioma cells were cultured in Dulbecco's modified Eagle medium (DMEM), high glucose supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/ml streptomycin. All cells were maintained in a humidified incubator at 37°C with 5% CO₂. SWOZ2 glioma cells were exposed to miRNA agomir vs. negative control (50 nM, RiboBio, Guangzhou, Guangdong, China) for 48 h. miRNA antagonist and negative control (100 nM, RiboBio, Guangzhou, Guangdong, China) were transiently transfected into SWOZ2-BCNU glioma cells which stably reduced miR-21 expression according to the manufacturer's instructions, respectively. The transfected efficiencies were verified by quantitative Real-time PCR (qRT-PCR). Besides, SWOZ2-BCNU glioma cells were co-transfected with miR-21 antagomir and Si-Spry2 (75 nM) for 48 h. Glioma cells were transfected at approximately 50-70% confluence according to the manufacturer's instructions after 24 h of culture.

RNA Extraction and Real-time Reverse Transcription-PCR for miRNA Detection

Total RNA was extracted from SWOZ2 and SWOZ2-BCNU glioma cells using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to manufacturer's instructions. The RNA concentration was measured by 260-280 nm absorbance using a Nanodrop Spectrophotometer (ND-100, Thermo Fisher, Waltham, MA, USA). cDNA from total RNA was synthesized using Bio-Rad Script™ cDNA synthesis Kit (Hercules, CA, USA). cDNA from miRNAs was generated using the Bulge-Loop™ miRNA qPCR primer Set (RiboBio, Guangzhou, Guangdong, China) with TaKaRa SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) in the ABI-7900 Real-time PCR Detection System (Foster City, CA, USA). PCR cycling conditions were 3

min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. The primers of hsa-miR-21 and U6 were synthesized by Applied Bio-Systems (Foster City, CA, USA). U6 were used as an internal normalized reference for miR-21 expressions, respectively. Expressions were normalized to endogenous controls and fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$.

Protein Extraction and Western Blotting

Total proteins were isolated after solubilization in lysis buffer. The concentration of protein samples was evaluated by bicinchoninic acid (BAC) Protein Assay Kit (Thermo Fisher, Waltham, MA, USA). The same amounts of protein (30 mg) were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride (PVDF) membranes. Immunoblots were visualized using the enhanced chemiluminescence (ECL) Kit (Thermo Fisher, Waltham, MA, USA) and the quantification of each band was performed using Image Lab Software (Bio-Rad, Hercules, CA, USA) with β -actin as a loading control.

Cell Viability Assay

Cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay. 3,000 cells per well were seeded in 96-well plates and allowed to attach overnight. After transfection, we incubated the cells with different dose of BCNU for 72 h, ranging from 2 mg/L to 128 mg/L. Next, 10 μ L of CCK-8 were added to the wells and the cells were incubated at 37°C for 4 h in 5% CO₂. Next, we tested cell viability by measuring the absorbance at 450 nm using a microplate reader (Infinite M1000 Pro, Tecan Group, Zurich, Switzerland) and comparing with the control cells. All experiments were performed in triplicate.

Statistical Analysis

Results were analyzed using SPSS software 11.0 (SPSS Inc., Chicago, IL, USA) and compared using one-way analysis of variance (ANOVA) with Fisher's post hoc test. Data were presented as mean \pm standard deviation (SD) of separate experiments ($n \geq 3$). p -value < 0.05 was considered to be significant.

Results

miR-21 Upregulated in SWOZ2-BCNU Glioma Cells

The expression levels of miR-21 and MDR-1 in SWOZ2 glioma cells as well as in SWOZ2-B-

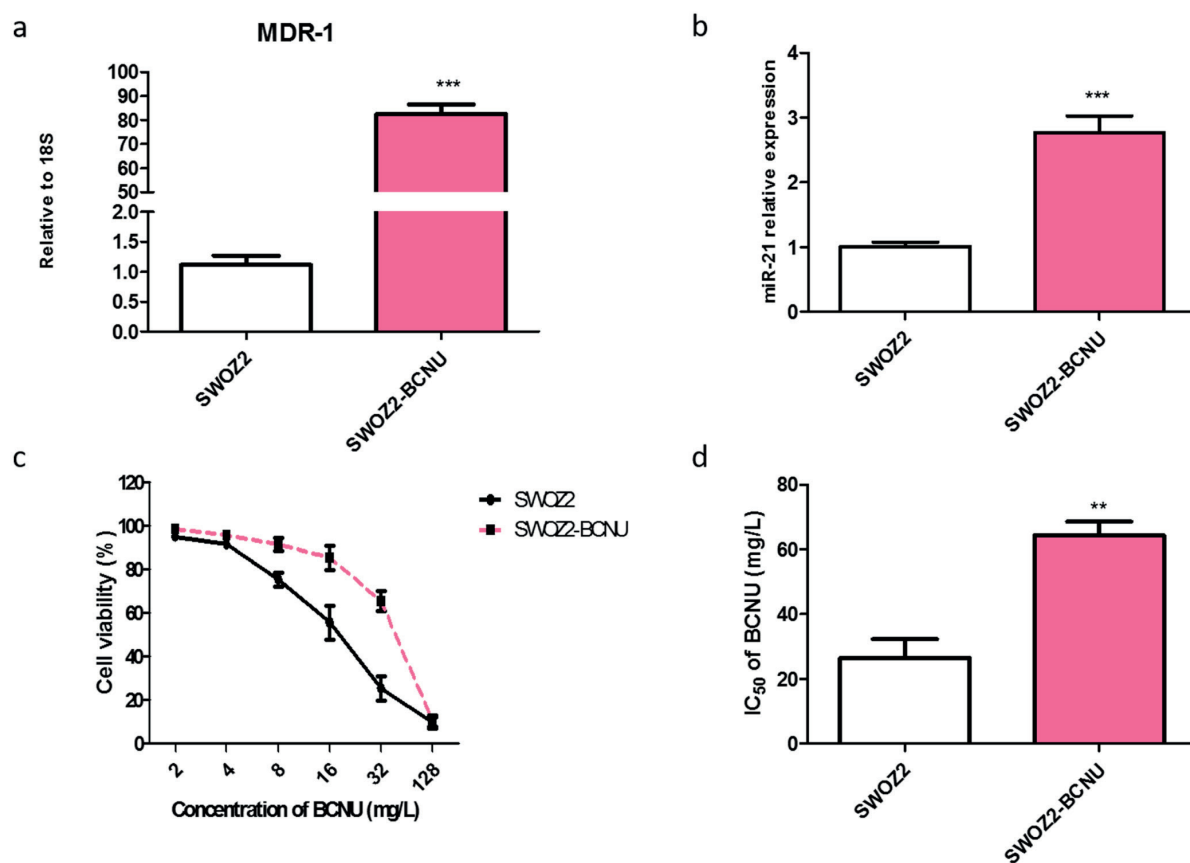


Figure 1. miR-21 upregulated in SWOZ2-BCNU glioma cells. (a) MDR-1 expression level was significantly upregulated in SWOZ2-BCNU glioma cells. (b) miR-21 level was increased in SWOZ2-BCNU glioma cells. (c) The viability of SWOZ2 and SWOZ2-BCNU cells treated with BCNU for 72 h measured by CCK-8 assay. (d) The IC₅₀ of BCNU for SWOZ2 and SWOZ2-BCNU cells detected by CCK-8 assay. *** $p < 0.001$, ** $p < 0.001$, * $p < 0.05$.

CNU glioma cells, were measured by qRT-PCR. As shown in Figure 1a, MDR-1 expression levels were significantly upregulated in SWOZ2-BCNU glioma cells compared with SWOZ2 group ($p < 0.01$). Furthermore, miR-21 level was increased in SWOZ2-BCNU glioma group (Figure 1b). To confirm SWOZ2-BCNU glioma cells were resistant to BCNU, we used CCK-8 to test cell viability and IC₅₀ of BCNU (Figure 1c-d). IC₅₀ of BCNU was (64.357±2.497) mg/L in SWOZ2-BCNU glioma cells, which was higher than that in SWOZ2 group. The resistance factor was 2.43.

miR-21 Involved in Glioma BCNU-Resistance

To elucidate the involvement of miR-21 in glioma BCNU-resistance, SWOZ2 glioma cells were transfected with miR-21 agomir, which could increase the level of miR-21 significantly ($p < 0.01$,

Figure 2a). Upregulation of miR-21 could enhance tolerance of SWOZ2 glioma cells. As shown in (Figure 2c), miR-21 overexpression increased cell viability and IC₅₀ of BCNU. IC₅₀ of BCNU in agomir control group was (20.484±3.135) mg/L and (52.357±2.076) mg/L in miR-21 agomir group ($p < 0.01$, Figure 2d), indicating that miR-21 was sufficient for glioma chemotherapy-resistance. Besides, SWOZ2-BCNU glioma cells were transfected with miR-21 antagomir to lower the expression of miR-21 ($p < 0.01$, figure 2b). Likewise, down-regulation of miR-21 weakened SWOZ2-BCNU glioma cells drug-resistance. IC₅₀ in antagomir control group was (66.024±2.671) mg/L and (31.421±3.163) mg/L in miR-21 antagomir group ($p < 0.01$, Figure 2e-f).

Taken together, these findings suggested that miR-21 has a vital role in BCNU-resistance of glioma cells.

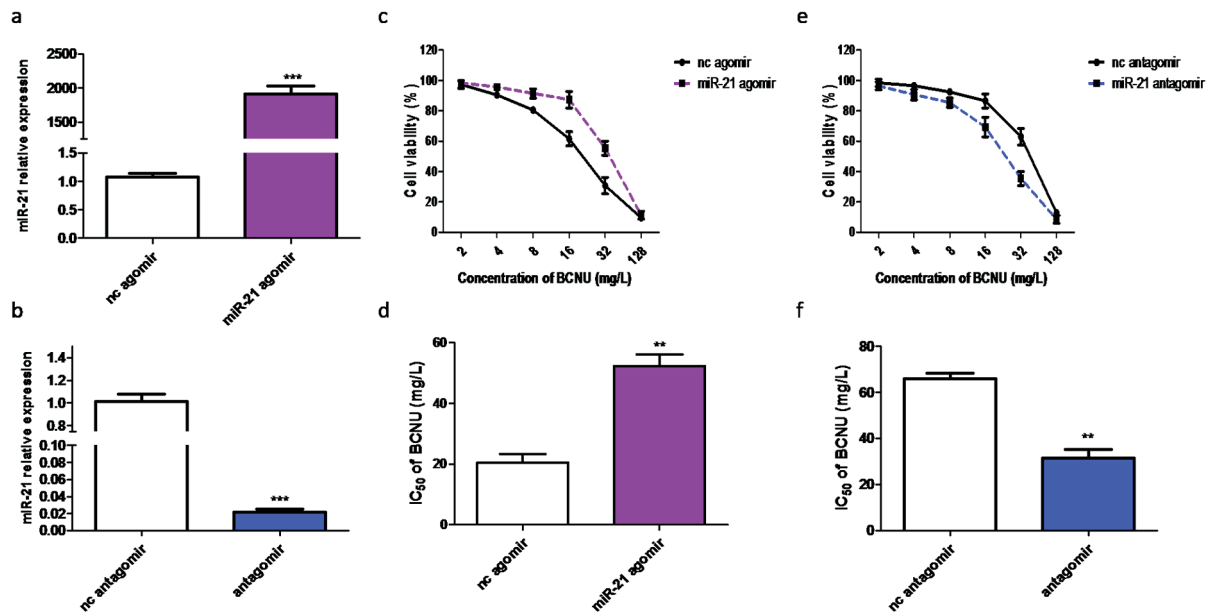


Figure 2. miR-21 involved in glioma BCNU-resistance. (a) The relative expression of miR-21 in SWOZ2 cells transfected with miR-21 agomir or miRNA negative control for 48 h detected by qRT-PCR. miR-21 expression was significantly increased in miR-21 agomir group. (b) The relative expression of miR-21 in SWOZ2-BCNU cells transfected with miR-21 antagomir or miRNA negative control for 48 h detected by qRT-PCR. miR-21 level was significantly decreased in miR-21 antagomir group. (c) The viability of SWOZ2 cells transfected with miR-21 agomir or miRNA negative control measured by CCK-8 assay after BCNU treatment. (d) The IC₅₀ of BCNU for SWOZ2 cells transfected with miR-21 agomir or miRNA negative control detected by CCK-8 assay. (e) The viability of SWOZ2-BCNU cells transfected with miR-21 antagomir or miRNA negative control measured by CCK-8 assay after BCNU treatment. (f) The IC₅₀ of BCNU for SWOZ2-BCNU cells transfected with miR-21 antagomir or miRNA negative control detected by CCK-8 assay. *** $p < 0.001$, ** $p < 0.001$, * $p < 0.05$.

miR-21 Targets *Spry2* for Glioma Chemotherapy-Resistance

To identify the exact target of miR-21 responsible for glioma BCNU-resistance, we uncovered about 200 potential targets using a miRNA target prediction algorithm, Target Scan. Among these targets, we chose *Spry2* as study object because it contains a miR-21 seed match in its 3' untranslated region (UTR), and it negatively regulates Ras signaling¹³. Besides, it has reported that miR-21 targets *Spry2* in cardiocytes and colon cancer in SW480 cells¹⁴ and down regulation of *Spry2* by miR-21 triggers malignancy in human gliomas¹⁵. In the present study, we found that *Spry2* was reduced in SWOZ2 cells transfected with miR-21 agomir (Figure 3a-b). Then, SWOZ2-BCNU glioma cells were transfected with Si-*Spry2* to detect cell viability and IC₅₀ of BCNU. It turned out that inhibition of *Spry2* could enhance glioma BCNU-resistance (Figure 3c-d). As mentioned above, miR-21 antagomir-transfected SWOZ2-BCNU glioma cells displayed decreased BCNU-resistance; however, this was partially rescued by si-*Spry2* co-transfection (Figure 4a-b). This result

suggests that the effect of miR-21 on glioma BCNU-resistance is mediated through *Spry2*.

Discussion

Cancer is a complicated disease closely related to the mutation and epigenetic alteration of genes. Therefore, novel target for therapy has been focused on the search for DNA modifications, although few genetic variants are confirmed to reproducibly influence human cancer¹⁶. Various signal transduction pathways involve in the chemotherapy resistance mechanisms of glioma, and miRNAs are candidate regulators of the response to antineoplastic agents.

miR-21 has been thought to be an oncogene that is upregulated in many cancers and is related to genes that modulate cell proliferation, apoptosis, drug resistance and invasiveness⁹⁻¹¹. Its expression has been associated with tumor grade and has been proposed as a marker for tumor progression^{17,18}. Previous research has elucidated that silencing of endogenous miR-21 significantly

increases cell cycle arrest in G2/M phase, phosphatase and tensin homologue (PTEN) gene expression and apoptosis associated caspase-3 expression in TMZ-treated cells¹⁹⁻²¹. In addition, it has also been shown that decreased miR-21 expression can inhibit the growth of glioma cell lines and induce apoptosis independently of PTEN status²⁰. Recently, miR-21 was also reported to regulate PDCD4, a tumor suppressor gene, in glioma cells²².

Besides, miR-21 seems to modulate drug resistance in some cancers²³. It has already been shown that increased miR-21 can induce doxorubicin-chemoresistance in T24 human bladder cancer cells while reduced miR-21 sensitized T24 cells to the drug²⁴. Likewise, miR-21 inhibition increased the chemo-sensitivity of glioma cells to taxol as well as etoposide^{25,26}. These results showed that miR-21 inhibition enhances chemo-sensitivity of temozolomide-resistance

glioma cells. However, its function in chemo-sensitivity of BCNU has not been discovered.

To validate the possible function of miR-21 in glioma BCNU-resistance, we transfected glioma cells with miR-21 agomir or antagomir and then treated with BCNU in this study. Our work provided evidence that miR-21 played important roles in BCNU-resistance of glioma, and Spry2 (one identified targets of miR-21) was involved.

Recent studies have demonstrated that Spry2 is also a tumor suppressor because it is reduced in hepatocellular carcinoma, lung cancer and breast cancer²⁷. Besides, knockdown of Spry2 expression also enhances Ras-induced lung cancer development in mice²⁸. Kwak et al¹⁵ has reported that regulation of Spry2 by miR-21 has an important role in the malignant progression and invasion of human gliomas. Although it has reported that miR-21 directly targets Spry2, it was still unclear whether the down-regulation of Spry2 has a vi-

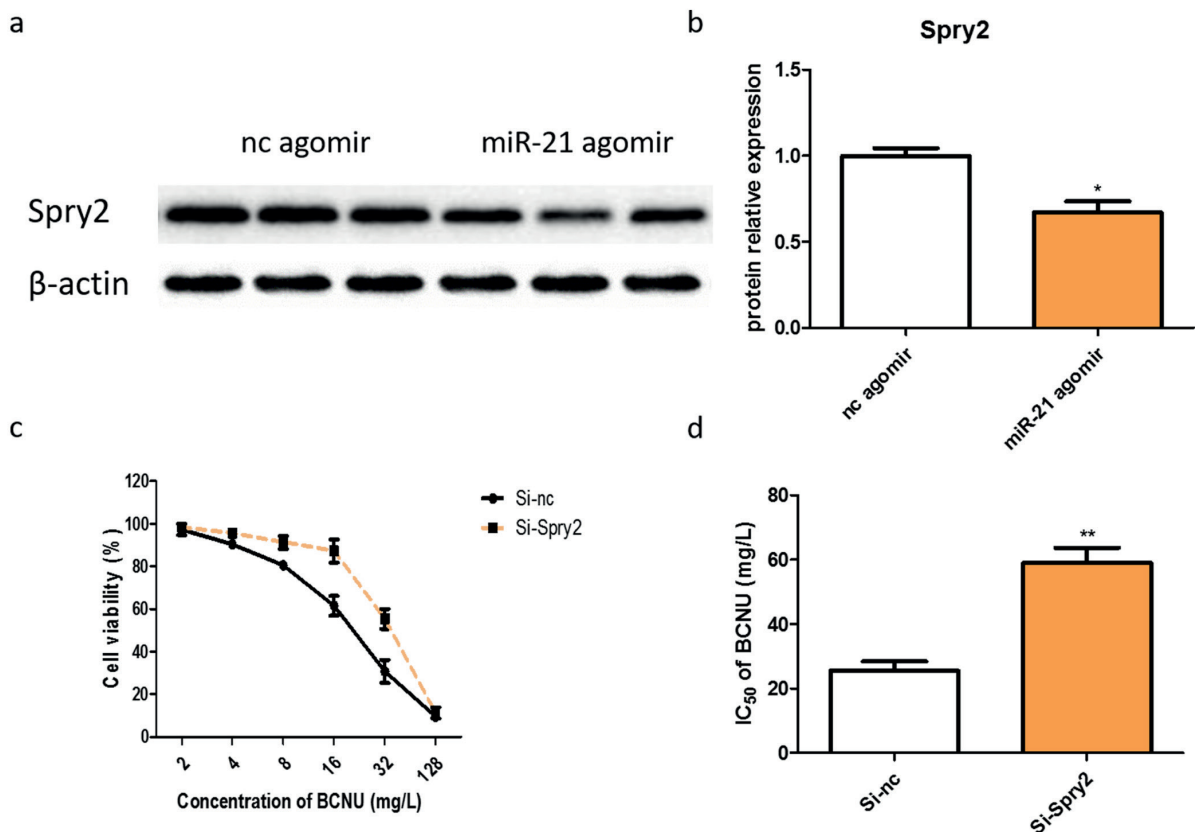


Figure 3. Decreased Spry2 was responsible for glioma BCNU-resistance. (a-b) The relative expression of Spry2 in SWOZ2 cells transfected with Si-Spry2 or SiRNA negative control for 48 h detected by Western blotting. (c) The viability of SWOZ2 cells transfected with Si-Spry2 or SiRNA negative control measured by CCK-8 assay after BCNU treatment. (d) The IC₅₀ of BCNU for SWOZ2 cells transfected with Si-Spry2 or SiRNA negative control detected by CCK-8 assay. *** $p < 0.001$, ** $p < 0.001$, * $p < 0.05$.

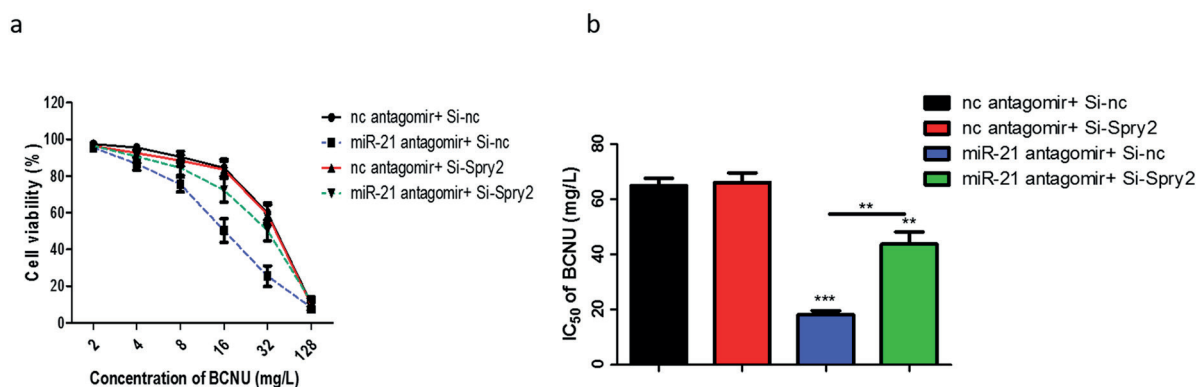


Figure 4. miR-21 targets Spry2 for glioma BCNU-resistance. (a) The viability of SWOZ2-BCNU cells co-transfected with Si-Spry2 and miR-21 antagomir measured by CCK-8 assay after BCNU treatment. (d) The IC₅₀ of BCNU for SWOZ2-BCNU cells co-transfected with Si-Spry2 and miR-21 antagomir detected by CCK-8 assay. *** $p < 0.001$, ** $p < 0.001$, * $p < 0.05$.

tal role in glioma chemotherapy-resistance. Our data demonstrated that Spry2 was decreased in SWOZ2 cells transfected with miR-21 agomir, and SWOZ2-BCNU glioma cells transfected with Si-Spry2 shown enhanced glioma BCNU-resistance. Most importantly, the improved BCNU-resistance of SWOZ2-BCNU glioma cells transfected with miR-21 antagomir was partially rescued by si-Spry2 co-transfection.

These results suggest the effect of miR-21 on glioma BCNU-resistance is mediated through Spry2. Therefore, the use of miR-21 inhibitors may function as an effective approach for reversing drug resistance in glioma.

Conclusions

We found that miR-21 was increased in BCNU-resistance glioma cells. Upregulation of miR-21 enhanced glioma drug resistance by targeting Spry2. Our results may suggest that the down-regulation of Spry2 by miR-21 plays a key role in glioma BCNU-resistance. Thus, Spry2 may be a novel therapeutic target for future treatment of glioma chemotherapy-resistance.

Conflict of interest

The authors declare no conflicts of interest.

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