miR-124-3p inhibits the viability and motility of glioblastoma multiforme by targeting RhoG

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Received August 5, 2020; Accepted January 18, 2021

DOI: 10.3892/ijmm.2021.4902

Abstract. The mortality rate of patients with glioma is increasing worldwide per annum. This is attributed to the poor disease prognosis, most notably for high-grade gliomas (grade III and IV), which does not improve the overall patient survival. The dysregulation of microRNA (miRNA/miR)-124-3p is found in a variety of tumors. However, the association between miR-124-3p expression and its target genes in glioma has not been thoroughly elucidated. The present study aimed to explore the possible effects of miR-124-3p and its proved target, Ras homology Growth-related (RhoG), on the oncogenic events associated with glioblastoma multiforme (GBM) development. The data demonstrated an inverse association between miR-124-3p and RhoG expression levels during GBM progression in GBM tissues and cells. U87 and U251 cells were employed for the *in vitro* assays. Luciferase reporter assays revealed that miR-124-3p interacted with RhoG at the RhoG 3' untranslated region and inhibited RhoG expression in GBM cells. Functionally, enriched miR-124-3p repressed RhoG transcription and suppressed GBM cell proliferation and migration, promoting apoptosis and altering the expression or activity of the apoptosis-related proteins of GBM cells. By contrast, the inhibition of miR-124-3p in GBM cells upregulated RhoG levels and promoted the proliferation of GBM cells. The knock down of RhoG expression by specific small interfering RNA sequences partially neutralized the effects induced by the miR-124-3p inhibitor. In conclusion, the present study demonstrated the crucial effects of miR-124-3p on the development and deterioration of GBM by targeting RhoG.

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Key words: small GTPase Ras homology Growth-related, microRNA-124-3p, glioblastoma multiforme, proliferation, apoptosis, migration, invasion

Introduction

As the most lethal pestilent primary tumor of the brain, gliomas constitute ~70% of primary malignant cancers in the human brain. According to the World Health Organization (WHO), gliomas are classified into an adverse group of tumors, including pilocytic astrocytomas and glioblastomas (1,2). Glioblastoma multiforme (GBM), ranks as the maximal grade of benign glioma. This type of cancer is prominently attributed to the composition of a highly distinctive type of cells and therefore displays phenotypic heterogeneity (1,3). Despite significant improvements in cancer therapeutic strategies and methods, the overall survival of patients with GBM has not been considerably improved (4,5). Therefore, it is required to develop novel biomarkers in order to improve the diagnostic potential of GBM.

MicroRNAs (miRNAs/miRs) consist of clusters of small non-coding RNAs of 17-27 nucleotides in length. By binding to target mRNAs, miRNAs regulate gene expression after transcription (6). Various studies have revealed that miRNAs affect the function of a novel cluster of tumor suppressor genes and oncogenes that contribute to tumorigenesis, apoptosis, angiogenesis and the invasion of cancer cells (6-8). miR-124 is the most well-identified miRNA in the brain and its aberrant expression has been observed in different malignancies, such as breast cancer (9), gastric cancer (10) and acute lymphoblastic leukemia (11), which indicates its potential roles in carcinogenesis (12). Previous studies indicated that miR-124 expression was downregulated in tissues of patients with glioma and that it exhibited a negative correlation with the glioma pathological grade. In addition, *in vitro* experiments demonstrated that miR-124 overexpression suppressed the proliferation and invasion of glioma cells, illustrating its important role as a tumor suppressor (13,14). The present study explored the possible effects of miR-124-3p and its potential target, Ras homology Growth-related (RhoG), in the oncogenic events and deterioration of GBM.

Materials and methods

Ethical approval. The research protocol was approved by the Medical Ethics Committee of Kunming Medical University (Kunming, China) and was performed in accordance with the Declaration of Helsinki (revised in 2000). GBM tumor tissues

were acquired from the patients following their permission at The Department of Neurosurgery, First Affiliated Hospital of Kunming Medical University Province (Kunming, China).

Tissues preparation and cell culture. A total of 73 patients were recruited from the clinic of the Department of Neurosurgery, First Affiliated Hospital of Kunming Medical University between February 2018 and December 2019. The patients provided informed consent for their participation in the study. The patients ranged in age from 41 to 76 years, with a median age of 48 years. There were 36 male and 37 female patients included. Age-specific expression levels of RhoG and miR-124-3p estimates are described in Tables II and III. Patients who underwent surgery at our hospital and had not received prior anticancer treatment were included. Patients excluded were those suffering from cerebral trauma, recurrent infections, systemic disease and other immunosuppression conditions as well as those that were HIV-infected. The resected sections were classified by three independent senior pathologists according to the histopathological data. The carcinoma tissues were not obtained for further analysis until they were verified as the primary GBM. Subsequently, the primary neoplasm and the adjacent tissue (≥3 cm away from the neoplasm) (15) were obtained and divided into two parts for gene or protein expression detection.

The human GBM cell lines, U87 and U251, and the human normal skin fibroblast cell line, HF, were provided by The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences (Shanghai, China). The U87 and U251 cells were verified by The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. No misidentified and/or contaminated cell lines were used in the present study. It must be emphasized that the cell line U87 has been reported to be misidentified/contaminated. Therefore, this cell line is considered a glioblastoma of unknown origin, according to the Cellosaurus database (https://web.expasy.org/cellosaurus/CVCL_0022). Therefore, in the present study, the identity of this cell line was validated using STR profile identification by the Cell Bank of the Chinese Academy of Sciences. The results revealed that the matching ratio between the test sample and the ATCC standard data was 94.4% and that this result confirmed the identity of the cell line used in this study as U87. The culture of the cells was performed by using the mixture medium, which contained Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Thermo Fisher Scientific, Inc.). The culture conditions were 37˚C with a humidified atmosphere containing 5% CO_2.

Cell treatment. The U87 and U251 cell lines were transiently transfected with either miR-124-3p (1.0 μl; 3 μM) or miR-124-3p inhibitor (2.5 μl; 3 μM) or the matched control using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The miR-124-3p, miR-124-3p inhibitor, miRNA mimics negative control (miR-NC) and scrambled siRNA negative control (miR-124 inhibitor NC) were provided by Guangzhou RiboBio Co., Ltd. The sequences of the miRNAs used were as follows: miR-124-3p (mimics), CCGUAAGUGGCGCACGGAAU; miR-NC, AAAGCCUUAUUCCUUCGUACG; miR-124-3p inhibitor, CAUUACGGCCAAUAUGUAAGGCA; and miR-124 inhibitor NC, AUGGUACGUGUAGGCCUACUAUG. The cells ($2x10^5$) were seeded in a plate and transfected with the aforementioned reagents for 48 h at 37˚C as recommended by the manufacturer's protocol. Subsequently, the treated cells were harvested for further analyses, including reverse transcription-quantitative PCR (RT-qPCR), western blotting, cell proliferation and apoptosis assays.

RT-qPCR. Samples from human GBM cells or from the tissue sections were used to evaluate the relative expression levels of miR-124-3p and RhoG by RT-qPCR. The extraction of total RNA was performed using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and an miRNeasy mini kit (Qiagen AB). The All-in-One™ miRNA quantitative RT-PCR Detection kit was used for the RT-qPCR, according to the manufacturer's protocol (GeneCopoeia, Inc.). The miRNA RT-PCR primers of hsa-miR-124-3p and U6 (reference gene) were provided by Applied Biosystems; Thermo Fisher Scientific, Inc. The expression levels of the examined samples were normalized to those of U6 (15). The sequences of the primers were as follows: U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; hsa-miR-124-3p, forward, 5'-AUUUCCCUCAAUCCUACUC-3' and reverse, 5'-UGAGCTGUCAACGCCUAUAUC-3'.

To assess the expression of RhoG mRNA, Moloney Murine Leukemia Virus Reverse Transcriptase (Takara Bio, Inc.) and 2X Mix SYBR Green I (Takara Bio, Inc.) were used. Each PCR reaction consisted of two parts, including the initial denaturation at 95˚C for 3 min and multiple cycles of amplification (39 cycles at 95˚C for 10 sec, 57˚C for 15 sec, and 72˚C for 30 sec), followed by a denaturation step at 95˚C for 10 sec, an annealing step at 65˚C for 5 sec, and a final extension step at 95˚C for 10 sec. GAPDH was used as the reference control. The primers were provided by Invitrogen; Thermo Fisher Scientific, Inc. and the sequences were described as follows: GAPDH forward, 5'-CGAGATCCCTCCAAAATCAA-3' and reverse, 5'-TTCACACCCATGACGAACAT-3'; and RhoG forward, 5'-CAGAGCATCAAGTGGTGGTGGTG-3' and reverse, 5'-GAGGATGCAGGACCGCCCACG-3'. The relative expressions levels of the genes examined were evaluated using the $2^{-\Delta\Delta Cq}$ method (16).

Western blot analysis. The BCA Assay kit (Beyotime Institute of Biotechnology) was used to determine the protein concentrations of each sample following total protein extraction by the cell lysis buffer (Beyotime Institute of Biotechnology). Subsequently, the expression levels of RhoG, Bcl-2, Bax, caspase-3 and caspase-9 proteins were assessed as previously described (17). Briefly, 40 μg protein per lane was separated via 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Beyotime Institute of Biotechnology). The membrane was soaked in 5% skimmed milk for 2 h at 37˚C and subsequently incubated with the corresponding primary antibody at 4˚C overnight. The details of each antibody are described as follows: RhoG (1:1,000; cat. no. sc-80015), Bax (1:500; cat. no. sc-7480) and Bcl-2 (1:800; cat. no. sc-7382; all from Santa Cruz Biotechnology, Inc.), caspase-9 (1:1,000; product code ab32539; Abcam), caspase-3 (1:500; product code ab13847; Abcam) and GAPDH (1:500; cat. no. sc-47724; Santa

cruz Biotechnology, Inc.). Following the primary antibody incubation, the membranes were washed three times with 0.01 M PBS buffer solution (Yunnan Labreal Biotechnology Co., Ltd.) and incubated with mouse IgG binding protein-HRP (1:2,000; cat. no. sc-516102) and mouse anti-rabbit IgG-HRP (1:1500; cat. no. sc-2357; both from Santa Cruz Biotechnology, Inc.) secondary antibodies at 37˚C for 1 h. ECL reagent (EMD Millipore) was utilized to observe the protein bands and GAPDH was used as the reference for protein expression.

Bioinformatics prediction for the target genes of miR-124-3p. The three computational algorithms, TargetScan (version 7.2; http://www.targetscan.org) (18), miRanda (http://www.microrna.org) (19) and PicTar (http://pictar.mdc-berlin.de) (20) were employed to investigate the candidate targets of miR-124-3p involved in GBM progression. According to the intersection, the target genes were predicted by the aforementioned databases and the RhoG gene was selected. Subsequent studies were performed to assess its function as a potential target of miR-124-3p.

Validation of the miR-124-3p target gene RhoG. Luciferase reporter assays were performed to validate the miR-124-3p target gene RhoG in GBM cells. The putative binding locations at the 3' untranslated region (3'-UTR) of the human RhoG mRNA were predicted and wild-type (wt) or the mutant (mut) of RhoG was established and packaged in the pLUC plasmid (Guangzhou RiboBio Co., Ltd.). Mutations were produced using the QuikChange Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc.) according to the manufacturer's instructions. The primers used for the construction of the luciferase reporters and mutations of binding sites are presented in Table I. A random control luciferase plasmid (Blank control) was also purchased (Guangzhou RiboBio Co., Ltd.). Briefly, $1x10^6$ U87 or U251 cells were seeded into 96-well plates and cultured at 70% confluence 24 h prior to the transfection. A total of 30 ng of the pLUC-UTR-wt or the pLUC-UTR-mut corresponding to the RhoG luciferase plasmid (pluc RhoG 3'-UTR-wt or pluc RhoG 3'-UTR-mut) was mixed with 40 nM miR-124-3p, miR-124-3p inhibitor or the matched control miR-NC (miR-NC or miRNA-124-3p inhibitor NC), and transfected with 10 ng *Renilla* plasmid into GBM cells using Lipofectamine 3000 at 37˚C for 4 h. Following 12 h of cell culture, the relative luciferase activity was detected using a Dual-Luciferase Reporter assay system (Promega Corporation) The firefly luciferase activity was normalized to the *Renilla* luciferase activity and collected for analysis using a luminometer (Centro LB 960; Berthold Technologies GmbH & Co. KG). Three independent examinations were conducted, which were completed in triplicate.

RhoG knockdown. To validate whether miR-124-3p exerts significant effects on GBM deterioration by regulating RhoG expression, the proliferative, migratory and invasive abilities of the tumor cells were quantified in miR-124-3p gain and loss-function cells. RhoG small interfering RNA (siRNA/si) was introduced in these cells. The siRNA sequences aimed at human RhoG (si-RhoG) and the matched negative control (si-NC) sequences were designed according to previously validated oligonucleotides (21) and purchased from Guangzhou RiboBio

Table I. Primers for RhoG luciferase reporter constructions.

Gene	Sequence of forward primer	Sequence of reverse primer
RhoG	5'-TTACTAGTCCCTGGCACTTGGCTTGGA-3'	5'-TAGAAGCTTGAGTCAGTCAGCAAATGCGT-3'
RhoG mutant		
Position 48-54	5'-CTTTTTCTCTGAATACATATTTCTCCTTAAG-3'	5'-CTTAAGGAGGAAATATGTATTCAGAGAAAAAG-3'
Position 56-62	5'-TCCGCCTCAGCTATACATAAAGGACTAATTC-3'	5'-GAATTAGTCCTTTATGTATAGCTGAGGCGGA-3'
Position 76-83	5'-GCTTATTCTCCGTTCCAAGGCATATCGCGTAA-3'	5'-ATATGGCCCTATATTTATAGCGCAGTTTCCAGC-3'
Position 139-145	5'-CCCACCAGTTATACATAGGTGCCTTGTCC-3'	5'-GGACAAGGCACCTATGTATAACTGGTGGG-3'

RhoG, Ras homology Growth-related.

Table II. Clinicopathologic features of GBM associated with RhoG levels.

	RhoG mRNA levels			
Features	Low expression level n=26	High expression level n=47	r_s	P-value
Ages (years)			0.113	0.21
40-49	9	14		
50-60	10	18		
>60	7	15		
Sex			0.128	0.19
Male	14	22		
Female	12	25		
Tumor size (cm)			0.612	**0.0015**[a]
<5 cm	23	11		
≥5 cm	3	36		
WHO grade			0.401	**0.002**[a]
I-II	15	8		
III	8	10		
IV	3	29		

[a]Values in bold indicate P<0.05. RhoG, Ras homology Growth-related; GBM, glioblastoma multiforme.

Co., Ltd. Lentiviral particles (lentivirus-GFP, 5 μg; Shanghai GeneChem Co., Ltd.) were packaged into 293T cells (Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) using Lipofectamine 3000 for RhoG gene knockdown. The 2nd generation system was employed. The ratio of the lentiviral plasmid: Packaging vector was 1:1 according to the manufacturer's instructions. The multiplicity of infection (MOI) used to infect cells was 10.45 U/ml penicillin (Thermo Fisher Scientific, Inc.) and was used to create stable cell lines. GBM cells and normal HF cells were transfected with recombinant $1x10^6$ lentivirus-transducing units. After 48 h of incubation at 37˚C, the transfection efficiency was conducted by siRNA knockdown and assessed using RT-qPCR. Subsequently, combined incubation of RhoG siRNA-transfected U87 and U251 cells with miR-124-3p or its inhibitor was performed at 37˚C. At 24 h post-transfection, the cells were harvested for further analysis.

Cell proliferation assay. Using an MTT assay kit (Sigma-Aldrich; Merck KGaA), cell viability was measured. Briefly, the cells were seeded in 96-well plates at a density of $2x10^4$ cells/well. The cells were cultured for 48 h and 0.1 mg MTT reagent was added per well and incubated at 37˚C. Following incubation for an additional 4 h, the culture medium was replaced with 150 μl of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The absorbance of each well was detected at 570 nm by using a microplate reader (Multiskan Mk3; Thermo Fisher Scientific, Inc.).

Apoptosis assay. The Annexin-PE/7AAD kit (BD Pharmingen; BD Biosciences) was utilized to assess the induction of early and late apoptosis of GBM cells following various treatments. Labeled cells were verified and analyzed by a flow cytometer (FACScan; BD Biosciences). The FACStation software V6.1X (BD Biosciences) was employed for data analysis. The experiments were performed in triplicate. In addition, the activities of caspases-3 and/or caspase-7 were evaluated using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega; Corporation) according to the manufacturer's instructions.

Migration and invasion detection. The scratch wound assay was performed to assess the migration of the GBM cells following certain treatments, as previously described (22). Briefly, at ~80% confluence, the tumor cells infected with different treatments were transferred into six-well plates and cultured at 37˚C. Subsequently a scratch wound was created through the middle of each well plate vertically using a 10-μl tip. The scratched cells were removed by PBS washing thrice, and fresh serum-free medium was replaced. At 48 h post-incubation, the cells were observed by a light microscope at a magnification of x200 (Olympus Corporation).

In addition, the detection of migration and invasion detection was accomplished in accordance with a previously described method (23). Briefly, for the migration assay, tumor cells were prepared in serum-free medium at a density of $1x10^5$ cells/ml and seeded in the upper chamber. A pre-coated chamber with 500 ng/ml Matrigel solution (BD Biosciences) was employed for the invasion assay. Following 48 h of incubation, the migratory cells on the surface of the upper membrane were washed. The remaining cells that had migrated/invaded to the lower membrane were collected for statistical analysis following staining with 0.1% crystal violet for 20 min and fixation with 20% methanol for 30 sec at 20-24˚C. The stained cells were observed, imaged and counted under an IBX3 inverted microscope (Olympus Corporation) at a magnification of x200.

Statistical analysis. Statistical analyses were performed using the SPSS version 21.0 software (IBM Corp.). The data are

Table III. Associations between miR-124-3p, RhoG levels and clinicopathologic features of GBM patients by multivariate analysis.

	miR-124-3p expression			RhoG expression		
Features	Low level (N=44) (≤ median) n (%)	High level (N=29) (> median) n (%)	Adjusted OR (95% CI)	Low level (N=26) (≤ median) n (%)	High level (N=47) (> median) n (%)	Adjusted OR (95% CI)
WHO grade						
I-II	5 (6.8)	22 (30.1)	2.24 (0.96, 4.02)	15 (20.6)	10 (13.7)	1.58 (0.98, 4.21)
III	21 (28.8)	5 (6.8)	**16.87 (12.36, 28.21)**[a]	8 (10.9)	29 (39.7)	**14.11 (9.45, 29.2)**[a]
IV	18 (24.7)	2 (2.8)	**21.87 (12.31, 31.58)**[a]	3 (4.2)		**18.42 (0.75, 24.5)**[a]
Tumor size (cm)						
<5	10 (18.9)	25 (16.3)	1.82 (1.13-5.01)	23 (31.5)	11 (15.1)	1.23 (0.73, 6.49)
≥5	34 (62.1)	4 (2.7)	**49.03 (29.92-58.12)**[a]	3 (4.1)	36 (49.3)	**29.63 (20.4, 42.9)**[a]

[a]Values in bold indicate P<0.05. miR-124-3p, microRNA-124-3p; RhoG, Ras homology Growth-related; GBM, glioblastoma multiforme; CI, confidence interval, OR, odds ratio.

expressed as the mean ± standard deviation (SD). Multivariate logistic regression analysis and Student t-test was employed to assess the association between miR-124-3p levels (low or high), RhoG gene levels (low or high), and the clinicopathological characteristics of GBM. The median expression levels of miR-124-3p or RhoG were used as the cutoff values (24). The odds ratios (ORs) with 95% confidence intervals were also measured. Logistic regression analysis was performed to estimate adjusted ORs for ordinal data. Significant differences between mRNA and proteins expression levels, cell viability, induction of apoptosis and migration and invasive activities, among multiple groups were determined using ANOVA and Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expressional changes of miR-124-3p and its potential target RhoG in GBM tissues and cells. The expression levels of miR-124-3p and its predicted target gene RhoG were evaluated in the samples obtained from patients with GBM by RT-qPCR. The results revealed that miR-124-3p expression in GBM carcinoma tissues was lower compared with the normal tissues (P<0.05; Fig. 1A). By contrast, the mRNA expression levels of RhoG in cancer samples were upregulated compared with those in the normal tissues (P<0.05; Fig. 1A).

In human GBM cells, the data indicated that the expression pattern of miR-124-3p and RhoG was similar to that observed in GBM carcinoma cells (vs. HF cells respectively; P<0.05; Fig. 1B).

Correlation analysis revealed that the downregulated miR-124-3p expression levels were negatively correlated with RhoG expression levels in patients with GBM (r=-0.821; P<0.05; Fig. 1C).

Expression levels of RhoG in tumor samples are associated with GBM progression. The expression levels of RhoG, a candidate target of miR-124-3p, were investigated in cancer tissues obtained from patients with GBM by RT-qPCR. The patients were divided into two groups according to the miR-124-3p and RhoG expression levels, as follows: Subjects with miR-124-3p/RhoG expression levels lower than the mean expression level were classified as the low-expression level group and subjects with miR-124-3p/RhoG expression levels higher than the mean expression level were classified as the high-expression level group (24). The association between the clinicopathological parameters of patients with GBM and RhoG mRNA expression levels were analyzed. The results indicated that increased levels of RhoG were positively associated with tumor size (r=0.612; P=0.0015) and WHO grade (r=0.401, P=0.002) (Table II). Regression analysis revealed that the downregulated expression levels of miR-124-3p were negatively associated with RhoG expression levels during GBM progression (Table III). The data indicated that the unusual alterations in miR-124-3p and RhoG expression levels were detrimental to the prognosis of GBM.

Assessment of the effects of miR-124-3p or miR-124-3p inhibitor transfection. The miR-124-3p expression levels were analyzed in U87 (Fig. 2A) and U251 (Fig. 2B) cells following various treat-

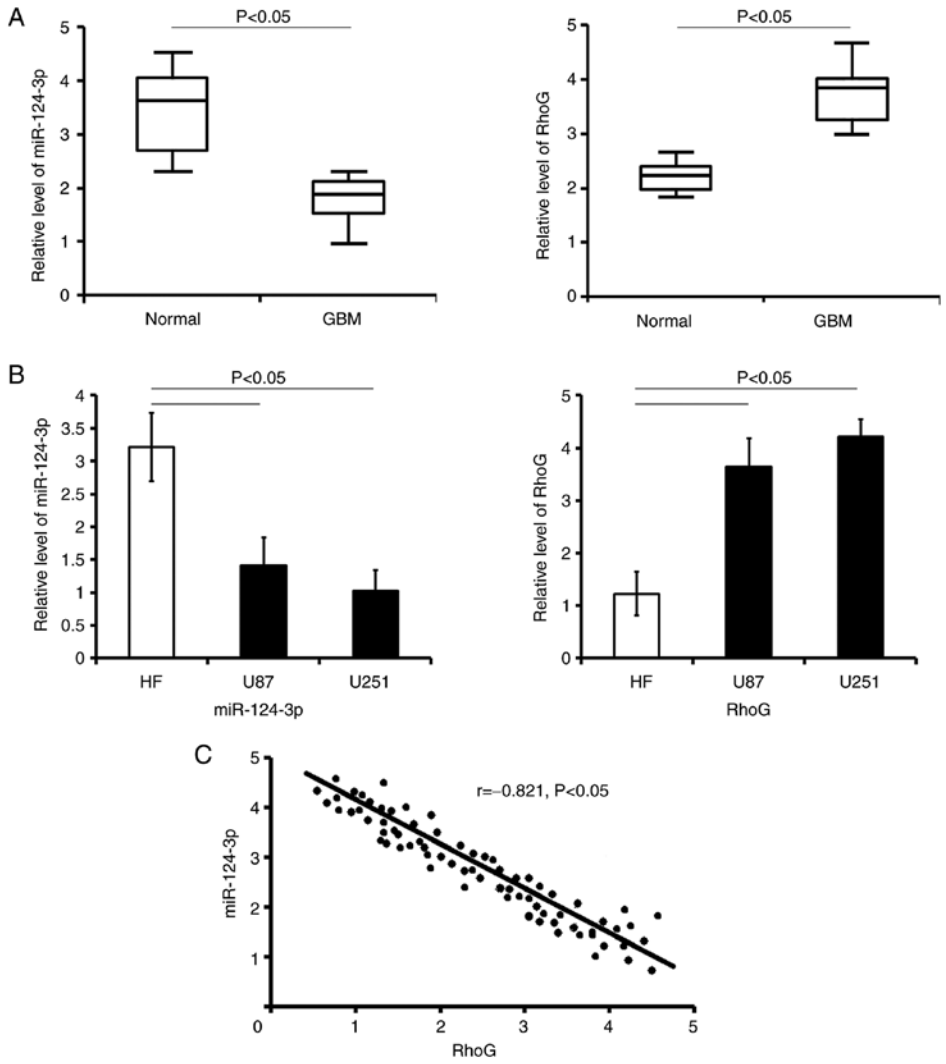

Figure 1. Aberrant changes of miR-124-3p and RhoG expression levels in GBM tissues and cells. (A) The tumor and matched adjacent tissues from patients with GBM were harvested, and the relative expression levels of miR-124-3p and RhoG were assessed by RT-qPCR (n=73). (B) The relative expression levels of miR-124-3p and RhoG were detected in healthy human HF cells and GBM cells (U87 and U251) by RT-qPCR. The data are presented as the mean ± SD. Five independent tests were carried out in triplicate (n=5). (C) Mutuality between miR-124-3p and RhoG levels in the tumor samples from patients with GBM (n=73). miR-124-3p, microRNA-124-3p; RhoG, Ras homology Growth-related; GBM, glioblastoma multiforme; RT-qPCR, reverse transcription-quantitative PCR; SD, standard deviation.

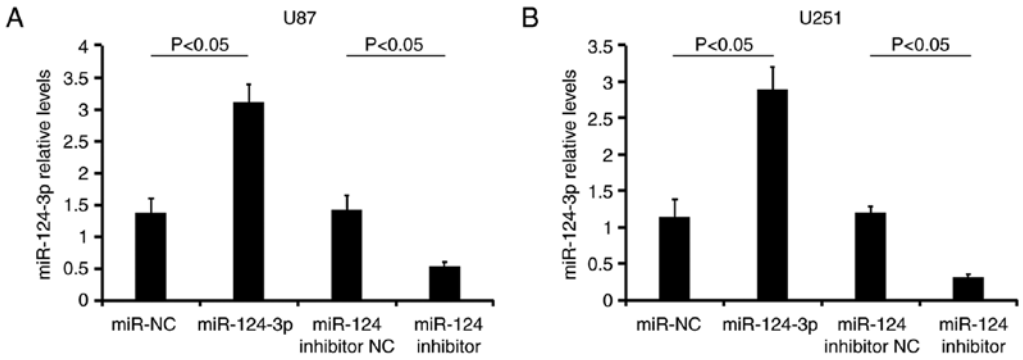

Figure 2. Effects of transfection with miR-124-3p or miR-124-3p inhibitor in U87 and U251 cells. The miR-124-3p expression levels were analyzed in (A) U87 and (B) U251 cells following various treatments using reverse transcription-quantitative PCR analysis. All the data are representative of five independent experiments (n=5). miR-NC, miRNA mimics negative control; miR-124-3p, miR-124-3p mimics; miR-124-3p inhibitor NC, miR-124-3p scrambled siRNA negative control; miR-124 inhibitor, miR-124-3p siRNA; miR-124-3p, microRNA-124-3p.

ments using RT-qPCR analysis. The transfection of miR-124-3p into the two types of GBM cells resulted in a significant increase in the miR-124-3p expression levels compared with miR-NC-transfected cells (P<0.05). In addition, the treatment of

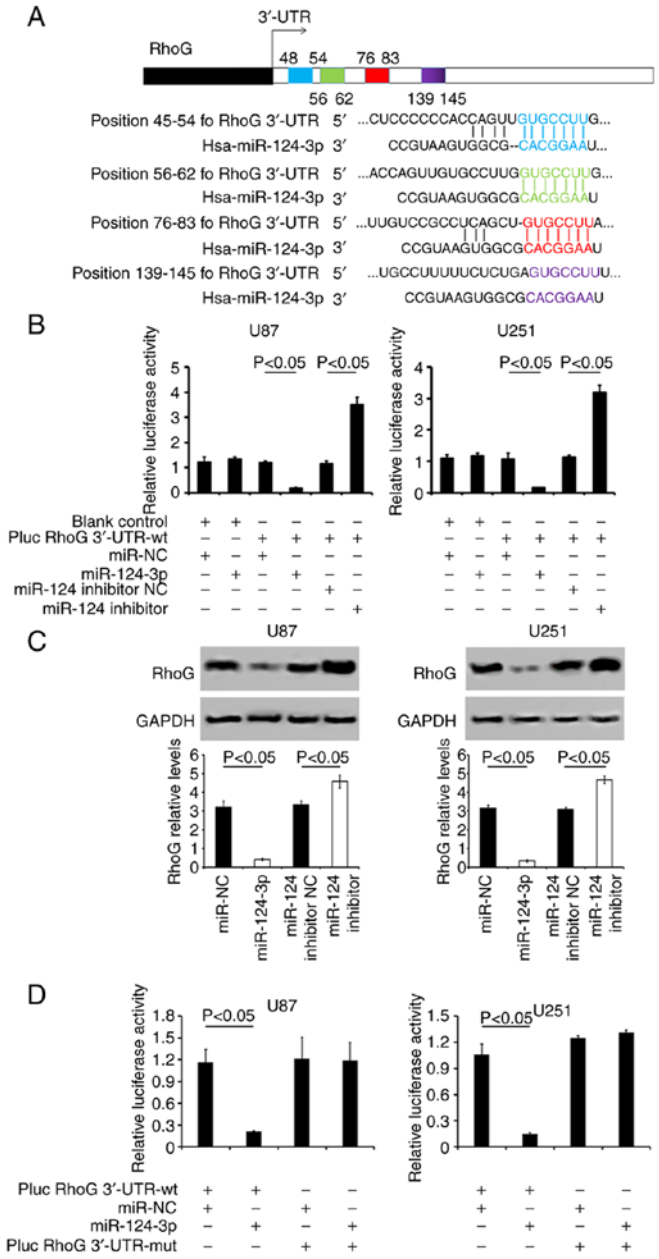

Figure 3. miR-124-3p targets the 3'-UTR seed site of RhoG. (A) Prediction of the 'seed regions' on RhoG 3'-UTR regulated by miR-124-3p. The 'seed' regions of the two miR-124-3p-binding sites were located at positions 48-54 (highlighted in blue) and 76-83 (highlighted in red) of the RhoG 3'-UTR. These regions are conserved between the species rat, mouse, and human. The poorly conserved binding site position 56-62 is highlighted in green, and another position 139-145 is highlighted in purple. (B) Examination of the relative luciferase activity in the cells co-transfected with miR-124-3p or miR-124-3p inhibitor and pluc RhoG 3'-UTR-wt, using a dual-luciferase reporter assay. (C) RhoG protein expression levels were analyzed in U87 and U251 cells following various treatments using western blot analysis. The representative lanes of RhoG protein expression are also presented. GAPDH served as the control. (D) Detection of the relative luciferase activity of miR-124-3p co-transfected with pLUC-RhoG 3'-UTR-wt or -mut using a dual-luciferase reporter assay. All the data are representative of five independent experiments (n=5). miR-124-3p, microRNA-124-3p; 3'-UTR, 3'-untranslated region; RhoG, Ras homology Growth-related; wt, wild-type; mut, mutant; SD, standard deviation; NC, negative control.

the cells with the miR-124-3p inhibitor decreased miR-124-3p levels (vs. the miR-124 inhibitor NC group; P<0.05; Fig. 2). The data revealed that the transfections were successful.

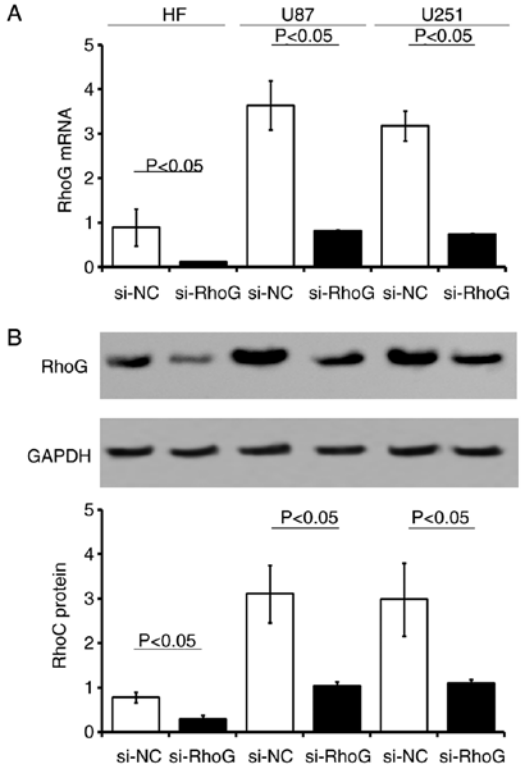

Figure 4. RhoG siRNA decreases the RhoG expression in GBM cells. (A) RhoG mRNA expression levels were assessed by reverse transcription-quantitative PCR in HF, U87 and U251 cells post RhoG siRNA transfection. (B) RhoG protein expression levels were evaluated using western blot analysis in HF, U87 and U251 cells post RhoG siRNA transfection. The data are presented as the mean ± SD. All the data are representative of five independent experiments (n=5). RhoG, Ras homology Growth-related; GBM, glioblastoma multiforme; siRNA, small interference RNA; SD, standard deviation; si-NC, RhoG siRNA negative control; si-RhoG, RhoG siRNA.

miR-124-3p targets RhoG at the 3'-UTR region. miR-124-3p exhibited four putative binding sites (two sites were conserved between the species rat, mouse, and human species and others were poorly conserved) for RhoG mRNA on the 3'-UTR (Fig. 3A). The co-transfection of miR-124-3p packaged with pLUC RhoG 3'-UTR-wt significantly decreased the relative luciferase activity. This was not observed in the pLUC RhoG 3'-UTR-mut or in the miR-NC transfected groups (Fig. 3B and D). The mutations in the putative miR-124-3p binding sites in the four 3'-UTRs abrogated the response to miR-124-3p (pluc RhoG 3'-UTR-mut + miR-NC vs. pluc RhoG 3'-UTR-mut + miR-124-3p; P>0.05; Fig. 3D). Moreover, the administration of the miR-124-3p inhibitor to the cells significantly reduced the relative luciferase activity (Fig. 3B). The transfection of miR-124-3p into the two types of GBM cells resulted in a notable reduction of RhoG expression levels compared with miR-NC-transfected cells (P<0.05). By contrast, the treatment of the cells with the miR-124-3p inhibitor increased RhoG levels (vs. the miR-124 inhibitor NC group; P<0.05; Fig. 3C).

Therefore, these results indicated that miR-124-3p was directly bound to RhoG 3' UTR and that it could downregulate RhoG expression.

Evaluation of the effects caused by RhoG siRNA transfection. The stable expression of three RhoG siRNA sequences in HF,

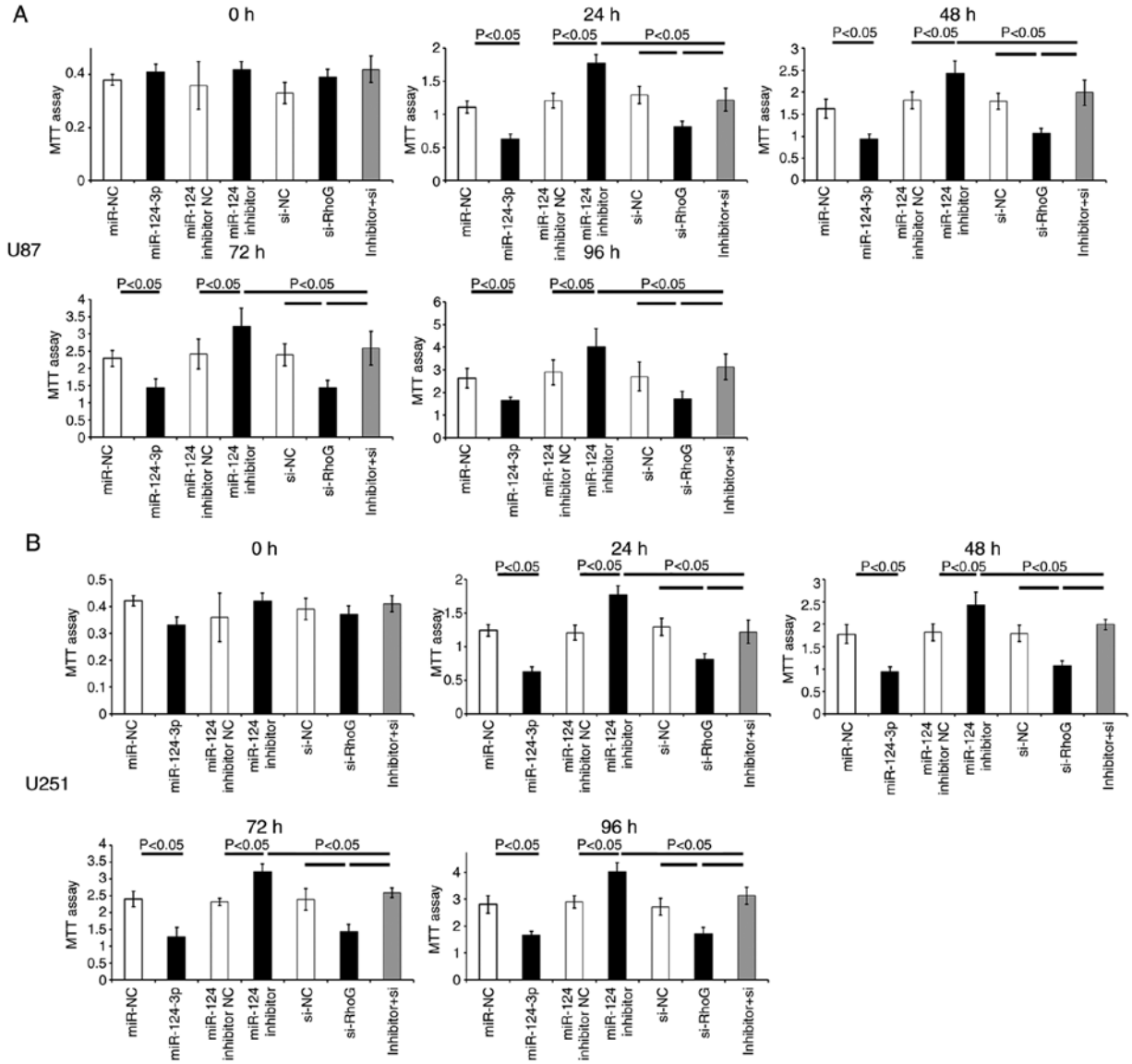

Figure 5. Effects of miR-124-3p/RhoG on the viability of GBM cells. Cell proliferation of (A) U87 and (B) U251 was detected using an MTT assay at 0, 24, 48, 72 and 96 h post-treatment. The data are presented as the mean ± SD. All the data are representative of five independent experiments (n=5). miR-124-3p, microRNA-124-3p; RhoG, Ras homology Growth-related; GBM; glioblastoma multiforme; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation; NC, negative control; si-NC, RhoG siRNA negative control; si-RhoG, RhoG siRNA; inhibitor+si, miR-124-3p inhibitor + RhoG siRNA.

U87 and U251 cells resulted in a >75% reduction in RhoG mRNA and protein expression levels (Fig. 4). The GBM cell lines and normal HF cells were stably transfected with RhoG siRNA-expressing or si-NC vector. The expression levels of RhoG mRNA or protein were significantly downregulated in cells transfected with si-RhoG compared with those transfected with the si-NC control sequences, respectively (P<0.05; Fig. 4).

miR-124-3p inhibits the viability and apoptosis of GBM cells by RhoG regulation. An MTT assay was performed to examine the possible influence of miR-124-3p or RhoG on the viability of GBM cells at 0, 24, 48, 72 and 96 h post-treatment. Transfection of the cells with miR-124-3p or RhoG siRNA significantly decreased the cell viability following 24, 48, 72 and 96 h of transfection in U87 (P<0.05; Fig. 5A) and U251 cells (P<0.05; Fig. 5B) compared with that observed in the NC groups. The functional inhibition of miR-124-3p by the application of its specific inhibitor resulted in a marked increase in the cell viability of U87 (P<0.05; Fig. 5A) or U251 cells (P<0.05; Fig. 5B).

In comparison with the control NC groups (miR-NC or si-NC), Annexin-PE/7AAD staining indicated that the transfection with either RhoG siRNA or miR-124-3p significantly increased the percentage of apoptotic U87 and U251 cells (P<0.05; Fig. 6). GBM cells transfected with miR-124-3p inhibitor indicated a lower apoptotic rate compared with the miR-124 inhibitor NC-treated cells (Fig. 6). The transfection of the cells with RhoG siRNA sequences and the subsequent treatment with the miR-124-3p inhibitor partially attenuated the effects produced by the inhibitor (Figs. 5 and 6).

Western blot analysis indicated that the miR-124-3p or RhoG siRNA-transfected cells had downregulated Bcl-2 expression levels and higher BAX expression levels compared with that of the miR-NC or si-NC cell groups (Fig. 7A). Transfection of the cells with either miR-124-3p or RhoG siRNA sequences promoted the cleavage of procaspase-9 and

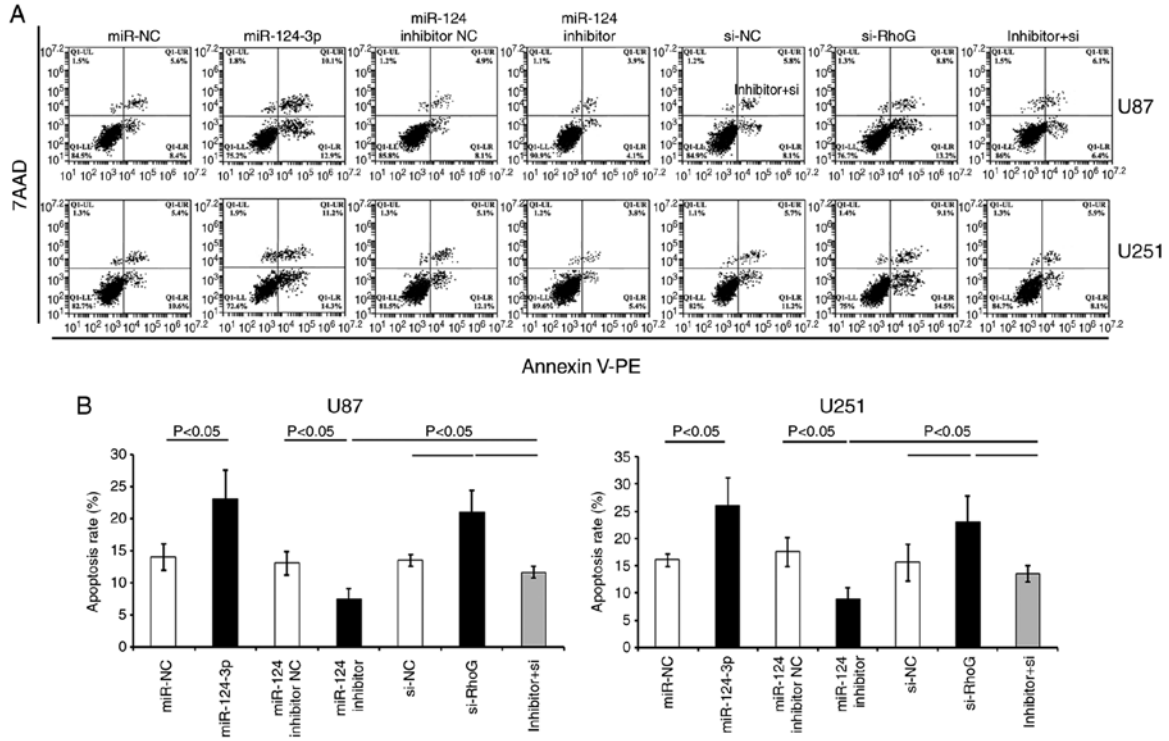

Figure 6. miR-124-3p suppresses apoptosis of GBM cells by targeting RhoG. (A) Apoptosis was assessed by flow cytometry. Annexin-PE/7AAD was used to label the cells following treatment with various reagents. (B) Quantitative analysis of the apoptotic rates in different groups. The data are presented as the mean ± SD. All the data are representative of five independent experiments (n=5). miR-124-3p, microRNA-124-3p; GBM, glioblastoma multiforme; RhoG, Ras homology Growth-related; SD, standard deviation; NC, negative control; si-NC, RhoG siRNA negative control; si-RhoG, RhoG siRNA; inhibitor+si, miR-124-3p inhibitor + RhoG siRNA.

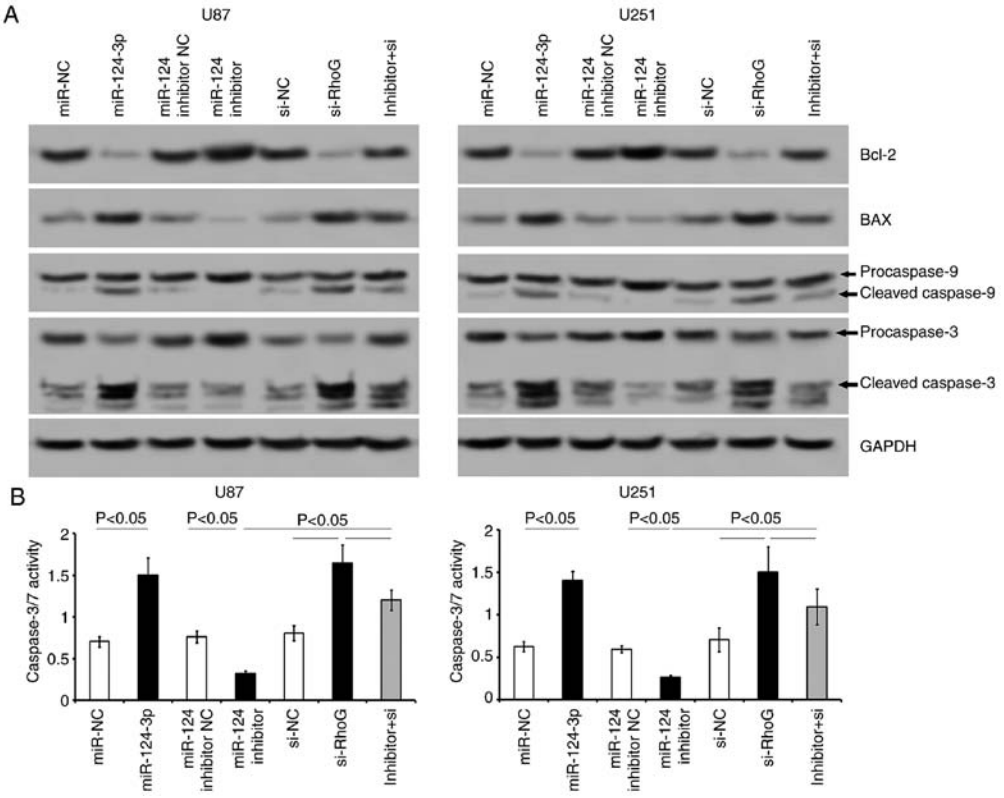

Figure 7. Targeting of RhoG by miR-124-3p affects the expression levels of apoptotic proteins in GBM cells. (A) The expression levels of anti-apoptotic proteins Bcl-2 and of the pro-apoptotic proteins BAX and Caspase-9/3 were examined by western blot analysis in GBM cells. (B) Caspase-3/7 activity was evaluated using an Apo-ONE Homogeneous Caspase-3/7 assay kit in U87 and U251 cells. The data are presented as the mean ± SD. All the data are representative of five independent experiments (n=5). RhoG, Ras homology Growth-related; GBM, glioblastoma multiforme; SD, standard deviation; NC, negative control; si-NC, RhoG siRNA negative control; si-RhoG, RhoG siRNA; inhibitor+si, miR-124-3p inhibitor + RhoG siRNA; miR-124-3p, microRNA-124-3p.

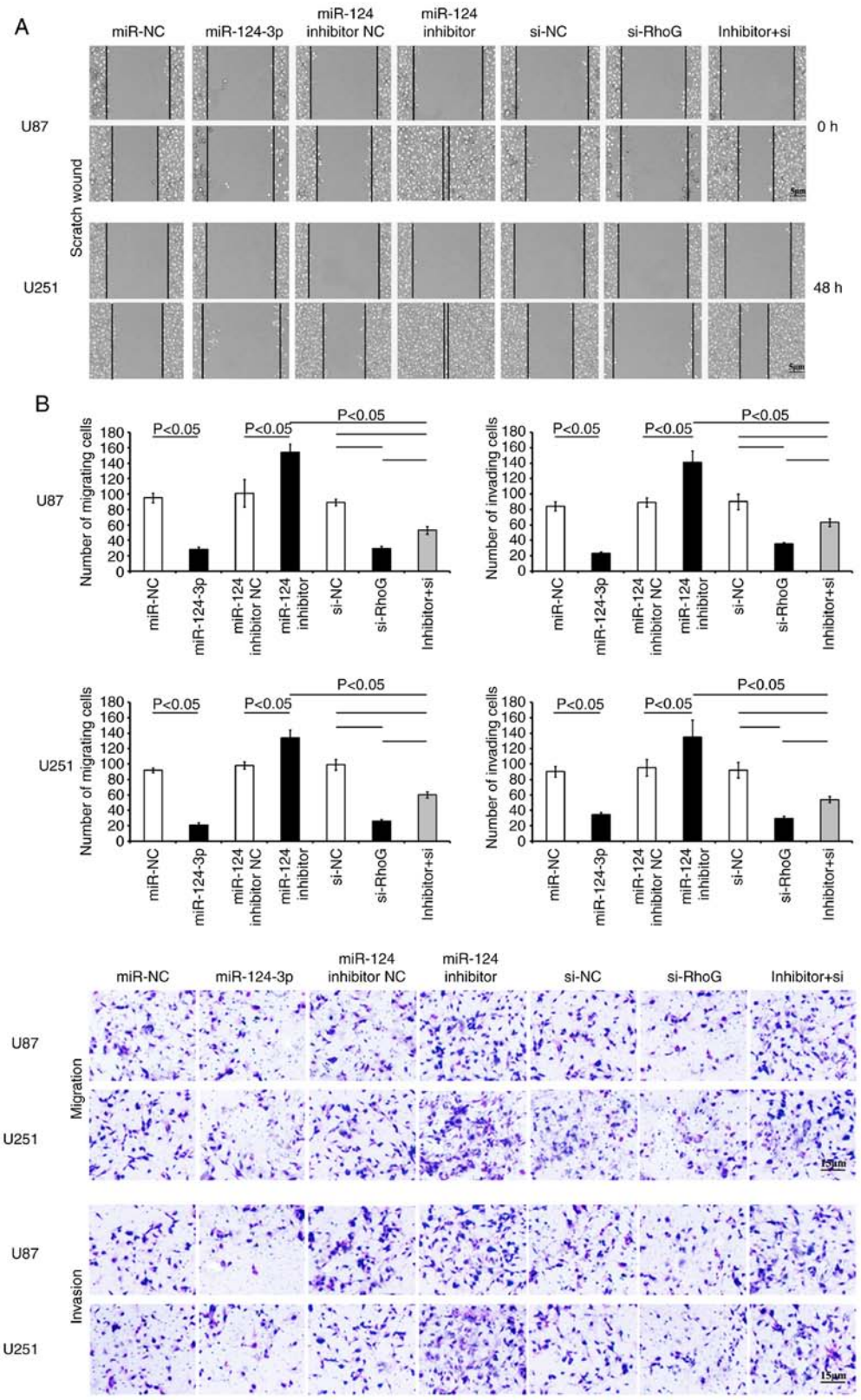

Figure 8. Effects of miR-124-3p on the migration of GBM cells by RhoG regulation. (A) Scratch wound assay was performed to assess the movement and migration of GBM cells. (B) Quantitative analysis of the migratory and invasive abilities of U87 and U251 cells following various treatments. The data are presented as the mean ± SD. All the data are representative of five independent experiments (n=5). miR-124-3p, microRNA-124-3p; GBM, glioblastoma multiforme; RhoG, Ras homology Growth-related; SD, standard deviation; NC, negative control; si-NC, RhoG siRNA negative control; si-RhoG, RhoG siRNA; inhibitor+si, miR-124-3p inhibitor + RhoG siRNA.

procaspase-3 into the corresponding active fragments in both GBM cell lines (Fig. 7A). Inhibition of miR-124-3p induced an increase in Bcl-2 levels and a decrease in BAX, cleaved caspase-9 and caspase-3 levels (Fig. 7A). Caspase-3/7 activity was significantly increased following the transfection of miR-124-3p or RhoG siRNA into the cells (P<0.05; Fig. 7B),

while the transfection of the cells with the miR-124-3p inhibitor suppressed the activity of caspase-3/7 ($P<0.05$; Fig. 7B). The co-transfection of miR-124-3p inhibitor and RhoG siRNA to the cells attenuated the apoptotic effects caused by the miR-124-3p inhibitor transfection alone (Figs. 6 and 7). The data indicated that the restoration of miR-124-3p levels in GBM cells was conducive for the prevention of GBM progression, possibly by regulating RhoG.

Targeting of RhoG by miR-124-3p inhibits the migration of GBM cells. Transfection of the cells with miR-124-3p resulted in a significant suppression of cell migration and invasion (Fig. 8). The scratch wound assay revealed that the transfection of the cells with miR-124-3p markedly reduced the migration of GBM cells compared with that of the control NC cells (Fig. 8A). The cells that were stained with crystal violet in the Transwell migration and invasion assays revealed considerably lower intensity in the miR-124-3p-transfected group compared with that of the NC control groups ($P<0.05$; Fig. 8B and C). However, the transfection of the cells with miR-124-3p inhibitor significantly increased the migration and invasion in both GBM cell lines (vs. NC control; $P<0.05$, respectively). RhoG siRNA-treated GBM cells presented a higher reduction in migration and invasion compared with the si-NC cells (Fig. 8). As anticipated, the co-transfection of the cells with both the miR-124-3p inhibitor and RhoG siRNA alleviated the effects on cell migration and invasion caused by single transfection of the cells with the miR-124-3p inhibitor (Fig. 8). The data indicated that miR-124-3p contributed to the reduction in the migration and invasion of GBM cells, which may be associated with the regulation of RhoG.

Discussion

The results of the present data revealed that the downregulated expression levels of miR-124-3p were negatively correlated with RhoG expression levels during tumor progression in samples from both patients with GBM and GBM cell lines. In addition, dual luciferase reporter assays verified that miR-124-3p bound to the 3'-UTR of RhoG and that this interaction could inhibit RhoG expression in GBM cells. The overexpression of miR-124-3p also suppressed cell viability and migration and induced the apoptosis of GBM cells by regulating RhoG expression. Therefore, these findings indicated the potential impact of miR-124-3p/RhoG on the development of GBM.

Glioma is a complicated neuroglial neoplasm, which consists of various homogeneous subtypes. These cancers are characterized by distinctive changes in expression levels of specific miRNAs and their corresponding targets (25). It has been revealed that the dysregulation of miRNAs contributes to the deterioration of GBM (26). The present study demonstrated that the aberrant decrease in miR-124-3p expression was detected in GBM human tissues and that it was associated with specific clinicopathological features and disease deterioration. *In vitro* assays further revealed that miR-124-3p upregulation suppressed the viability and migration of GBM cells. The results indicated the potential role of miR-124-3p in GBM progression. Similar observations have been reported by other previous studies, which have revealed the function of miR-124 as a tumor suppressor. miR-124 has been demonstrated to downregulate the expression levels of various targets, including Smad2 (13) and p62 (14), and exhibits high potential as a therapeutic target of glioma.

The Argonaute (AGO) proteins, interact with mature miRNAs and can form the RNA-induced silencing complex for silencing, the expression of target genes following transcription (27,28). It is well known that a single cohort of miRNAs can target a large number of varied target mRNAs via interacting with the AGO proteins (29,30).

Various genes have been identified and validated as targets of miR-124. These genes are involved in different pathological progresses. RhoG, which is regulated by miR-124, is involved in neuronal development (31,32). It has also been revealed that miR-124 is silenced at the post-transcriptional level in a variety of types of cancer cells and that this process modulates their proliferation via the regulation of CDK6, CTGF, ITGB1, RhoG and ROCK1 (9-12).

In the present study, RhoG was identified and validated as a miR-124-3p target gene implicated in the progression of GBM. Rho family small GTPases exert essential effects on cell migration and actin cytoskeleton formation. Moreover, they regulate various pathways involved in the development of tumors (33-35). Due to its crucial role in the cytoskeletal reorganization, the small GTPase RhoG has attracted considerable attention. It has been revealed that RhoG is critical to phagocytosis and that it has the ability to alter gene transcription and cell vability (34,35). It is also involved in cell migration and invasion of pathogens (32). As a vital upstream target of Rac, RhoG plays a crucial role in the regulation of cell migration by activating Rac through its effector ELMO (36-38). Previous studies have also revealed that Src homology 3 domain-containing guanine nucleotide exchange factor (SGEF)-RhoG is an essential downstream regulator of migration and invasion in TWEAK/Fn14-induced glioblastoma cells (39). In the present study, correlation analysis revealed that the endogenous downregulated expression levels of miR-124-3p were correlated with RhoG expression levels in patients with GBM. High levels of RhoG were associated with the deterioration of GBM. The results of luciferase reporter assays indicated that miR-124-3p was bound to the 3'-UTR sequence of the RhoG gene and that it could inhibit RhoG transcription in GBM cells. The treatment of cells with RhoG siRNA or miR-124-3p markedly reduced their expansion, migration and invasion, while it promoted the induction of apoptosis. In contrast to these findings, the treatment of the cells with the miR-124-3p inhibitor enhanced the viability and migration of GBM cells. Furthermore, RhoG siRNA relieved the effects induced by the miR-124-3p inhibitor, which led to a marked increase in the viability and migration of GBM cells. The present study demonstrated that RhoG was involved in the survival, proliferation, migration and invasion of GBM. This effect was mediated by miR-124-3p. The data indicated that miR-124-3p acted as a tumor suppressor and delayed the deterioration of GBM by targeting RhoG.

Furthermore, miR-124-3p and/or RhoG siRNA increased in the apoptotic rates of GBM cells and the expression of activated caspases 3/7, whereas they altered the transcriptions of the proteins associated with apoptosis. It has been revealed that phosphatidylinositol 3-kinase (PI3K) plays a vital role in malignant transformation (40). The PI3K-induced inhibition of

apoptosis is performed by several proteins regulated by protein kinase B (PKB) and/or the PKB-enzyme-dependent (such as GSK-3 and ILK) signaling pathway (40). The direct interaction of these proteins with PI3K and the activation of Akt and RhoG may facilitate the induction of apoptosis (41). Therefore, the present study proposed that the inhibition of tumor cell proliferation induced by RhoG siRNA may be associated with the anchorage deficit by means of a PI3K-dependent mechanism. However, further investigation is required to confirm this hypothesis (41,42).

In addition, the present study demonstrated that the miR-124-3p inhibitor influenced GBM cell proliferation, viability and migration, even in the absence of RhoG. These results suggested that miR-124-3p influenced the carcinogenic transformation of GBM cells through a mechanism involving RhoG inhibition. Combined with the research reported by other groups, the present findings demonstrate that miR-124 may target multiple proteins, including CTGF, ITGB1, RhoG, ROCK1, CTGF, ROCK2 and/or EZH2, that function spatiotemporally or in cooperation with different cellular processes (9,43). These observations provide evidence that miR-124 acts via the regulation of various proteins, which are upregulated in several types of human cancer (13,14,43-45). The overexpression of these proteins is positively correlated with tumor metastasis and/or poor disease prognosis (13,14,43-45). In addition to the regulation of RhoG, miR-124-3p was synchronously involved in GBM tumorigenesis through other regulatory mechanisms. However, further investigations are necessary to confirm these findings.

Moreover, since the increased expression level of RhoG was detected in the glioma carcinomas and cell lines, specific siRNA targeting RhoG was used to suppress the endogenous RhoG expression and to explore the potential therapeutic effects of RhoG knockdown in GBM cases. It was also verified whether RhoG was a target gene for miR-124-3p. Therefore, miR-124-3p inhibitor and RhoG siRNA were co-transfected to determine whether RhoG siRNA attenuated the effects induced by miR-124-3p inhibitor. The miR-124-3p inhibitor induced the recovery in endogenous RhoG expression, while RhoG siRNA transfection led to RhoG expression inhibition. These results further verified that miR-124-3p was involved in the carcinogenic events of GBM by targeting RhoG. In a future study, the effects of overexpression of RhoG may be investigated since this is a limitation of the present study.

In conclusion, the findings of the present study indicated that the downregulated expression levels of miR-124-3p were conversely correlated with RhoG expression levels during glioma progression in GBM tissues and cells. In addition, the results of the dual luciferase reporter assays confirmed that miR-124-3p interacted with the 3'-UTR of RhoG and inhibited RhoG transcription in GBM cells. Finally, the overexpression of miR-124-3p suppressed the expansion and infiltration of GBM cells and increased their apoptotic rate by RhoG transcriptional regulation. The present study demonstrated the possible effects of miR-124-3p on the development of GBM by targeting RhoG.

Acknowledgements

Not applicable.

Funding

The present study was supported in part by grants from the Health Commission of Yunnan Province Training Plan for Medical Reserve Talents (grant no. H-2017029), the Yunnan Clinical Medical Center of Nervous System Diseases (grant no. ZX2019-03-05), and the Research Innovation Team of Yunnan province (grant no. 2019HC022).

Availability of data and materials

The datasets used and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

SC, CJS and XPW conceived, designed, wrote, submitted and replied to review comments of the manuscript. SC, JXL and YPW collected the tumor tissues and prepared the cell cultures. CJS, YPW, TY and XPW performed cell treatment and luciferase reporter assays. SC, CJS, TY, and XPW performed the RT-qPCR, apoptosis assay and cell viability assay. SC, CJS, JXL and XPW conducted the western blot detection and scratch wound assay. JXL, YPW and TY performed the statistical analysis. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study complied with the Declaration of Helsinki (revised in 2000) and approved by the Medical Ethics Committee of Yunnan province. GBM tumor tissues were acquired from patients with permission of the Department of Neurosurgery, First Affiliated Hospital of Kunming Medical University Province, Kunming, China. The patients provided informed consent for their participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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