MiR-124 governs glioma growth and angiogenesis and enhances chemosensitivity by targeting R-Ras and N-Ras

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Background. Glioma is one of the most aggressive and lethal human brain tumors. Accumulating evidence shows that microRNAs play important roles in cancers, including glioma. Previous studies reported that miR-124 levels were downregulated in glioma specimens. Here, we further investigate the potential role of miR-124 in glioma.

Methods. The expression levels of miR-124 were detected in glioma specimens by quantitative reverse transcriptase PCR. The direct targets of miR-124 were identified by bioinformatics analysis and were further validated by immunoblotting and luciferase reporter assay. The effects of miR-124 on glioma cell proliferation and chemosensitivity to temozolomide were analyzed by Cell-Counting Kit 8 assay. Apoptosis was evaluated by fluorescence activated cell sorting analysis. A xenograft model was used to study the effect of miR-124 on tumor growth and angiogenesis.

Results. Expression levels of miR-124 were greatly downregulated in glioma specimens. related Ras viral oncogene homolog (R-Ras) and neuroblastoma Ras viral oncogene homolog (N-Ras) were identified as direct targets of miR-124. MiR-124 inhibited glioma cell growth, invasion, angiogenesis, and tumor growth and increased chemosensitivity to temozolomide treatment by negatively regulating the Ras family and its downstream signaling pathways: phosphatidylinositol-3 kinase/Akt and Raf/extracellular signal-regulated kinase 1/2. Furthermore, overexpression of R-Ras rescued the inhibitory effects of miR-124. Meanwhile, overexpression of R-Ras and N-Ras restored miR-124–inhibited vascular endothelial growth factor (VEGF) transcription activation. In clinical glioma specimens, protein levels of R-Ras and N-Ras were upregulated and inversely correlated with miR-124 expression levels.

Conclusions. Taken together, these results revealed that miR-124 levels in tumor tissues are associated with glioma occurrence, angiogenesis, and chemoresistance and that miR-124 may be used as a new diagnostic marker and therapeutic target for glioma in the future.

Keywords: carcinogenesis, glioma, miR-124, N-Ras, R-Ras.

Glioma is the most common type of primary brain tumor, originating in glial cells, with poor prognosis during the past 40 years. Approximately 20,000 new cases of glioma are diagnosed in the United States every year. The median survival from glioblastoma multiforme, the most aggressive type of malignant glioma, is only 12 to 14 months, even treated with surgery, chemotherapy, and/or radiotherapy. Temozolomide (TMZ), a DNA alkylating antineoplastic drug, is a promising chemotherapeutic agent that readily crosses the blood–brain barrier for treating glioblastoma. It can efficiently inhibit the proliferation of glioma cells and induce apoptosis. However, median overall survival has not significantly increased in patients treated by chemotherapy and radiotherapy. Thus, further studies that aim to enhance the therapeutic effect of chemotherapy drugs are urgently required.
MicroRNAs (miRNAs), small noncoding RNA molecules, are known to play vital roles in the progression of various cancers, including glioma, and have been proposed as novel targets for anticancer therapies in recent years. More than 50% of miRNAs are known to be involved in human tumorigenesis or angiogenesis by directly targeting oncogenes or tumor suppressor genes. MiR-21 accelerated tumorigenesis by targeting several tumor suppressor genes, such as phosphatase and tensin homolog and programmed cell death 4. MiR-195 was downregulated in glioblastoma and inhibited invasiveness of primary glioblastoma cell lines by targeting EZF transcription factor 3 and cyclin D3. According to previous studies, one miRNA can potentially regulate hundreds of genes at posttranscriptional levels, and one gene can be targeted by multiple miRNAs, leading to complex regulatory networks. Thus, dysregulation of these miRNAs may directly lead to subsequent abnormal expression of their targets, resulting in tumorigenesis. Among these miRNAs, miR-124 has been shown to be downregulated in human glioma tissues versus normal brain tissues. However, the role and mechanism of miR-124 in regulating tumorigenesis are still to be further elucidated.

Rat sarcoma (Ras) genes are the most frequently activated oncogenes in human cancer. Oncogenic point mutations in 1 of the 3 Ras isoforms—Kirsten Ras (K-Ras), neuroblastoma Ras viral oncogene homolog (N-Ras), and Harvey Ras (H-Ras)—have been detected in ~30% of human tumors. K-Ras is most frequently mutated among the 3 isoforms in malignancies; its mutation rate in all tumors is estimated to be 25%–30%. Knobbe et al. found that activating mutations in N-Ras as a molecular alteration contributes to aberrant Ras signaling in a small fraction of glioblastomas. Ras viral oncogene homolog (R-Ras) is another member of the Ras family, originally cloned through its homology to the well-known oncogene H-Ras. The R-Ras protein is 55% identical to H-Ras and regulates glioma cell proliferation, growth, and invasion. R-Ras expression and phosphorylation levels correlate with increasing grade of gliomas in human brain tumor samples. Recent studies have identified some miRNAs posttranscriptionally regulating K-Ras and N-Ras, such as Let-7a, miR-622, miR-145, miR-146b, and miR-146a. However, fewer studies focused on how miRNAs regulate R-Ras and affect tumorigenesis. Here, we identified that R-Ras was a novel direct target of miR-124 and found that levels of R-Ras and N-Ras in glioma samples were inversely correlated with miR-124 expression levels. Previous studies showed that activation of Ras signaling caused cell growth, differentiation, and survival. Oncogenic R-Ras and N-Ras promote tumorigenesis through activating the signaling pathways of both phosphatidylinositol-3 kinase (PI3K)/Akt and RAF/extracellular signal-regulated kinase (ERK) 1/2. Thus, silencing Ras signaling may be an efficient therapeutic strategy in glioma and other cancers, but the optimal and novel therapeutic strategies remain to be elucidated.

In the present study, we address several important questions: (i) whether miR-124 overexpression inhibits cell proliferation, cell invasion, tumor growth, and angiogenesis; (ii) what functional target(s) of miR-124 is(are) involved in tumor growth and angiogenesis; (iii) whether miR-124 inhibits expression of vascular endothelial growth factor (VEGF) via its direct target(s); and (iv) whether miR-124 increases chemosensitivity to TMZ and its underlying mechanism. The answers to these questions would provide new insights into a better understanding of the role of miR-124 in glioma development and provide a novel therapeutic strategy for treatment of glioma as well as other cancers driven by Ras family oncogenes.

Materials and Methods

Human Tissue Samples

The human glioma tissue samples were obtained from patients undergoing standard surgical procedure at the Department of Neurosurgery of the First Affiliated Hospital of Nanjing Medical University, China. Normal brain tissues were collected as negative controls from patients undergoing decompressive craniectomy for traumatic brain injury. Samples were collected with informed consent of the patients and were immediately frozen in liquid nitrogen until analysis. All experiments were approved by the ethics committee of Nanjing Medical University. The human glioma tissue samples used in this study were from 8 cases of World Health Organization (WHO) grade II diffuse astrocytoma, 8 cases of WHO grade III anaplastic astrocytoma, and 8 cases of WHO grade IV glioblastoma. All samples were histologically classified and graded according to WHO guidelines and validated by a clinical pathologist (H.L.). No information regulated by the US Health Insurance Portability and Accountability Act was included in the study, which qualified for exemption #4 of the National Institutes of Health.

Cell Culture and Antibodies

Human U87 and U251 glioma cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/mL, and 100 ng of streptomycin/mL. Human embryonic kidney 293T cells were cultured in DMEM supplemented with 10% FBS, 100 units of penicillin/mL, 100 ng of streptomycin/mL, and 2 mmol/L glutamine. All the cells were incubated at 37°C supplemented with 5% CO₂. Antibodies against mammalian target of rapamycin (mTOR), phosphorylated (p)-Akt (Ser473), total Akt, p-ERK1/2, and total ERK1/2 were purchased from Cell Signaling Technology. Antibodies against c-Raf, hypoxia-inducible factor (HIF)–1α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Bioworld. Temozolomide was obtained from Sigma.

Lentiviral Packaging and Stable Cell Line Establishment

The lentiviral packaging kit was purchased from Open Biosystems. Lentivirus carrying hsa-miR-124 or hsa-miR-negative control (miR-NC) was packaged in human embryonic kidney 293T cells and collected from the supernatant as instructed by the manufacturer’s manual. Stable cell lines were established by infecting lentivirus into U87 and U251 cells, followed by puromycin selection.

RNA Isolation and Quantitative reverse transcriptase PCR

Total RNAs of cells or human tissues were extracted using TRIzol reagent according to the manufacturer’s instructions. To determine the quantity of the mRNA levels of VEGF, total RNAs were reverse transcribed by oligodeoxythymidine primer using the PrimeScript RT Reagent Kit. The housekeeping gene GAPDH was used...
as an internal control. The primers were as follows: VEGF forward primer, 5'-TCGCGCCCTCCGAACCATGA-3'; VEGF reverse primer, 5'-CCCTGTTGAAGATCTGGTTC-3'; GAPDH forward primer, 5'-CCA CCATGGCAAATTCCATGGCA-3'; GAPDH reverse primer, 5'-TCTAGA CGGCAGCTCAGGTCCACC-3'. To measure expression levels of miR-124, the stem-loop specific primer method was used as described previously.27,28 Quantitative reverse transcriptase (qRT) PCR primers were the following: miR-124 RT primer, 5'-ACACTCCACGTGGAAGGCACGCCTG-3' antisense; 5'-TGTTGCAGGATGGTGTCG-3'. U6 RT primer: 5'-TGTTGCAGGATGGTGTCG-3'; U6 PCR primers, sense: 5'-CTGGCT CGGCAGCACA-3'; antisense: 5'-AACGCTTCCAAGATTTGCCTG-3'. Quantitative RT-PCR was performed using SYBR Premix Dimer Eraser on a 7900HT system. GAPDH or U6 levels were used as an internal control, and fold changes were calculated by relative quantification (2- ΔΔCt).

Protein Extraction and Immunoblotting

Cells or tissues were lysed on ice for 30 min in radioimmunoprecipitation assay buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM peptatin A. The lysates were centrifuged at 12 000 rpm at 4°C for 15 min, the supernatants were collected, and protein concentrations were determined using bicinchoninic acid assay. Protein extracts were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes in transfer buffer (20 mM Tris, 150 mM glycine, 20% [volume/volume] methanol). Membranes were blocked with 5% nonfat dry milk for 2 h and incubated with primary antibodies. An electrochemiluminescence detection system (Thermo Scientific) was used for signal detection.

Dual-luciferase Reporter Assay

For dual-luciferase assay, 3' untranslated regions (UTRs) of R-Ras and N-Ras containing predicted miR-124 seed-matching sites and corresponding mutant sites were amplified by PCR from human cDNA and inserted into the SacI and HindIII restriction enzyme sites of a pmIR-REPORT vector (Ambion). These constructs were validated by DNA sequencing. U87 cells were seeded in a 24-well plate and cotransfected with the wild-type (WT) or mutated (mut) reporter plasmid, plasmid renilla luciferase–thymidine kinase (pRL-TK) plasmids, and miR-124 or miR-NC. Luciferase activities were analyzed 24 h after transfection using the Promega Dual Luciferase Reporter Assay System.

To determine the effects of miR-124 on transcriptional activation of VEGF, VEGF reporter plasmid mitogen activated protein 11 (pMAP11)–WT or pMAP11-mut was cotransfected into U87 cells with pRL-TK plasmid and equal amounts of miR-124 or miR-NC precursor. Luciferase activities were measured from the cells 24 h after the culture. To determine the effect of R-Ras and/or N-Ras on miR-124–regulating transcriptional activation of VEGF, U87 cells were cotransfected with miR-124 or miR-NC, VEGF reporter, and pRL-TK plasmid, together with plasmid cytomegalovirus 6 (pCMV6) vector alone or with pCMV6–R-Ras and/or pCMV6–N-Ras plasmid (OriGene Technologies) using Lipofectamine 2000 (Invitrogen). Luciferase activities were measured 48 h after the transfection.

Cell Proliferation and Apoptosis Assay

Cells in the logarithmic phase of growth were seeded at 3 000 per well and cultured in 96-well plates. Cell proliferation was assayed using the Cell-Counting Kit 8 (CCK8; Dojindo Laboratories) according to the manufacturer’s instructions at indicated time points. Three independent experiments were performed in triplicate. The apoptosis assay was tested in U87 cells 72 h after transfection using the annexin V–fluorescein isothiocyanate Apoptosis Detection Kit I (BD Biosciences) and analyzed by fluorescence-activated cell sorting.

Invasion Assays

Cell invasion was determined using 24-well Matrigel invasion chambers (Becton Dickinson) in accordance with the manufacturer’s instructions. Cells (5 x 10⁵) were seeded per well in the upper well of the invasion chamber in DMEM without serum. The lower chamber well contained DMEM supplemented with 10% FBS to stimulate cell invasion. After incubation for 24 h, noninvading cells were removed from the top well with a cotton swab, while the bottom cells were fixed with 3% paraformaldehyde, stained with 0.1% crystal violet, and photographed in 3 independent 10× fields for each well. Membrane was air-dried and soaked for 15 min at room temperature with 33% acetic acid decolorization (200 μL/well). The stained solution was transferred to 96-well plates, and the absorbance value was read at an optical density of 570. Three independent experiments were conducted in triplicate.

In vitro Chemosensitivity Assay

Cancer cells were seeded at a density of 3 000 cells per well in a 96-well plate overnight. Freshly prepared TMZ solution was added at different concentrations as indicated, and cell survival was assayed by CCK8 48 h later. The percentages of live cells were calculated after normalization to the controls (cells without prior drug treatment). The cell viability in 100 μM TMZ treatments was tested every 24 h in both U87 and U251 cells overexpressing miR-124 and miR-NC. The relative percentages of surviving cells were calculated and normalized to that of the control (cells with 100 μM TMZ at day 0).

Caspase-3 Activity Assay

The activity of caspase-3 was determined using the Beyotime caspase-3 activity kit. Cell lysates were prepared and incubated with reaction buffer containing caspase-3 substrate (Ac-DEVD-pNA) after the treatment as indicated. Assays were performed on 96-well plates by incubating 10 μL protein of cell lysate per sample in 80 μL reaction buffer containing 10 μL caspase-3 substrate (Ac-DEVD-pNA; 2 mM) at 37°C for 2 h according to the manufacturer’s protocol. The reaction was then measured at 405 nm for absorbance.
Matrigel Plug Assay
Male Bagg albino/Ca-nu nude mice (6 wk old) were purchased from Shanghai Experimental Animal Center and maintained in specific pathogen-free conditions. Eight mice were randomly divided into 2 groups. U87 cells stably expressing miR-124 were harvested and resuspended in serum-free medium. Aliquots of the cells (2 × 10^6 cells in 100 μL) were mixed with 200 μL Matrigel. The mixture was immediately injected into both flanks of the nude mice. U87 cells stably expressing miR-NC plasmid were used as control. On day 11 after the implantation, mice were euthanized, and Matrigel plugs were trimmed out—half of the plugs were used for measuring hemoglobin content using Drabkin’s Reagent Kit (Sigma-Aldrich) according to the manufacturer's instructions, and the remaining tissues were formalin fixed and paraffin embedded. Tissue sections at 5 μm were stained and analyzed using antibody against CD31 (Abcam).

Tumor Growth Assay in Mice
Eight nude mice were randomly divided into 2 groups. U87 cells stably expressing miR-124 were injected subcutaneously into both flanks of the nude mice (5 × 10^6 cells in 100 μL). U87 cells stably expressing miR-NC were used as negative control. Tumor sizes were measured using a vernier caliper every 2 days when tumors were visible, and tumor volumes were calculated according to the formula: volume = 0.5 × length × width^2. Mice were euthanized 23 days later, and tumors were trimmed and weighed. Total proteins were extracted for immunoblotting assays to test specific protein expression levels.

Statistical Analysis
All experiments were performed 3 times, and data were analyzed using GraphPad Prism 5 software. The correlations between miR-124 expression and R-Ras or N-Ras levels in glioma tissues were analyzed using Spearman’s rank test. Statistical evaluation for data analysis was determined by t-test. The differences were considered to be statistically significant at P < .05.

Results
Downregulation of MiR-124 Expression in Human Gliomas
We measured expression levels of miR-124 in 6 normal brain tissues and 24 glioma tumor samples. Quantitative reverse transcriptase (qRT) PCR assay showed that miR-124 expression levels were significantly decreased in glioma samples compared with the normal brain tissues (Fig. 1A). Then, we divided all glioma samples into grade II, grade III, or grade IV according to WHO classification. We found that miR-124 levels were downregulated in these 3 groups compared with the normal brain group (P < .01; Fig. 1B). Moreover, the levels of miR-124 expression in high-grade tumors (WHO grades III and IV) were significantly lower than those in low-grade tumors (WHO grade II) (P < .05; Fig. 1B). These results indicate that the expression levels of miR-124 are downregulated in glioma and that miR-124 suppression levels inversely correlate with higher grades of glioma malignancy.

MiR-124 Directly Targets R-Ras and N-Ras
To understand the potential role and mechanism of miR-124 in glioma, we adopted the bioinformatic algorithm TargetScan to identify potential target genes of miR-124. Among the candidates, we found that seed sequence of miR-124 matched 3′-UTRs of 2 members of the Ras family, R-Ras and N-Ras (Fig. 2A). To verify whether miR-124 directly targets both R-Ras and N-Ras, 3′-UTR sequences containing putative binding sites of WT or mut were cloned into the pMIR-REPORT vector. U87 cells were cotransfected with reporter plasmid (R-Ras–WT or N-Ras–WT) and miR-124 or negative control (miR-NC). MiR-124 transfected cells showed a remarkable reduction of luciferase activities of both R-Ras and N-Ras reporters (Fig. 2B). The similar assay was performed using the mutant reporters containing mutated R-Ras or N-Ras 3′-UTR in miR-124 binding sites as indicated (Fig. 2A). As expected, miR-124 overexpression did not affect the luciferase activities of R-Ras or N-Ras 3′-UTR mut reporter (Fig. 2B). To determine whether R-Ras and N-Ras expression was indeed regulated by miR-124 at the protein level, we established U87 and U251 cells that stably expressed miR-124 or miR-NC. Immunoblotting results revealed that both R-Ras and...
Fig. 2. MiR-124 directly targets related Ras viral oncogene homolog (R-Ras) and neuroblastoma Ras viral oncogene homolog (N-Ras). (A) The sequence of miR-124 binding sites within R-Ras and N-Ras. The reporter constructs of the R-Ras and N-Ras 3′-UTR sequences and the mutated 3′-UTR sequences are shown in the schematic diagram. (B) Luciferase reporter assay was performed to detect the relative luciferase activities of WT and mut R-Ras or N-Ras reporters; * and ** indicate significant difference at $P < .05$ and $P < .01$, respectively. (C) Total proteins from U87 and U251 cells overexpressing miR-124 or miR-NC were subjected to immunoblotting to detect R-Ras and N-Ras expression levels. (D) The expression levels of R-Ras and N-Ras in normal brain tissues and glioma specimens were determined by immunoblotting; the fold changes were normalized to GAPDH. (E) Spearman’s correlation analysis was used to determine the corrections between the R-Ras/N-Ras expression and miR-124 levels in human glioma specimens. (Spearman’s correlation analysis, $r = -0.4701$, $r = -0.4597$, respectively.)
Fig. 3. MiR-124 overexpression regulates Ras signaling; R-Ras and N-Ras have synergistic effects on restoring miR-124–inhibited VEGF transcriptional activation. (A) The expression levels of c-Raf, Akt, ERK1/2, mTOR, and HIF-1α, the downstream molecules of R-Ras and N-Ras, were detected by immunoblotting analysis. (B) VEGF mRNA levels were determined by qRT-PCR, normalized to GAPDH level; * indicates significant difference at \( P < .05 \). (C) MiR-124 overexpression inhibits VEGF transcriptional activation in U87 cells. U87 cells were cotransfected with miR-124 or miR-NC, pRL-TK plasmid, pMAP11–VEGF WT reporter, or pMAP11 mut reporter. Luciferase activities were determined by the dual-luciferase reporter assay system and normalized to the miR-NC group. ** indicates significant difference at \( P < .01 \). (D) Forced expression of R-Ras and N-Ras restored miR-124–inhibited VEGF transcriptional activation. U87 cells were cotransfected with miR-124 or miR-NC, VEGF reporter, and pRL-TK plasmid, together with pCMV6 vector alone or with pCMV6–R-Ras and/or pCMV6–N-Ras plasmid at 500 ng (1/4 dose) or 1 μg (1/2 dose). The relative luciferase activities of VEGF reporter were assayed 48 h later. ** indicates significant difference compared with control (\( P < .01 \)); ### indicates significant difference compared with miR-124 treatment alone (\( P < .01 \)).
N-Ras expression levels were downregulated in U87 and U251 cells by overexpression of miR-124 (Fig. 2C). These results suggest that miR-124 directly targets R-Ras and N-Ras by binding its seed region to their 3′-UTRs in glioma cells.

To further determine the correlation between miR-124, R-Ras, and N-Ras levels, we measured the levels of R-Ras and N-Ras proteins in glioma specimens and normal brain tissues. The results showed that the average expression levels of both R-Ras and N-Ras were significantly higher in tumor tissues than in the normal brain tissues (Fig. 2D, S1A–B). Then, we determined the correlation between R-Ras and N-Ras levels and miR-124 expression levels in the same glioma tissues. As shown in Fig. 2E, Spearman’s correlation analysis demonstrated that both R-Ras and N-Ras levels in glioma samples were inversely correlated with miR-124 expression levels (Spearman’s correlation \( r = -0.4701 \) and \( -0.4597 \), respectively). Thus, lower levels of miR-124 in glioma are associated with induction of R-Ras and N-Ras, which may in turn induce tumorigenesis.

**MiR-124 Overexpression Inhibits Akt and Raf/ERK1/2 Signaling Pathways**

Activation of the R-Ras and N-Ras pathway has been well documented in various tumor types. Previous studies have shown that Akt and ERK1/2 pathways are major downstream effectors of R-Ras and N-Ras signaling in the regulation of cell proliferation and survival; and several downstream molecules, such as c-Raf, mTOR, HIF-1α, and VEGF, have been linked to this pathway. Therefore, we investigated whether the Akt and Raf/ERK1/2 pathways are affected by miR-124/R-Ras/N-Ras in glioma cells. In U87 and U251 cells stably expressing miR-124, the expression levels of c-Raf were significantly reduced compared with cells stably expressing miR-NC (Fig. 3A). As expected, the levels of p-Akt and p-ERK1/2 were also downregulated, while no significant reduction in total protein levels of Akt or ERK was detected. Meanwhile, we observed that levels of mTOR, HIF-1α, and VEGF were also downregulated and were associated with lower expression levels of p-Akt and p-ERK1/2 (Fig. 3A and B). Therefore, miR-124 can directly target R-Ras and N-Ras and subsequently suppress the Akt and ERK1/2 signaling pathways in glioma cells.

VEGF is a critical regulator of tumor angiogenesis and may be activated by HIF-1α. To determine whether miR-124 inhibits VEGF expression via HIF-1, we introduced a VEGF reporter containing an HIF-1 binding site and its mut counterpart as we described previously. As shown in Fig. 3C, forced expression of miR-124 inhibited VEGF WT but not mut reporter activities, suggesting that miR-124 inhibited VEGF transcriptional activation via the HIF-1 DNA binding site in the VEGF promoter region, and the mutation of the HIF-1α binding site abolished the inhibitory effect of miR-124.

**R-Ras and N-Ras Have Synergistic Effects to Restore MiR-124–inhibited VEGF Transcriptional Activation**

To better understand the synergistic effects of R-Ras and N-Ras, U87 cells were cotransfected with miR-124 or miR-NC, VEGF reporter, and pRL-TK plasmid, together with pCMV6 vector alone or with pCMV6–R-Ras and/or pCMV6–N-Ras cDNA plasmid, and subsequently subjected to luciferase activity analysis. As shown in Fig. 3D, transfection of R-Ras or N-Ras alone at low dose did not increase VEGF reporter activities, while the combination of R-Ras and N-Ras significantly restored VEGF transcriptional...
MiR-124 increases chemosensitivity of glioma cells to TMZ treatment, and overexpression of R-Ras and N-Ras partially abolished the apoptotic induction effect of miR-124 in the presence of TMZ. (A) Cell proliferation was evaluated in glioma cells stably expressing miR-NC or miR-124, with or without the TMZ treatments at different doses. CCK8 assay was performed 48 h after treatment. * and ** indicate significant difference at $P<0.05$ and $P<0.01$, respectively.
activation. We also observed that transfection of R-Ras or N-Ras cDNA alone at higher dose was sufficient to rescue miR-124–inhibiting VEGF transcriptional activation (Fig. 3D). These results indicated that miR-124 inhibited VEGF transcriptional activation through targeting of both R-Ras and N-Ras expression in the cells.

**Overexpression of R-Ras Reverses the Inhibitory Effects of MiR-124**

As shown above, R-Ras and N-Ras are direct targets of miR-124. To test whether overexpression of R-Ras reverses the expression of miR-124–inhibiting downstream molecules, expression vector containing R-Ras cDNA without a 3′-UTR was transfected into U87 cells overexpressing miR-124. As shown in Fig. 4A, the decreased level of c-Raf, a member of the Raf kinase family, was restored by overexpression of R-Ras. Similarly, forced expression of R-Ras also restored miR-124–suppressed Akt and ERK1/2 activation. Furthermore, overexpression of R-Ras in miR-124–treated cells rescued miR-124–inhibited HIF-1α and VEGF expression (Fig. 4A and B). These results indicate that miR-124 inhibits Akt and Raf/ERK1/2 pathways and downstream HIF-1α and VEGF expression via targeting R-Ras.

**Overexpression of MiR-124 Increases Chemosensitivity of Glioma Cells to Temozolomide and Its Apoptotic Induction Effect in the Presence of Temozolomide Through R-Ras and N-Ras**

To explore the potential role of miR-124 in chemotheraphy, we treated U87 and U251 cells stably expressing miR-124 or miR-NC with different concentrations of TMZ. As shown in Fig. 5A, overexpression of miR-124 in U87 and U251 cells significantly increased chemosensitivity to TMZ treatment, and cell viability was significantly suppressed by TMZ treatment with inverse correlation of the drug concentrations compared with miR-NC group cells. Furthermore, cell viability in the presence of TMZ (100 μM) was assayed by CCK8 at different time points. The results showed that overexpression of miR-124 significantly inhibited cell survival of both U87 and U251 cells in the presence of TMZ (Fig. 5B). In order to test whether miR-124 and its targets R-Ras and N-Ras may play a role in cell apoptosis in the presence of TMZ treatment, fluorescence activated cell sorting analysis was performed to detect cell apoptosis rates. The combination treatment of miR-124 + TMZ significantly induced cell apoptosis, whereas forced expression of R-Ras or N-Ras partially abolished the effect induced by miR-124 + TMZ treatment (Fig. 5C). Moreover, we found that the activity of caspase-3, a key executor of cell apoptosis, was significantly upregulated upon treatment by miR-124 + TMZ compared with miR-124 or TMZ treatment alone, whereas R-Ras or N-Ras overexpression attenuated the activation of caspase-3 induced by miR-124 + TMZ treatment (Fig. 5D). Similarly, overexpression of R-Ras or N-Ras in miR-124–overexpressing cells with TMZ treatment restored the expression of c-Raf (Fig. 5E). These results indicate that miR-124 renders glioma cells more sensitive to TMZ treatment and that the miR-124 + TMZ combination induces an apoptotic effect through targeting of R-Ras and N-Ras in the cells.

**MiR-124 Suppresses Tumor Angiogenesis in Nude Mice**

Our previous study demonstrated that miR-124 downregulated HIF-1α and VEGF expression levels that are important for angiogenesis as previously described.38 We further tested whether miR-124 may inhibit tumor angiogenesis in vivo. U87 cells stably expressing miR-124 or miR-NC were mixed with Matrigel and injected into both flanks of nude mice. Representative Matrigel plugs from the mice are shown in Fig. 6A, and hemoglobin levels of plugs were measured to analyze the relative angiogenesis responses. The angiogenesis responses in the miR-124 overexpression group were decreased by 60% compared with the negative control group (Fig. 6B). Meanwhile, the number of CD31-positive microvessels was also significantly inhibited by miR-124 overexpression in U87 cells (Fig. 6C and D).

**Overexpression of MiR-124 Inhibits Cell Proliferation, Invasion, and Tumor Growth**

Given the important role of R-Ras and N-Ras in the regulation of cell proliferation, migration, and invasion,39,40 miR-124–overexpressing U87 cells were used to analyze cell growth and invasion. The results showed that cell growth and invasion were attenuated in miR-124–overexpressing U87 cells compared with U87 cells expressing miR-NC (Fig. 7A and B). To test the role of R-Ras or N-Ras in cellular function, we showed that forced expression of R-Ras or N-Ras cDNAs restored miR-124–inhibited cell proliferation and invasion (Fig. 7A and B). To further clarify the inhibitory effect of miR-124 on tumor growth in vivo, we subcutaneously injected U87 cells stably expressing miR-124 or miR-NC into both flanks of nude mice. After 4 weeks, we found that tumor growth was significantly slower in mice injected with U87 cells overexpressing miR-124 than in mice with U87 cells overexpressing miR-NC. As early as 7 days postimplantation, the growth of tumor xenographs between 2 groups exhibited statistical significance (P = .008; Fig. 7C). In agreement with the tumor growth curve, the size of tumors from the miR-124 overexpression group was significantly smaller than that from the miR-NC overexpression group, showing 80% reduction of tumor weight (Fig. 7D and E). We also performed immunoblotting to detect the expression levels of R-Ras or N-Ras in randomly selected tumor tissues derived from miR-NC– or miR-124–overexpressing...
U87 cells. The tumors from the miR-124–overexpressing group expressed much lower levels of R-Ras or N-Ras than those from the control group (Fig. 7F). Thus, our results suggest that overexpression of miR-124 suppresses cell proliferation, invasion, and tumor growth by inhibiting its targets R-Ras and N-Ras.

Discussion

MicroRNAs, a class of small regulatory RNAs, have been demonstrated to play important roles in a wide variety of oncogenic activities, such as proliferation, invasion, and angiogenesis. Dysregulated miRNAs have been observed in various kinds of tumors, including brain tumors such as glioma and its aggressive glioblastoma subtype. Accumulating data indicate that miRNAs are involved in advanced stages of cancer progression and may act as activators or suppressors of tumorigenesis. In our study, we found that miR-124 was downregulated in human glioma tissues compared with normal brain tissues. Moreover, we demonstrated that overexpression of miR-124 in glioma cells led to the downregulation of p-Akt, ERK1/2, and the angiogenesis inducer VEGF via directly targeting the 3′-UTRs of R-Ras and N-Ras.

As members of the Ras family, R-Ras and N-Ras have been recognized as oncogenes. It has been reported that the expression levels of R-Ras and N-Ras are related to the malignant degree of cancers, including glioma, breast cancer, and melanoma, among others. Recently, accumulating evidence has indicated that expression levels of the Ras family can be regulated by miRNAs. For example, overexpression of Let-7 can inhibit lung tumor cell line growth, and the expression of the Ras oncogene is regulated by Let-7. A recent study found that miR-124 directly targets N-Ras and pim-3 in glioblastoma stem cells, but there is a lack of further functional analysis of miR-124. Our present study confirmed that R-Ras as well as N-Ras were downregulated by miR-124 not only in vitro, but also in vivo. Furthermore, R-Ras and N-Ras were upregulated in glioma tissue specimens, and the increased levels of R-Ras and N-Ras protein expression levels were inversely correlated with the decreased miR-124 levels, further confirming that R-Ras and N-Ras were downregulated by miR-124 in clinical glioma tumor tissues.

R-Ras and N-Ras are known to interact with a number of effectors, including c-Raf, PI3K/Akt, and ERK1/2, and to play important roles in cancer cell growth and tumorigenesis. Our results show that miR-124 acts as a tumor suppressor through various mechanisms, including inhibition of tumor cell growth, invasion, angiogenesis, and suppression of the Ras/Akt and Ras/Raf/ERK1/2 signaling pathways. Furthermore, we found that VEGF levels were markedly downregulated in miR-124–overexpressing glioma cancer cells, while forced expression of R-Ras restored miR-124–inhibited VEGF transcriptional activation. Angiogenesis is a key process in tumor growth. Here, we report that miR-124 functions as an antiangiogenic regulator in glioma tumorigenesis. Overexpression of miR-124 in glioma cells led to a reduced number of microvessels and impaired tumor growth in a tumor xenograft. Meanwhile, overexpression of miR-124 enhanced the chemosensitivity of human glioma cells to TMZ treatments, and miR-124 induced glioma cell apoptosis through targeting R-Ras and N-Ras when the cells were treated by TMZ. Thus, the miR-124 + TMZ combination could be an effective therapeutic strategy for suppressing the growth of glioma.

In summary, the present study provides novel evidence of an important link between miR-124 and tumorigenesis in human glioma.
Fig. 7. Overexpression of miR-124 inhibits cell growth, invasion, and tumor growth in vivo. (A) U87 cells stably expressing miR-NC or miR-124 were transfected with pCMV vector, pCMV-R-Ras, or pCMV-N-Ras cDNA plasmid; CCK8 proliferation assay was performed every 24 h. MiR-124 overexpression decreased U87 cell growth, while overexpression of R-Ras or N-Ras restored miR-124–inhibition effect. (B) Transwell invasion assay of U87 overexpressing miR-124 cells with or without R-Ras or N-Ras overexpression. After being fixed, stained, and photographed, the cells in the bottom of the invasion chamber were measured by the absorbance at 570 nm. Scale bar, 20 μm. ** and ## indicate significant difference compared with control and miR-124 treatment alone, respectively (P < .01). (C) U87 cells stably expressing miR-124 or NC were injected subcutaneously into nude mice. Each treatment group contained 8 tumors, and the tumor volumes were measured after 7 days, when the xenografts were visible. (D) On day 23, xenografts were removed from the nude mice. The photograph shows the representative tumors from each graph. Scale bar, 2 mm. (E) Tumors from 2 groups were weighed and analyzed; ** indicates significant difference at P < .01. (F) The levels of R-Ras and N-Ras from the tumor tissues in 2 groups were analyzed by immunoblotting.
glioma. Our findings suggest that miR-124 functions as a tumor suppressor by negatively regulating R-Ras and N-Ras expression. MiR-124 impairs tumor growth and angiogenesis through inhibiting Ras/Akt and Ras/Raf/ERK1/2 pathways at multiple levels. Interestingly, we demonstrate that miR-124 renders glioma cells more sensitive to TMZ treatment. Although miRNA-based therapeutics are still in the initial stages of development, our findings are encouraging and suggest the potential of miR-124 as a diagnostic/prognostic marker and novel therapeutic target for glioma in the future.

**Conflict of interest statement.** None declared.

## References


