

Original Article

Ginsenoside Rh2 inhibits glioma cell proliferation by targeting microRNA-128

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Aim: To examine the influence of ginsenoside Rh2 (Rh2), a triterpene saponin extracted from the traditional medicinal plant ginseng, on the expression of miRNAs in human glioma cells.

Methods: The expression profile of miRNA (miR) was analyzed in human U251, T98MG and A172 glioma cells using a miRNA array and quantitative real-time PCR. Cell viability was assessed using a colorimetric assay (cell counting kit-8). Transfection of miR-128 was performed using Lipofectamine 2000. Caspase 3 activity was determined using a caspase colorimetric assay kit. Apoptosis was assessed using annexin V and propidium iodide staining. Protein expression was determined with Western blot analysis. miRNA-128 targeting activity was measured using a luciferase reporter assay.

Results: In U251 cells treated with Rh2 (12 µg/mL), 14 of 452 human miRNAs were up-regulated and 12 were down-regulated as detected with the miRNA array assay. The up-regulation of miR-128 by Rh2 was further verified in human U251, T98MG and A172 cells using quantitative real-time PCR. In U251 cells, transfection of a miR-128 inhibitor (50 nmol/L) prevented the overexpression of miR-128 by Rh2, and significantly blunted Rh2-induced cytotoxicity, apoptosis, caspase 3 activation, transcriptional activation of E2F3a, a miR-128 target gene, as well as E2F3a protein expression.

Conclusion: The anti-proliferative effect of Rh2 in human glioma cells was mediated in part through up-regulation of miRNA-128 expression.

Keywords: ginsenoside Rh2; triterpene saponin; glioma cell; microRNA; miR-128; apoptosis; apoptosis cell proliferation; caspase 3; E2F3a

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Introduction

Gliomas are the most common brain tumors in humans. Even when treated with surgery, radiotherapy, chemotherapy, and other intensive regimens, malignant gliomas are incurable^[1]. The tumors are characterized by rapid cell growth and diffuse cellular infiltration into adjacent normal tissues^[2]. Although a number of genetic and molecular lesions have been correlated with glioma progression, a complete understanding of the molecular basis and therapy of these tumors remains elusive.

One novel approach to characterizing the molecular and therapeutic targets of gliomas is based on the expression profiling of miRNAs^[3]. These small RNA molecules are a class of endogenously expressed small noncoding RNA, 18-25 nucleotides in length^[4]. To date, more than 700 miRNAs have been identified in humans^[5]. miRNAs are known to be important in the regulation of many fundamental cellular processes, such as cell proliferation, differentiation and apoptosis^[6, 7]. Follow-

ing binding to the 3'-untranslated regions (UTRs) of specific mRNAs, miRNAs regulate target gene expression by inducing translational repression or mRNA degradation^[8]. It is estimated that up to 30% of human genes may be regulated by miRNAs^[8]. Moreover, approximately 50% of the known miRNAs were reported to be located in cancer-associated genomic regions^[9, 10] and miRNA dysregulation has been detected in various cancer cells^[11]. Therefore, aberrations in miRNA expression patterns are thought to be involved in the progression of human cancers^[12]. Since the first report of abnormal miRNA expression in glioblastomas in 2005, there has been an increasing number of reports each year describing miRNA dysregulation and function in various brain tumors^[13-17]. These findings not only provide new insights into the molecular pathogenesis of gliomas, but also are useful in identifying miRNAs as potential targets in therapeutic intervention.

Ginsenoside Rh2 is a biologically active phytochemical extracted from Ginseng, a commonly used alternative drug taken orally in traditional herbal medicines in China, Korea, Japan and some Western countries^[18]. It is a triterpene saponin, consisting of a steroid nucleus and a sugar moiety^[18].

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Rh2 has been reported to have a variety of biological effects, such as reducing blood glucose^[19] and ameliorating ischemic brain injury^[20]; in addition, it has antiallergic activity^[21] and antiproliferative effects^[22]. The ability of Rh2 to suppress cell growth has also been observed in glioma cells^[22, 23]. Because Rh2 promotes neoplastic cells to return to a normal cell phenotype, it is expected to be a new type of anticancer agent^[23]. It displays low toxicity, is associated with only a few side effects and is generally regarded as an anticancer nutrient^[23]. Although extensive investigations have shown that Rh2 exerts its antiproliferative effects through induction of an apoptotic pathway^[24], the role of miRNAs in this process has not yet been explored.

Using an miRNA array to examine miRNA expression in Rh2-treated human glioma cells, we found that Rh2 altered the miRNA expression in human glioma U251 cells. We verified the observed up-regulation of the brain-enriched miR-128 by quantitative real-time PCR in human U251, T98MG and A172 glioma cells. To further investigate the role of miR-128 in Rh2-mediated antiproliferation, we transfected miR-128 inhibitor into glioma cells and observed an abrogation of Rh2-induced miR-128 overexpression, causing significant inhibition of Rh2-induced cytotoxicity, apoptosis, caspase-3 activation, transcriptional activation of E2F3a, a miR-128 target gene, and the expression of E2F3a protein.

Materials and methods

Reagents

Ginsenoside Rh2 (20R-form, >99% purity, HPLC pure) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The chemical structure of ginsenoside Rh2 is shown in Figure 1.

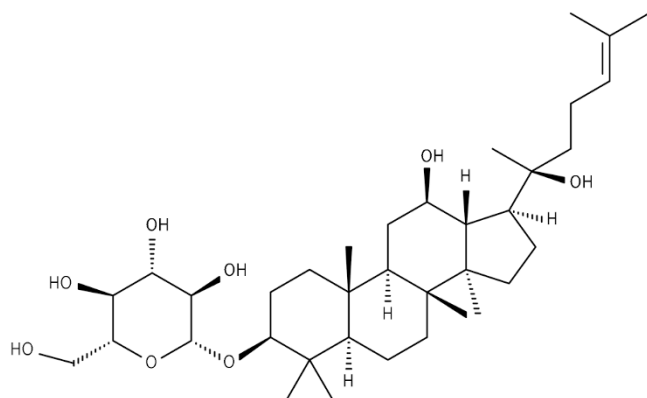


Figure 1. Chemical structure of ginsenoside Rh2 (20R-form).

Cell lines and culture conditions

Human U251, T98MG and A172 glioma cells were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium (Gibco Life Technologies, Grand Island, NY, USA), supplemented with 10%

fetal bovine serum (Sijiqing, Hangzhou, China), 100 U/mL penicillin and 100 mg/mL streptomycin. All cells were routinely passaged and maintained in a humidified incubator of 5% CO₂ at 37 °C.

Cell proliferation assay

Cell viability was assessed colorimetrically using the cell counting kit-8 (CCK-8, Dojindo Laboratories, Tokyo, Japan)^[25]. Human glioma U251 cells (1×10⁵) were seeded in each well of a 96-well plate and incubated for 24 h prior to treatment with different dosages of Rh2 or vehicle. After treatment, 10 μL of the CCK-8 solution was added into each well, and the cells were incubated for an additional 2 h. The absorbance value (A) at 470 nm was read using a microplate reader (Bio-Rad, CA, USA) with a reference wavelength of 630 nm.

miRNA microarray analysis

The U251 cells were cultured for 24 h and then incubated with 12 μg/mL Rh2 for 24 h. Total cellular RNA was isolated from Rh2-treated and -untreated cells using TRIzol reagent (InvitrogenTM, Carlsbad, CA, USA) according to the manufacturer's protocol. Five micrograms of total RNA was labeled at the 3'-end with Hy3TM using miRCURY Array Labeling kit (Exiqon, Vedback, Denmark) for 1 h at 0 °C.

The labeled RNA was purified using a Miniprep Kit (QIAGEN, Valencia, CA, USA). The resulting sample was diluted with hybridization buffer, and 20 μL of the diluted sample was loaded onto the spotted area of the array and covered with a Bioarray LifterSlip coverslip. The chamber was rotated in a 60 °C water bath for 14 h. The miRCURY LNA miRNA Array 8.1 contained probes for 452 mature human miRNAs printed in quadruplicate. Next, the slides were washed in wash buffer A (2×SSC/0.2% SDS) at 60 °C until the Bioarray LifterSlip coverslip fell off. Then the slides were washed with wash buffer B (1×SSC) twice and wash buffer C (0.2×SSC) once. After drying by centrifugation for 5 min at 1000 round per minute, the slides were scanned with a Genepix 4000B laser scanner (Molecular Devices) and digitized by Genepix Pro 6.0. The resulting ratios of miRNA spots to background were calculated. The data were analyzed by subtracting out the background and normalizing the signals using global Lowess (locally weighted scatter plot smoothing) regression algorithm. All miRNAs having a ratio of 2.0 or greater were included.

Quantitative real-time PCR for miRNA expression

To confirm the miRNA levels obtained from the microarray results, miRNA expression was assessed using quantitative real-time PCR (qRT-PCR). Total cellular RNA was extracted from each of the experimental groups. Reverse transcription was performed using gene specific primers. The sequences of the primer pairs were as follows: miR-128: forward 5'-CGCGCTCACAGTGAACCG-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'; miR-15b: forward 5'-TAGCAGCATCATGGTTTACA-3' and reverse 5'-TCGTGCCA-GCGGCTCG-3'; miR-21: forward 5'-CGGGATCCTGGGGT-TCGATCTTAACAGGC-3' and reverse 5'-CGGAATCCCA-

CAATGCAGCTTAGTTTTCC-3'; miR-25: forward 5'-GTGT-TGAGAGGGCGGAGACTT-3' and reverse 5'-TCAGAC-CGAGACAAGTGCAA-3^[14-17]. U6 was taken as an internal control (forward 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse 5'-CGCTTCACGAATTTGCGTGCAT-3'). qRT-PCR was performed using a Rotor-Gene 3000 real-time SYBR-green PCR system. The reactions were carried out in a 96-well optical plate at 95 °C for 10 min and then amplified for 10 s at 90 °C, followed by 1 min at 60 °C for 40 cycles. The relative value of each miRNA to U6 RNA was calculated using the $2^{-\Delta\Delta Ct}$ method, where Ct is the number of cycles at which the application reaches a threshold, as determined by SDS software v1.2 (Applied Biosystems Inc). Thermal denaturation was administered at the conclusion of the PCR to determine the number of the products that were present in the reaction. Each reverse transcription and PCR assay were performed in triplicate.

miR-128 inhibitor transfection

The miR-128 inhibitor 5'-AAAGAGACCGGUUCACU-GUGA-3' and miR-128 inhibitor control 5'-CAGUACUUUUGUGUAGUACAAA-3' were purchased from RiboBio Co Ltd, China. U251 cells in a volume of 100 μ L DMEM medium were plated in each well of a 96-well plate. After 24 h of culture, the cells were transfected with 50 nmol/L miR-128 inhibitor or inhibitor control for 48 h using Lipofectamine 2000 (Invitrogen). After transfection, the wells were treated with Rh2 for different lengths of time in order to study cell proliferation, apoptosis, as well as caspase 3 and E2F3a expression.

Luciferase assay

A E2F3a 3'-untranslated region (UTR) reporter plasmid (pmiR-RB-REPORTTM 3'-UTR) was purchased from RiboBio Co Ltd, China. This vector was constructed by ligating a 1127-bp fragment to the type E2F3a 3'-UTR, which encompassed the target sequence for miR-128. The E2F3a 3'-UTR (accession number: NM0019490) was amplified from the genomic DNA using the following primers: forward 5'-AAACAATGCCAGGGT-GTCTC-3', reverse 5'-GCTCACACACGAAATGGCTA-3'. The empty plasmid containing only the E2F3a 3'-UTR was used as a control. The U251 cells were transfected with 50 nmol/L E2F3a 3'-UTR plasmid or empty E2F3a 3'-UTR plasmid for 48 h using Lipofectamine 2000 (Invitrogen). After transfection, the cells were treated with Rh2 for 24 h, and the luciferase assay was performed using TECAN Genios multifunctional microplate reader.

Western blotting

Cells were harvested and lysed in a buffer containing 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% NP40 and protease inhibitors (2 μ g/mL leupeptin, 2 μ g/mL pepstatin, 2 μ g/mL aprotinin and 2 μ g/mL PMSF, Sigma) for 30 min on ice. Lysates were centrifuged at 12 000 round per minute at 4 °C for 20 min, and the supernatants were collected. The protein concentrations were determined using the Bradford method. The proteins were separated using SDS-PAGE and

transferred to a nitrocellulose membrane. The membranes were incubated with rabbit anti-human E2F3a (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-human β -actin antibody (Sigma, USA).

Apoptosis assay

Apoptosis was determined by annexin V and propidium iodide (PI) staining using the apoptosis detection kit (Beyotime, China) according to the manufacturer's instructions. Briefly, U251 cells were transfected with 50 nmol/L miR-128 inhibitor or miR-128 inhibitor control for 48 h, and then with 12 μ g/mL Rh2 for 48 h. After treatment, the cells were washed with 50 mmol/L cold phosphate buffer (pH 7.5), centrifuged at 1200 \times g for 5 min, and suspended in binding buffer. The treated cells were incubated with annexin V and propidium iodide for 15 min at room temperature, after which the samples were analyzed for annexin V binding affinity within 1 h by flow cytometry^[26].

Caspase 3 activity assay

Caspase 3 activity was determined using a caspase colorimetric assay kit (BioVision Research Products, USA) according to manufacturer's protocol. The assay is based on spectrophotometric detection of the chromophore ρ -nitroanilide (ρ NA) after cleavage from the labeled substrate DEVD- ρ NA. Briefly, 1×10^6 cells were first treated with 50 nmol/L miR-128 inhibitor or miR-128 inhibitor control for 48 h and then with a different dosage of Rh2 for various time. The cells were collected, washed with ice-cold PBS, and lysed in a lysis buffer. Cell lysates were measured for protease activity using a caspase-specific peptide conjugated with the color molecule ρ NA. The caspase-cleaved chromophore ρ NA was quantitated with a spectrophotometer at a wavelength of 405 nm^[27].

Statistical analysis

The results were analyzed by one-way ANOVA or Student's *t* test. Both analyses were performed using SPSS 13.0 statistical software. A value of $P < 0.05$ was considered statistically significant. All values were expressed in terms of mean \pm SEM or mean \pm SD.

Results

Effects of ginsenoside Rh2 on miRNA expression in human glioma U251 cells

To identify the miRNAs involved in Rh2-induced cytotoxicity, total RNA was extracted from U251 cells treated with 12 μ g/mL Rh2 for 24 h for miRNA microarray analysis. The Exiqon microarray (Exiqon, Vedback, Denmark) containing 452 mature human miRNA probes was used to identify the cellular miRNA expression profile. miRNAs up-regulated more than 2-fold or down-regulated by 2-fold by Rh2 treatment were classified as being up-regulated or down-regulated miRNAs, respectively. Table 1 summarizes the results of Rh2-regulated miRNAs and their chromosomal locations are summarized. Of the 452 human miRNAs tested the microarray, 14 miRNAs were up-regulated and 12 miRNAs were down-

Table 1. Microarray analysis of miRNA expression in human gliomas U251 cells treated with ginsenoside Rh2.

miRNA	Fold (Mean±SEM)	P value	Localization	Up-down regulation
miR-15b	3.15±0.18	0.015	17q23.1	up
let-7c	2.03±0.15	0.010	21q11.2	up
let-7d	2.97±0.25	0.024	7q22.1	up
miR-29b	2.32±0.21	0.017	6q23.31	up
miR-106b	2.26±0.17	0.005	7q22.1	up
miR-125b	2.88±0.30	0.020	11q24.1	up
miR-128	3.04±0.19	0.013	2q21.3	up
miR-129	2.54±0.24	0.014	7q32.1	up
miR-137	2.13±0.14	0.008	1q21.3	up
miR-138	2.29±0.11	0.009	3q21.33	up
miR-181a	2.41±0.22	0.011	1q32.1	up
miR-181b	2.62±0.26	0.021	1q32.1	up
miR-181c	2.35±0.23	0.017	19p13.3	up
miR-323	2.57±0.18	0.013	14q32.31	up
miR-16	0.41±0.07	0.001	21q11.2	down
miR-18	0.37±0.10	0.007	9q22.2	down
miR-21	0.17±0.04	0.000	17q23.1	down
miR-25	0.23±0.09	0.003	7q22.1	down
miR-32	0.43±0.18	0.009	11q24.1	down
miR-92	0.39±0.11	0.005	21q11.2	down
miR-107	0.37±0.13	0.006	7q32.1	down
miR-155	0.28±0.09	0.002	3p21.33	down
miR-210	0.45±0.11	0.005	11p15.5	down
miR-218	0.37±0.21	0.012	4p15.31	down
miR-328	0.42±0.12	0.007	16q22.1	down
miR-370	0.38±0.07	0.003	14q32.2	down

1. The results presented fold change of signal ratio of 12 µg/mL Rh2-treated cells to untreated control cells.
2. The raw data were normalized and analyzed with software of MatLab version7.4, which produced an average value of the four spot replications of each miRNAs.
3. Chromosome localization of microRNAs as referred in miRBase sequences (<http://microrna.sanger.ac.uk>).

regulated in the Rh2-treated U251 cells. To further confirm the miRNA array results, we randomly selected the following four miRNAs: the up-regulated miR-128 and miR-15b as well as the down-regulated miR-21 and miR-25, and measured their expression levels by quantitative RT-PCR. As shown in Figure 2A, the results of quantitative RT-PCR for the four selected miRNAs were consistent with the miRNA array results, indicating that the results of miRNA array are reliable. To determine whether Rh2-induced miRNA expression occurred in other glioma cells, we measured the expression of miR-128 and miR-21 in human glioma cell lines, T98MG and A172. We observed consistent results in these two glioma cells as compared to U251 cells (Figure 2B).

miRNA-128 mediated Rh2-induced growth inhibition in glioma cells

Given that miR-128 has been reported to inhibit glioma cell

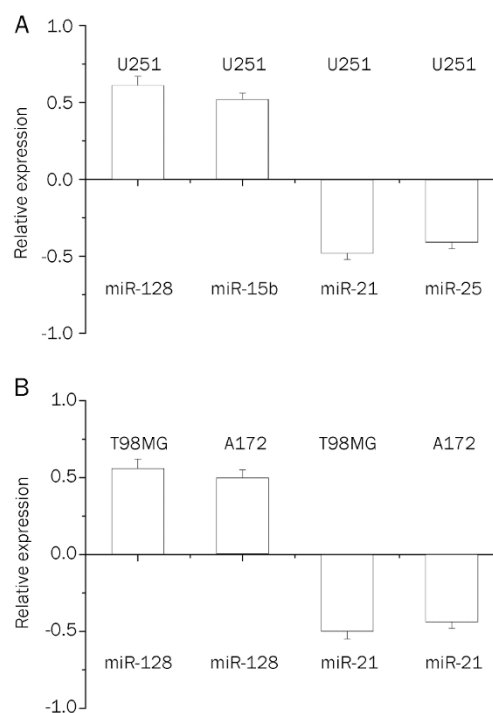


Figure 2. Validation of miRNA expression by quantitative real-time PCR. (A) The expressions of up-regulated miR-128 and miR-15b, as well as the down-regulated miR-21 and miR-25 in U251 cells were verified. The results of quantitative RT-PCR for the four selected miRNAs were consistent with the miRNA array results. (B) The expressions of miR-128 and miR-21 in human T98MG and A172 glioma cells were determined. The consistent results in these two glioma cells were observed as compared to U251 cells. The cells were treated with 12 µg/mL Rh2 for 24 h. Data were expressed as means±SEM of 3 independent experiments. Fold change of miRNA expression was presented in log₂ scale.

proliferation^[17], we sought to further investigate its role in Rh2-induced cytotoxicity. We first investigated the effect of miR-128 inhibitor on miR-128 overexpression induced by Rh2 in human U251, T98MG and A172 glioma cells with qRT-PCR. As shown in Figure 3, inhibition of miR-128 significantly suppressed Rh2-induced miR-128 overexpression, as compared with miR-128 inhibitor control. These results demonstrate that anti-miR-128 treatment was effective for inhibition of miR-128 overexpression.

We next examined the antiproliferative effect of Rh2 in human glioma U251 cells. As shown in Figure 4, Rh2 treatment (6, 12, and 25 µg/mL) of U251 cells resulted in a dose-dependent inhibition of cell growth. In a time course study, 12 µg/mL Rh2 resulted in increasing levels of inhibition of cell growth up to 72 h, as measured by the cell counting kit-8 assay.

To investigate whether miR-128 could be involved in Rh2-induced growth inhibition, we transfected U251 cells with 50 nmol/L miR-128 inhibitor or inhibitor control for 48 h and then examined the alteration of Rh2-induced cell growth inhibition. Knockdown of miR-128 following transfection of miR-128 inhibitor significantly increased the proliferation of U251

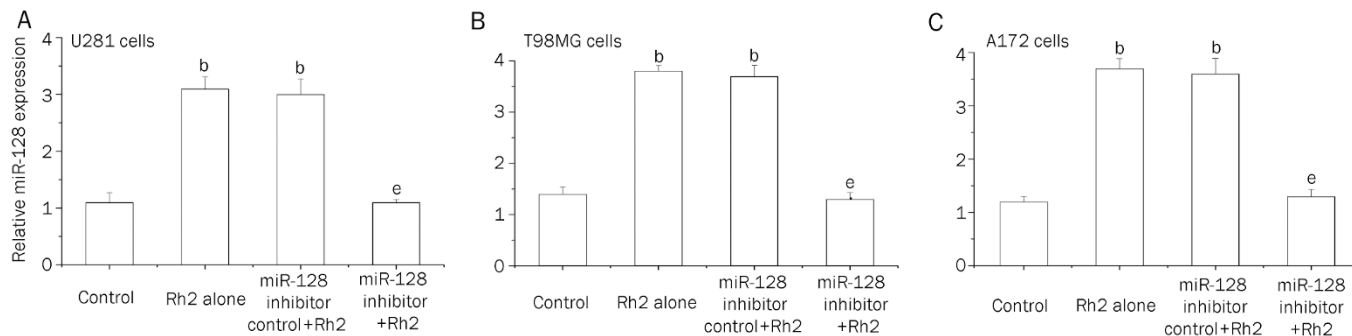


Figure 3. Transfection of miR-128 inhibitor significantly suppressed Rh2-induced miR-128 over-expression in U281 cells (A), T98MG cells (B), and A172 cells (C). The effects of miR-128 inhibitor transfection on Rh2-induced miR-128 overexpression in human U251, T98MG and A172 glioma cells were determined using qRT-PCR. The cells were transfected with 50 nmol/L miR-128 inhibitor or inhibitor control for 48 h, and then with Rh2 (12 μ g/mL) for 24 h. The experiment was performed three times, each in triplicate. Data were expressed as means \pm SEM. ^b P <0.05 compared with control. ^e P <0.05 compared with Rh2 alone or miR-128 inhibitor control+Rh2.

cells treated with Rh2, as compared with the cells transfected with a miR-128 inhibitor control (Figure 5).

miRNA-128 mediated Rh2 induction of apoptosis in glioma U251 cells

Rh2 is known to exert its tumor growth inhibitory effect through the induction of apoptosis^[28, 29]. We further examined whether Rh2-induced overexpression of miR-128 was able to inhibit apoptosis induced by this compound. The rate

of apoptosis was measured by flow cytometry with annexin V and PI staining following Rh2 treatment. U251 cells were treated with 50 nmol/L miR-128 inhibitor or miRNA-128 inhibitor control for 48 h prior to treatment with 12 μ g/mL Rh2 for 48 h. Transfection of the miR-128 inhibitor significantly prevented Rh2-induced apoptosis in U251 cells. The percentages of early apoptotic cells were 6.78% \pm 2.11%, 25.77% \pm 4.10%, 27.36% \pm 5.33% and 11.25% \pm 2.89% in the control, Rh2 alone, Rh2+miR-128 inhibitor control, and Rh2+miR-128 inhibitor group, respectively (Figure 6 A-D, the lower right quadrant). The percentages of late apoptotic cells were 7.62% \pm 2.34%, 27.84% \pm 3.56%, 28.07% \pm 5.92% and 14.77% \pm 3.62% in the control, Rh2 alone, Rh2+miR-128 inhibitor control, and Rh2+miR-128 inhibitor group, respectively (Figure 6 A-D, the upper right quadrant).

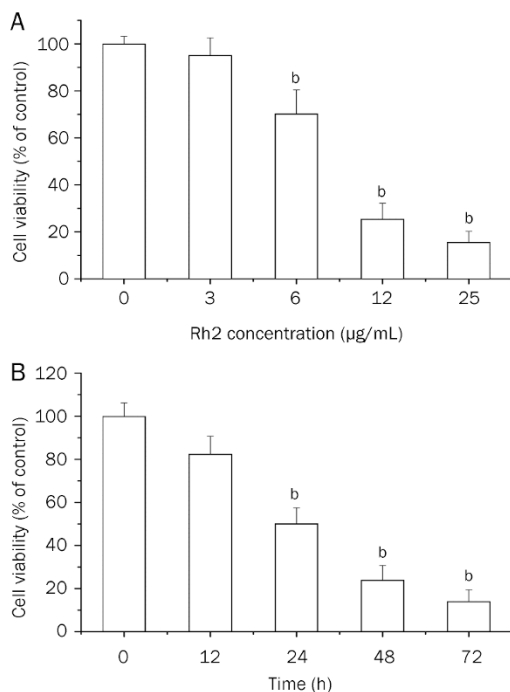


Figure 4. Rh2 inhibited viability of human glioma U251 cells determined using a CCK-8 assay. (A) Dose-dependent response. U251 cells were treated with Rh2 (3, 6, 12 and 25 μ g/mL) for 48 h. (B) Time-dependent response. U251 cells were treated with Rh2 (12 μ g/mL) for 12, 24, 48 and 72 h. The results represented three independent experiments. Data were expressed as means \pm SEM. ^b P <0.05 compared with control.

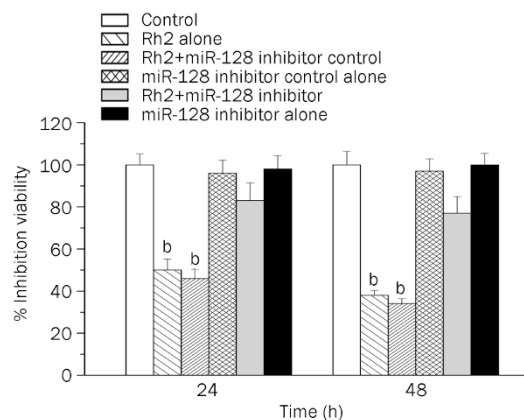


Figure 5. Transfection of miR-128 inhibitor prevented Rh2-induced cytotoxicity in glioma U251 cells. U251 cells were transfected with 50 nmol/L miR-128 inhibitor to knockdown miR-128. A miR-128 inhibitor control was used as negative control. At 48 h after transfection, the cells were treated with 12 μ g/mL Rh2 for 24 or 48 h. Cell proliferation was determined using CCK-8 assay. ^b P <0.05 compared with Rh2+miR-128 inhibitor. The experiment was performed three times, each in triplicate.

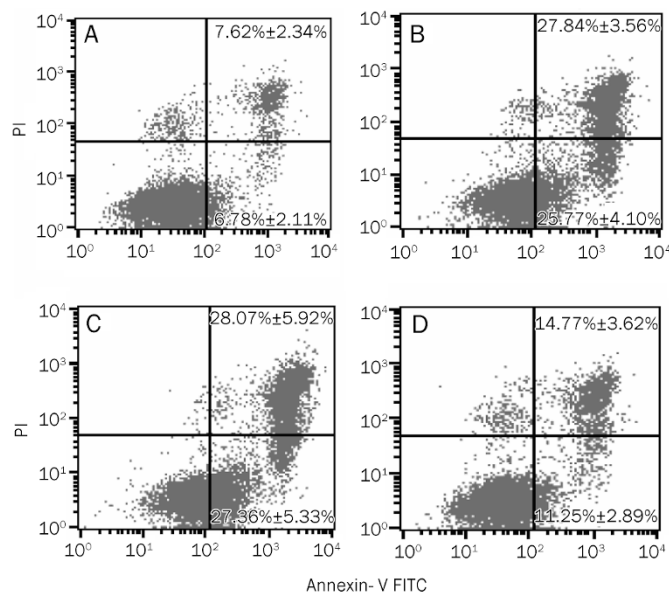


Figure 6. Transfection of miR-128 inhibitor inhibited Rh2-induced apoptosis in U251 cells. The cells were transfected with 50 nmol/L miR-128 inhibitor or inhibitor control for 48 h and then with Rh2 (12 $\mu\text{g}/\text{mL}$) for 48 h. Apoptosis was evaluated by annexin V and propidium iodide staining. The LR and UR quadrants indicated the percentage of early and late apoptotic cells, respectively, in control (A), Rh2 alone (B), Rh2+miR-128 inhibitor control (C) and Rh2+miR-128 inhibitor group (D). The experiment was performed three times, each in triplicate. Each point represented the means \pm SEM. There were significant differences between Rh2 alone or Rh2+miR-128 inhibitor control and control group, as well as between Rh2 alone and Rh2+miR-128 inhibitor group ($P<0.05$).

miRNA-128 mediated Rh2 induction of caspase 3 activity in glioma U251 cells

The previous studies have shown that the process of Rh2-induced apoptosis requires the activation of caspase 3 protease^[28]. To assess whether miR-128 overexpression is involved in Rh2-induced caspase 3 activation in U251 cells, we measured caspase 3 activities after the cells were treated with Rh2 alone or Rh2 plus miR-128 inhibitor. Results from the colorimetric assay demonstrated that caspase 3 activity increased significantly in a dose- and time-dependent manner in Rh2-treated U251 cells (Figure 7). We also found that the knockdown of miR-128 by transfection of a miR-128 inhibitor significantly inhibited Rh2-induced caspase 3 activation, as compared to cells treated with miR-128 inhibitor control (Figure 7).

Rh2 suppressed the activity of luciferase reporter containing the E2F3a-3'-UTR and the level of E2F3a protein in glioma U251 cells

E2F3a is a validated target of miR-128, and has been reported to mediate miR-128-induced glioma cell growth inhibition^[17]. To investigate whether overexpression of miR-128 induced by Rh2 affects E2F3a expression, the activity of a luciferase

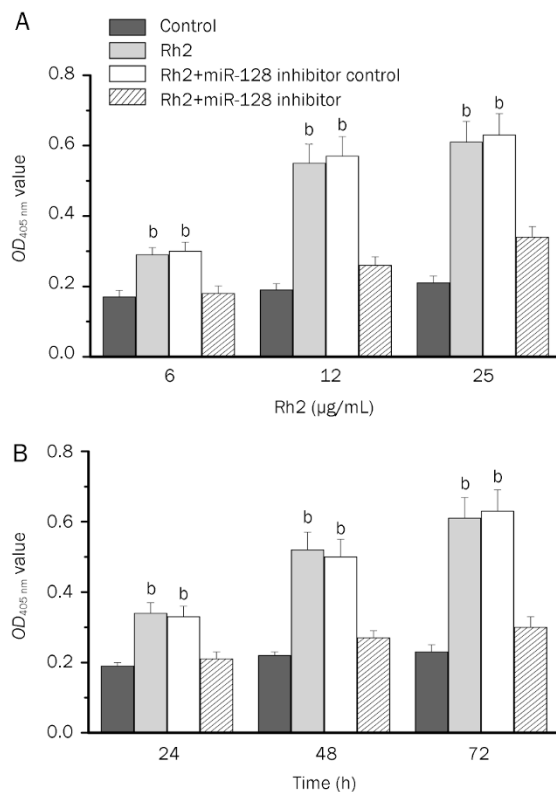


Figure 7. Transfection of miR-128 inhibitor inhibited Rh2-induced caspase 3 activation in U251 cells. (A) Transfection of miR-128 inhibitor inhibited the caspase 3 activation induced by treatment with 6, 12, and 25 $\mu\text{g}/\text{mL}$ Rh2 for 48 h. The cells were transfected with 50 nmol/L miR-128 inhibitor or inhibitor control for 48 h, and then with Rh2 for 48 h. (B) Transfection of miR-128 inhibitor inhibited the caspase 3 activation induced by treatment with 12 $\mu\text{g}/\text{mL}$ Rh2. The cells were transfected with 50 nmol/L miR-128 inhibitor or inhibitor control for 48 h, and then with Rh2 for 24, 48, and 72 h. Caspase 3 activity was determined using colorimetric assay. The experiment was performed three times each in triplicate. Mean \pm SEM. ^b $P<0.05$ compared with control or miR-128 inhibitor+Rh2.

reporter containing the E2F3a-3'-UTR sequence was determined in U251 cells. The protein level of E2F3a was also assessed in U251 cells. We found that cells treated with 12 and 25 $\mu\text{g}/\text{mL}$ Rh2 had suppressed luciferase activity (Figure 8A), indicative of transcriptional inhibition, as well as decreased the E2F3 protein expression in U251 cells (Figure 8B). To further explore whether miR-128 inhibitor prevents Rh2-induced inhibition of E2F3a expression, cells were transfected with 50 nmol/L miR-128 inhibitor for 48 h prior to 12 $\mu\text{g}/\text{mL}$ Rh2 for 24 h. We also found that transfection of miR-128 inhibitor prevented Rh2-induced inhibition of E2F3a expression, as compared with cells transfected with miR-128 inhibitor control (Figure 8B).

Discussion

Many miRNAs have been reported to have an oncogenic or a tumor suppressor function and to be involved in cell prolifer-

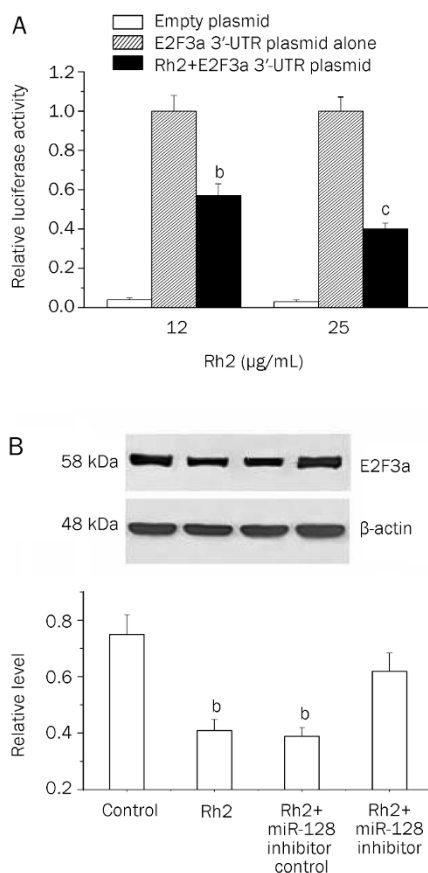


Figure 8. Rh2 suppressed the activity of luciferase reporter containing the E2F3a-3'-UTR and the level of E2F3a protein in U251 cells. (A) Rh2 inhibited the luciferase activity of E2F3a-3'-UTR. The activity of luciferase reporter containing the E2F3a-3'-UTR sequence was measured by TECAN Genios multifunctional microplate reader. The cells were transfected with 50 nmol/L E2F3a 3'-UTR plasmid or empty E2F3a 3'-UTR plasmid for 48 h using Lipofectamine 2000 (Invitrogen). After transfection, the cells were treated with Rh2 for 24 h, and the luciferase assay was performed. The experiment was performed three times, each in triplicate. Mean±SEM. ^b $P<0.05$, ^c $P<0.01$ compared to E2F3a 3'-UTR plasmid alone. (B) Transfection of miR-128 inhibitor prevented Rh2-induced inhibition of E2F3a protein expression in U251 cells, as compared with Rh2+miR-128 inhibitor control. The cells were transfected with 50 nmol/L miR-128 inhibitor for 48 h and then with 12 µg/mL Rh2 for 24 h. β -actin was used as a loading control in Western blot. ^b $P<0.05$ as compared to control or Rh2+miR-128 inhibitor.

eration, growth and apoptosis^[11, 30]. However, little is known about the relationship between miRNA expression and Rh2-induced anti-proliferation. The present study demonstrated that Rh2, a natural glycoside from the Ginseng, altered miRNA expression in human glioma U251 cells, including 14 up-regulated and 12 down-regulated miRNAs. We verified that miR-128 was up-regulated in human U251, T98MG and A172 glioma cells by quantitative real-time PCR. Among the miRNAs regulated by Rh2, some have been reported to be tumor suppressors. For instance, miR-128 and miR-181 function as tumor suppressors and trigger growth inhibition in

human glioma cells. In addition, knockdown or reduction of miR-21 led to glioma cell apoptosis and cell growth inhibition, reduced invasiveness and suppressed tumorigenicity^[31]. Our findings indicate that miRNAs play important roles in Rh2-induced antiproliferation in glioma cells.

miR-128 is a brain-enriched miRNA whose overexpression inhibits glioma cell proliferation^[17]. In our study, miR-128 was up-regulated by Rh2. To further investigate miR-128's role in Rh2-induced cytotoxicity, a miR-128 inhibitor was used to diminish miR-128 overexpression induced by Rh2 and led to an increase in U251 cell proliferation. Our study demonstrated that Rh2 could inhibit the proliferation of glioma U251 cells in a dose- and time-dependent manner. Moreover, we found that miR-128 inhibitor significantly inhibited miR-128 up-regulation and restored cell viability in Rh2-treated cells, implying that miR-128 overexpression is one of the principal mechanisms by which Rh2 mediates anti-proliferation in U251 cells.

Activation of apoptotic pathways is a key mechanism by which chemotherapeutic drugs kill cancer cells. Rh2 has been reported to induce apoptosis in glioma cells^[28]. Here, we demonstrated that Rh2 could induce apoptosis of glioma U251 cells in a dose- and time-dependent manner, illustrated by annexin V and propidium iodide staining. Moreover, we found that treatment with a miR-128 inhibitor markedly inhibited Rh2-induced apoptosis in these cells. Previous studies have shown that Rh2 could induce apoptosis through activation of the caspase pathway in human neuroblastoma^[28]. Consistent with the previous finding, our results showed that Rh2 significantly increased caspase 3 activity in U251 cells. We also found that knockdown of miR-128 expression prevented Rh2-induced caspase 3 activation. These results suggest that miR-128-mediated apoptosis is a key mechanism in Rh2-induced cytotoxicity in U251 cells.

In addition, our results showed that knockdown of Rh2-induced miR-128 overexpression by transfection of miR-128 inhibitor only partially prevented Rh2-induced anti-proliferation in glioma U251 cells. However, in our initial experiments, we transfected a miR-128 mimic into U251 cells and observed a reduced proliferation of the cells. In cells treated with a miR-128 mimic in addition to Rh2, the compound did have an anti-proliferation effect, although there was no significant difference compared with miR-128 mimic control (data not shown). These results suggest that there may be additional miRNAs that also mediate Rh2-induced cytotoxicity in glioma cells. For instance, miR-21 has been found to function as an oncogene and to be abundant and up-regulated in glioma cells^[32, 33]. Taken together with our findings, these results suggest that the fold change values of miR-21 was larger than those of miR-128. In fact, the expression levels of miR-128 were lower than those of miR-21 in our microarray results. Therefore, miR-21 appears to be a better candidate for study. In the present study, we focused on miR-128 because it is a brain-enriched miRNA and has strong anti-proliferative roles in glioma development. However, our results do not exclude the involvement of miR-21 in Rh2-induced cytotoxicity. Further

studies are needed to investigate the molecular mechanism of miR-21 in Rh2-induced anti-proliferative effects.

Computational algorithms showed several hundreds of putative targets of miR-128 (<http://www.microrna.org>). A recent study also showed that the transcription factor E2F3a is a direct target of miR-128^[17]. Considering that overexpression of miR-128 can inhibit proliferation of glioma cells by targeting E2F3a, we wondered whether Rh2 could affect E2F3a via induction of miR-128 overexpression. We found that Rh2 inhibited E2F3a protein expression using Western blotting, an effect that was reversed following treatment with an miR-128 inhibitor. These results suggest that Rh2-induced inhibition of glioma cell proliferation may be mediated by negatively regulating the expression of E2F3a, a miR-128 target gene. E2F3a has been found to stimulate cell proliferation, apoptosis and carcinogenesis in a transgenic mouse model^[34]. E2F3a protein expression negatively correlated with the expression level of miR-128 in glioma cells^[17]. The sequence of miR-128 and its target site in the 3'-UTR of E2F3a is shown in Figure 9, as predicted by Miranda software (<http://www.microrna.org>). The 5'-end of miR-128 is complementary to the 3'-UTR of E2F3a, which mediates post-transcriptional negative regulation of E2F3a via RNA duplex formation.

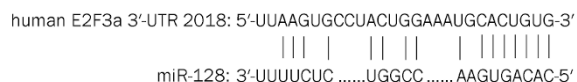


Figure 9. Sequence of miR-128 and its target site in the 3'-UTR of E2F3a.

Except for E2F3a, miR-128 has been reported to exert its anti-proliferative and stem cell self-renewal effects in glioma cells through targeting Bmi-1, a stem cell renewal factor^[33]. In our study, we could not investigate the effect of Rh2 on Bmi-1 and stem cell self-renewal effects in glioma cells. Therefore, it would be interesting to examine the effects of Rh2 has on stem cell self-renewal through miR-128-mediated Bmi-1 pathway in human glioma neurosphere cultures that possess features of glioma "stem-like" cells.

In conclusion, the present study shows that ginsenoside Rh2 exerts its anti-proliferative effect in U251 glioma cells in part by up-regulation of miRNA-128 expression. This finding provides new insight into the understanding of the molecular mechanism of Rh2-mediated cytotoxicity.

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Author contribution

Hua FENG and Nan WU designed research; Nan WU and Guo-cai WU performed research; Rong HU and Mei LI contributed new analytical tools and reagents; Rong HU and Mei LI analyzed data; and Nan WU wrote the paper.

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