European Review for Medical and Pharmacological Sciences 2015; 19: 3593-3604

Overexpression of miR-98 inhibits cell invasion in glioma cell lines via downregulation of IKKε

Y.-H. FAN, M.-H. YE, L. WU, S.-G. LV, M.-J. WU, B. XIAO, C.-C. LIAO, Q.-K. JI, Y. CHAI, X.-G. ZHU

Department of Neurosurgery, The Second Affiliated Hospital, Nanchang University, Nanchang, Jiangxi, People's Republic of China

Yang-hua Fan, *Min-hua Ye* and *Lei Wu* contributed equally to this wor

Abstract. – OBJECTIVE: MicroRNAs (miRNAs) function as negative regulators for the expression of genes involved in cancer metastasis. The aim of this study was to investigate the potential role of miR-98 in gliomas and validate its regulatory mechanism.

PATIENTS AND METHODS: Cell viability assays are used to measure proliferation of cell. mRNA expression is measured by qRT-PCR. Western blot analysis is used to measure protein expression.

RESULTS: Functional studies showed that miR-98 overexpression inhibited glioma migration and invasion, but had no effect on the cell viability. An enhanced green fluorescent protein reporter assay, quantitative RT-PCR, and a western blot analysis confirmed that miR-98 suppressed the expression of IκB kinase (IKKε) by directly targeting its 3′-untranslated region, also, the NF-κB p65 nuclear translocation and matrix metalloproteinase (MMP)-9 expression were significantly arrested in glioma cells treated with miR-98 mimics. Accordingly, the overexpression of IKKε or NF-κB p65 can restore cell migration and invasion after being inhibited by miR-98, and can restore NF-κB p65 nuclear translocation as well as increase MMP-9 expression.

CONCLUSIONS: These findings demonstrated that miR-98 functions as a tumor suppressor in gliomas. Furthermore, miR-98 may act as a potential therapeutic biomarker for glioma patients.

Key Words:
miR-98, IKKε, NF-κB p65, Glioma.

Introduction

Glioma is the most common and lethal type of intracranial tumor in adults[1]. Despite major therapeutic improvements based on combinations of neurosurgery, chemotherapy, and radiotherapy techniques, the prognosis and survival rate of glioma patients are still extremely poor[2], especially for the glioblastoma multiforme patients (GBM, World Health Organization grade IV astrocytoma). The median survival time is only 12-15 months for patients with GBM[3]. The poor prognosis in glioma patients is largely attributed to its invasiveness and high rate of recurrence[4]. Therefore, it is essential to investigate the mechanisms involved in glioma initiation and progression.

MicroRNAs (miRNAs) are a family of small non-coding, endogenous, single RNA molecules that play important roles in gene expression by binding to the 3′-untranslated regions (3′-UTRs) of target gene mRNA, leading to mRNA cleavage or translational repression[5]. They have a profound impact on many processes that are frequently disrupted during malignant transformation, including cell proliferation, apoptosis, stress responses, maintenance of stem cell potency, and metabolism[6]. To date, approximately 60% of protein-coding genes have been found to be regulated by miRNAs[7]. Approximately 50% of miRNAs are located in fragile sites or cancer-related gene regions[8], suggesting that abnormal miRNA expression is related to cancer pathogenesis. For instance, miR-324 inhibits glioma cell proliferation via GLI1, functioning as a tumor suppressor[9]. Additionally, miR-221 promotes the tumorigenesis of hepatocellular carcinoma *in vitro* and *in vivo*[10,11]. Recently, researchers have discovered that miR-98 is dysregulated in many tumors, such as lung cancer[12], breast cancer[13], and esophageal squamous cell carcinoma[14]. However, little is known about the role of miR-98, especially its regulatory mechanism in glioma cells.

In the present study, we found that miR-98 plays an anti-invasion role by inhibiting glioma cell migration and invasion. Furthermore, we determined that the IκB kinase IKKε is a direct target of miR-98 in glioma cells.

Corresponding Author: Xin-Gen Zhu, MD; e-mail: zxg2008vip@163.com

Patients and Methods

Patients

Patients with glioma (n = 53) who underwent an initial surgery at The Second Affiliated Hospital of Nanchang University between 2010 and 2013 were retrospectively selected for this study. No patients had received therapy before resection. The normal brain tissues (n = 10) were obtained from epileptic resections. Each patient participated after providing informed consent and the use of the samples for research was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Nanchang University. All tumors were classified based on the WHO criteria for tumors of the central nervous system and were quick frozen at the time of resection until analysis. The study consisted of 23 low-grade astrocytoma (WHO grade II) samples and 30 high-grade samples [including 11 cases of anaplastic astrocytoma (WHO grade III) and 19 cases of glioblastoma multiforme (WHO grade IV)]. The clinical characteristics of the glioma patients are listed in Table I.

Cancer Cell Lines

The human glioma cell lines U87 and U251 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO_2.

Table I. Distributions of characteristics among glioma patients (n = 53) included in the study.

Characteristics	No. of cases	
	N	(%)
Gender		
Male	29	(54.7)
Female	24	(45.3)
Age (years)		
≤ 50	31	(58.5)
> 50	22	(41.5)
Body mass index		
< 20	10	(18.9)
20-28	37	(69.8)
> 28	6	(11.3)
Family history		
Positive	7	(13.2)
Negative	46	(86.8)
Smoking		
Positive	33	(62.3)
Negative	20	(37.7)
Drinking		
Positive	29	(54.7)
Negative	24	(45.3)
WHO grade		
I/II	23	(43.4)
III/IV	30	(56.6)

Cell Transfection

The miR-98 mimics and their negative controls, pcDNA3/IKKε, pcDNA3/NF-κB p65, and control vector were purchased from RiboBio Company (Guangzhou, China). Transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. At 48 h after transfection, the cells were harvested for subsequent experiments.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA was extracted from glioma tumor tissue, adjacent normal tissue, and glioma cell lines using the Trizol Total RNA Reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) synthesis was performed with 2 μg of total RNA using the RevertAid™ HMinus First Strand cDNA Synthesis Kit (Takara, Otsu, Shiga, Japan). The miR-98 primers were obtained from RiboBio Company, and RNU6B (U6 small nuclear B non-coding RNA) was used as an internal control. The U6 RT primer sequence was 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAAC-3′. The *IKKε* primer sequences were as follows: forward primer 5′-TGCGTGCAGAAGTATCAAGC-3′ and reverse primer 5′-TACAGCAGCCACAGAACAG-3′. The matrix metalloproteinase-9 (*MMP-9*) primer sequences were as follows: forward primer 5'-TTGGTCCACCTGGTTCAACT-3′ and reverse primer 5′-ACGACGTCTTCCAGTACCGA-3′. Quantitative PCR was performed using the SYBR PrimeScript RT-PCR Kit (Takara) using the Applied Biosystems 7300 Fluorescent Quantitative PCR System (Foster City, CA, USA). The reaction mixtures were incubated at 95°C for 30 s, followed by 40 amplification cycles of 95°C for 5 s and 60°C for 34 s. Gene expression was quantified using the $^{\Delta\Delta}$CT calculation where CT was the threshold cycle.

Cell Viability Assays

Transfected cells were plated on a 96-well plate at a density of 2,000 cells per well. At 48h after the transfection, 20 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide] was added to each well and incubated for 4 h at 37°C in a CO_2 incubator. Dimethyl sulfoxide (DMSO) (200 µl) was added to each well and the optical density was measured at 570 nm. The data are presented as means ± SD from triplicate samples of three independent experiments.

Transwell Invasion Assays

The migration and invasion assays were performed using a Transwell chamber (Millipore, Billerica, MA, USA). For the migration assay, the transfected cells were seeded in the upper chamber with serum-free medium (1×10^5 cells), and the lower chamber contained Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. For the invasion assay, the chamber was coated with Matrigel. After 24 h of cell migration or invasion, the cells were fixed and stained with hematoxylin and eosin. The invading cells were photographed under a microscope and the cell numbers were counted.

Wound Healing Assays

U87 and U251 cells were seeded in 6-well plates (2×10^5 cells/well). At 48 h after transfection (~90% confluency), a wound area was carefully created by scraping the cell monolayer with a sterile 10 µl pipette tip, washed with phosphate-buffered saline (PBS) to remove detached cells, and supplemented with fresh growth media. Cells were then incubated at 37°C and the width of the wound area was monitored with an inverted microscope at various time points. The normalized wound area was calculated using TScratch software.

Enhanced Green Fluorescent Protein Reporter Assay

The *IKKε* 3′-UTR was amplified and inserted in the downstream region of the pcDNA3/EGFP vector. The mutant *IKKε* 3′-UTR was amplified using the pcDNA3/EGFP-*IKKε* 3′-UTR as a template. The cells were co-transfected with miR-98 mimics and the wild-type or mutant *IKKε* 3′-UTR, and the construct expressing the fluorescent protein was used as the spiked-in control. At 48 h after transfection, the cells were lysed with RIPA buffer and the fluorescence intensity was measured using an ELX800 microplate reader.

Western Blot Analysis

The cells were harvested at 48 h after transfection and lysed with RIPA buffer for 30 min at 4°C. Nuclear NF-κB p65 protein sub-fractions were prepared using a commercial extraction kit (Active Motif, Tokyo, Japan) according to the manufacturer's instructions. The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The protein (20 µg) was analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The first antibodies were polyclonal rabbit anti-IKKε (1:800 dilutions; Cell Signaling Technology, Beverly, MA, USA), anti-NF-κB p65 antibody (1:1000 dilution; Boster, Wuhan, China), and anti-β-actin antibody (1:5000 dilution; Abcam, San Francisco, CA, USA). The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase at a dilution of 1:1000. The bound antibodies were detected using the Enhanced Chemiluminescence Plus Western Blotting Detection System (GE Healthcare, Bethesda, MD, USA). β-actin was used as an internal control to normalize IKKε and MMP-9 expression levels. Histone H3 was used as a marker for the nuclear loading control.

Statistical Analysis

All statistical analyses were implemented in the SPSS 20.0 statistical software package. All data are presented as means ± standard deviations (SD) and the experiments were repeated three times. All data were statistically analyzed using a one-way analysis of variance with a Bonferroni correction. Bivariate correlations were calculated based on Spearman's rank correlation coefficients. A p-value < 0.05 was considered statistically significant.

Results

Significant Differences in miRNA-98, IKKε, and NF-κB P65 Expression Between Resected Glioma Tissues and Normal Brain Tissues

We examined the expression of miRNA-98 by quantitative RT-PCR and that of IKKε and NF-κB P65 by western blot analyses using resected glioma tissues and normal brain tissues. We detected relationships among these factors. Consistent with the results of previous reports, we found a significant decrease in miRNA-98 expression (Figure 1A, $F = 381.1$; $p < 0.0001$) and significant increases in *IKKε* (Figure 1B, $F = 88.88$; $p < 0.0001$) and NF-κB P65 (Figure 1C, $F = 138.9$; $p < 0.0001$) expression in the resected glioma tissues compared with normal brain tissues.

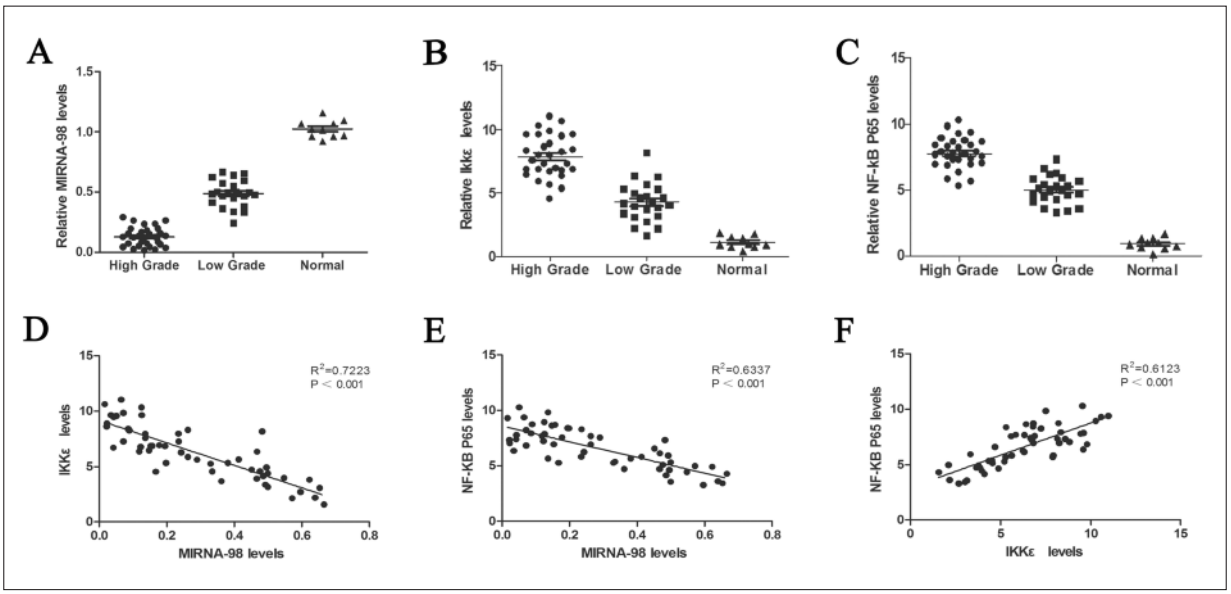

Figure 1. miRNA-98 levels were negatively correlated with IKKε and NF-κB P65 levels in patient gliomas. ***A,-C,*** Examination of miRNA-98 levels by quantitative RT-PCR ***(A)*** and IKKε ***(B)*** and NF-κB p65 levels ***(C)*** by western blots for resected glioma tissues and normal brain tissues. ***D,-F,*** Correlation tests for miRNA-98, IKKε, and NF-κB P65 levels in the resected glioma tissues from 53 patients. ***D,*** A strong negative correlation was detected between miRNA-98 and IKKε expression ($R^2 = 0.7223$; $p < 0.001$). ***E,*** A strong negative correlation was detected between miRNA-98 and NF-κB P65 expression ($R^2 = 0.6337$; $p < 0.001$). ***F,*** A strong positive correlation was detected between IKKε and NF-κB P65 expression ($R^2 = 0.6123$; $p < 0.001$).

Strong Correlations Between miRNA-98, IKKε, and NF-κB P65 were Detected in the Resected Glioma Tissues

We examined the causal link between miRNA-98, IKKε, and NF-κB P65 expression levels, which may be positively correlated with cancer invasiveness. We performed correlation tests to examine the relative miRNA-98, IKKε, and NF-κB P65 expression levels in resected glioma tissue samples obtained from 53 patients. Strong negative correlations were detected between miRNA-98 and IKKε expression (Figure 1D, $R^2 = 0.7223$; $p < 0.001$) and between miRNA-98 and NF-κB P65 expression (Figure 1E, $R^2 = 0.6337$; $p < 0.001$), while a strong positive correlation was detected between IKKε and NF-κB P65 expression (Figure 1F, $R^2 = 0.6123$; $p < 0.001$), suggesting that NF-κB P65 levels are positively affected by IKKε activation and negatively controlled by miRNA-98 in gliomas.

IKKε Was a Direct Target of miR-98 in U87 and U251 Cells

We extended our test to one human glial cell line and two human glioma cell lines. These cell lines showed much lower miR-98 expression levels and higher IKKε expression levels than those of control human glial cells (Figure 2A-2B). In addition, three bioinformatics tools, TargetScan, miRanda, and miRwalk, were used to predict the miR-98 target genes that mediated the anti-metastatic effects of miR-98 in U87 and U251 cells. Considering the functions of potential target gene and miR-98 binding sites, IKKε was identified as a candidate target. To confirm the direct binding sites of IKKε and miR-98, we constructed an enhanced green fluorescent protein (EGFP) reporter gene with the *IKKε* 3′-UTR and the mutant *IKKε* 3′-UTR. The sequences are shown in Figure 2C. We performed an EGFP reporter assay to detect the effects of miR-98 on GFP intensity controlled by the *IKKε* 3′-UTR or mutant 3′-UTR. We found that miR-98 reduced *IKKε* expression intensity by approximately 40% (Figure 2D), but had no effect on the mutant *IKKε* 3′-UTR (Figure 2E). Overall, these results indicate that miR-98 suppresses *IKKε* expression via direct binding to its 3′-UTR.

miRNA-98 Overexpression Inhibits Cell Migration and Invasion n U87 and U251 Cells

We transfected U87 and U251 cells with miR-98 mimics or negative controls to investigate the

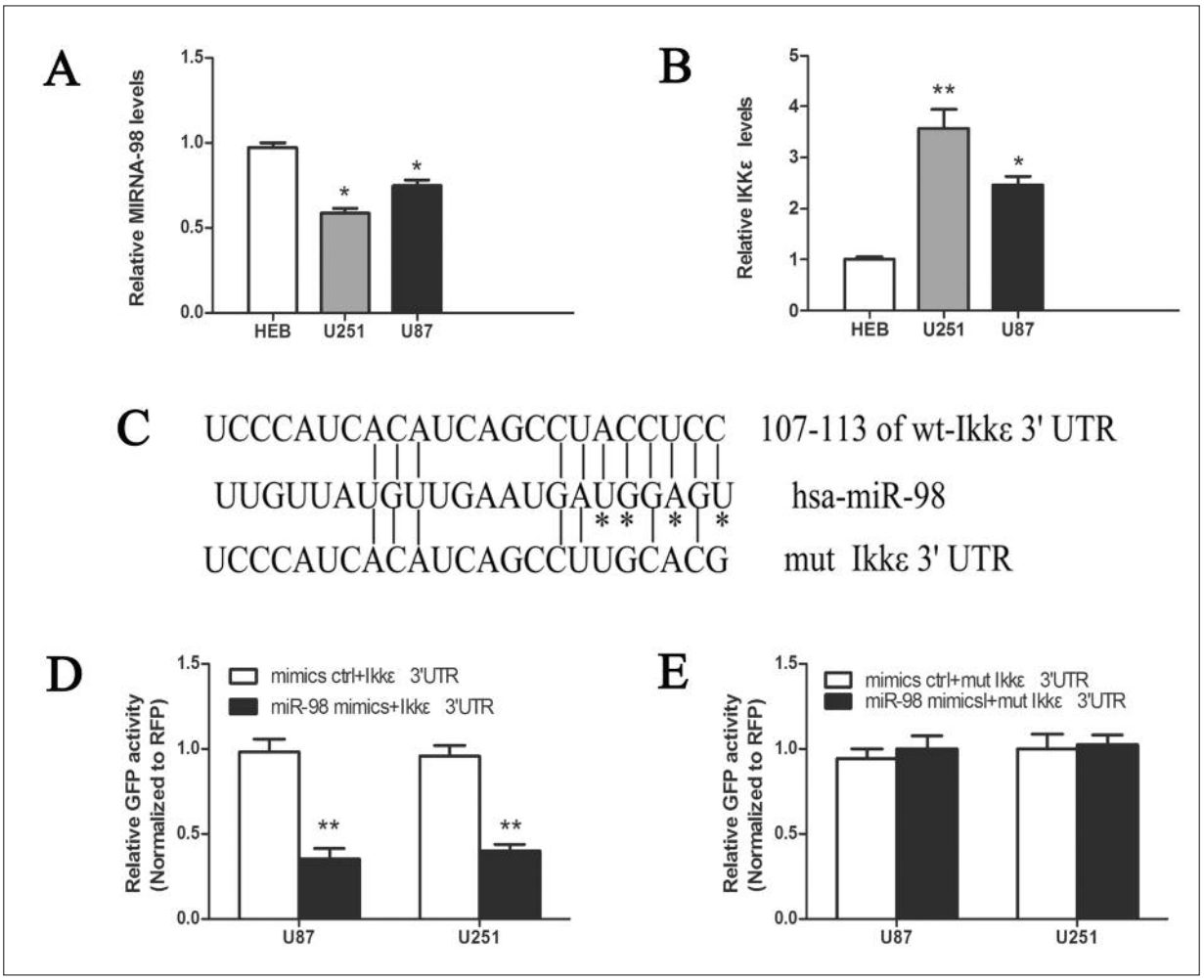

Figure 2. MiR-98 inhibits IKKε expression by directly targeting its 3′-UTR. ***A,*** Decreased expression of miR-98 was examined by quantitative RT-PCR in glioma cells compared to expression in the human glial cell line HEB. ***B,*** Increased expression of IKKε was examined by quantitative RT-PCR in glioma cells compared to expression in the human glial cell line HEB. ***C,*** The predicted miR-98 binding site within the IKKε 3′-UTR and its mutated version by site mutagenesis are shown. ***D,-E,*** Cells were co-transfected with miR-98 mimics and wild-type or mutant IKKε 3′-UTR, and the GFP intensity was measured by the EGFP reporter assay. *$p < 0.05$, **$p < 0.01$.

function of miR-98. The expression of miR-98 was analyzed by quantitative RT-PCR. The cells transfected with miR-98 mimics had a higher miR-98 expression level compared to the cells transfected with negative controls (Figure 3A). Based on the MTT assay, miR-98 overexpression had no effect on cell growth in U87 or U251 cells. Furthermore, we compared the invasion abilities of the transfected cells. As shown in Figure 3B, miR-98 overexpression reduced the number of invading cells by about 50% in U87 and U251 cells compared with the negative controls based on Transwell assays. Consistent with the cell invasion results, cell migration was also inhibited by miR-98 in U87 and U251 cells based on wound healing assays (Figure 3C). Since migration and invasion are two essential aspects for cancer cell metastasis, these data suggested that miR-98 plays an important role in inhibiting glioma metastasis.

miR-98 Inhibits IKKε Expression

To determine if the expression of IKKε is regulated by miR-98, we performed quantitative RT-PCR and western blot analyses in miR-98 mimic- or control-transfected U87 and U251 cells. As shown in Figure 4A-4B, miR-98 overexpression suppressed IKKε expression at both the mRNA and protein levels compared with the controls.

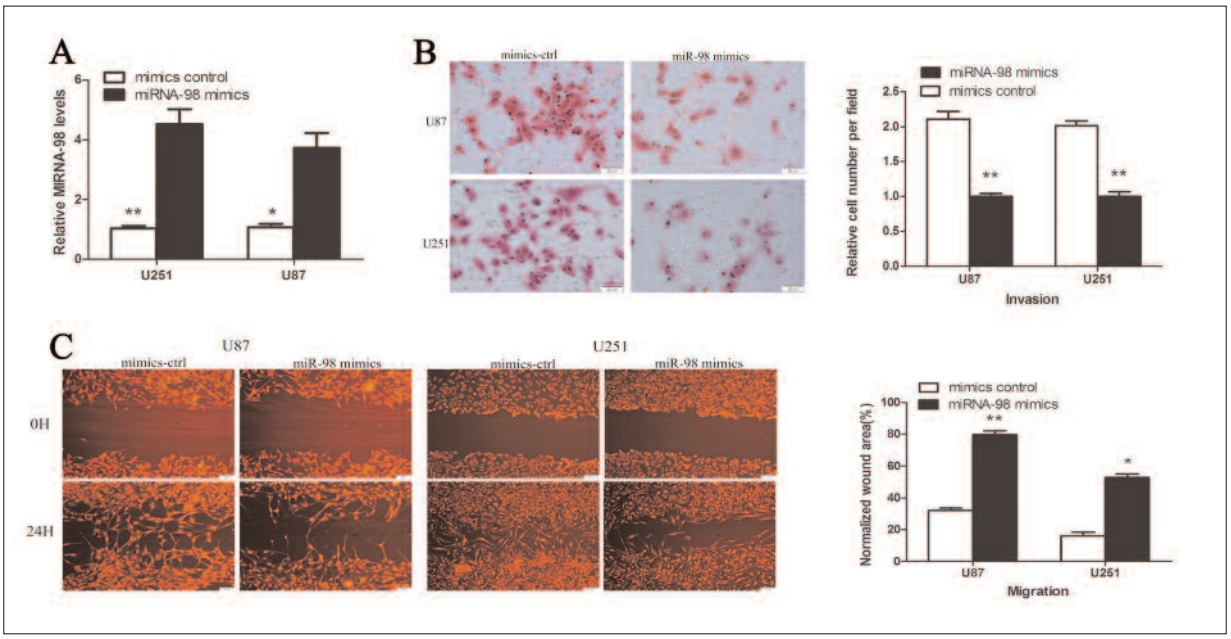

Figure 3. MiR-98 suppresses cell migration and invasion in U87 and U251 glioma cell lines. ***A,*** Quantitative RT-PCR of miR-98 expression in U251 or U87 cells transfected with miR-98 mimics or controls; U6 was used as an internal control. ***B,*** Cell invasion assays using Transwell-coated membranes with Matrigel. A significant reduction in invasion was observed after increasing miR-98 expression in U87 and U251 cells. ***C,*** miR-98 overexpression inhibited the migration of U87 and U251 cells. Compared with cells transfected with control mimics, cells treated with miR-98 mimics showed a wider wound area at 24 h after wound generation. The normalized wound area was calculated using the software Tscratch. The relative levels of the controls in all experiments were set at 1. $*p < 0.05$, $**p < 0.01$.

Overexpression of miR-98 Impairs Nuclear Translocation of NF-κB and Affects its Downstream targets

To determine whether the overexpression of miR-98 affects constitutive NF-κB activity in glioma cells, we analyzed the expression of NF-κB p65 in total cells and nuclear extracts of transfected cells by western blot analyses. As shown in Figure 4D, nuclear translocation of the NF-κB p65 protein was markedly lower in both U87 and U251 cells treated with miR-98 mimics than in control cells. However, total cell NF-κB p65 protein expression for both U87 and U251 did not differ between miR-98-treated and control cells (Figure 4A). These results indicated that increased miR-98 impairs NF-κB p65 nuclear translocation, and does not reduce the overall quantity of NF-κB.

Overexpression of miR-98 in glioma cells reduced expression of the NF-κB target gene *MMP-9* at the mRNA (Figure 4C) and protein levels (Figure 4A). Our results demonstrated that miR-98 modulates the expression of NF-κB target genes by impairing NF-κB p65 nuclear translocation. Since the target gene *MMP-9* has an important role in promoting cancer cell metastasis, these data also suggested that miR-98 plays an important role in inhibiting glioma metastasis.

IKKε Overexpression Rescues Cell Migration and Invasion in U87 and U251 Cells Inhibited by miR-98

To clarify the important role of miR-98 in the inhibition of cell migration and invasion via the regulation of Ikkε, as opposed to other genes, we performed a rescue experiment by co-transfecting cells with miR-98 mimics and pcDNA3/IKKε. IKKε was overexpressed in pcDNA3/IKKε-transfected cells based on quantitative RT-PCR and western blots (Figure 5A-5B). In addition, we found that IKKε restored cell migration and invasion abilities that were reduced by miR-98 mimics (Figure 6A-6B). Our results suggested that IKKε plays an important role in glioma metastasis reduction, and miR-98 overexpression inhibits cell migration and invasion in U87 and U251 cells via reduced IKKε expression.

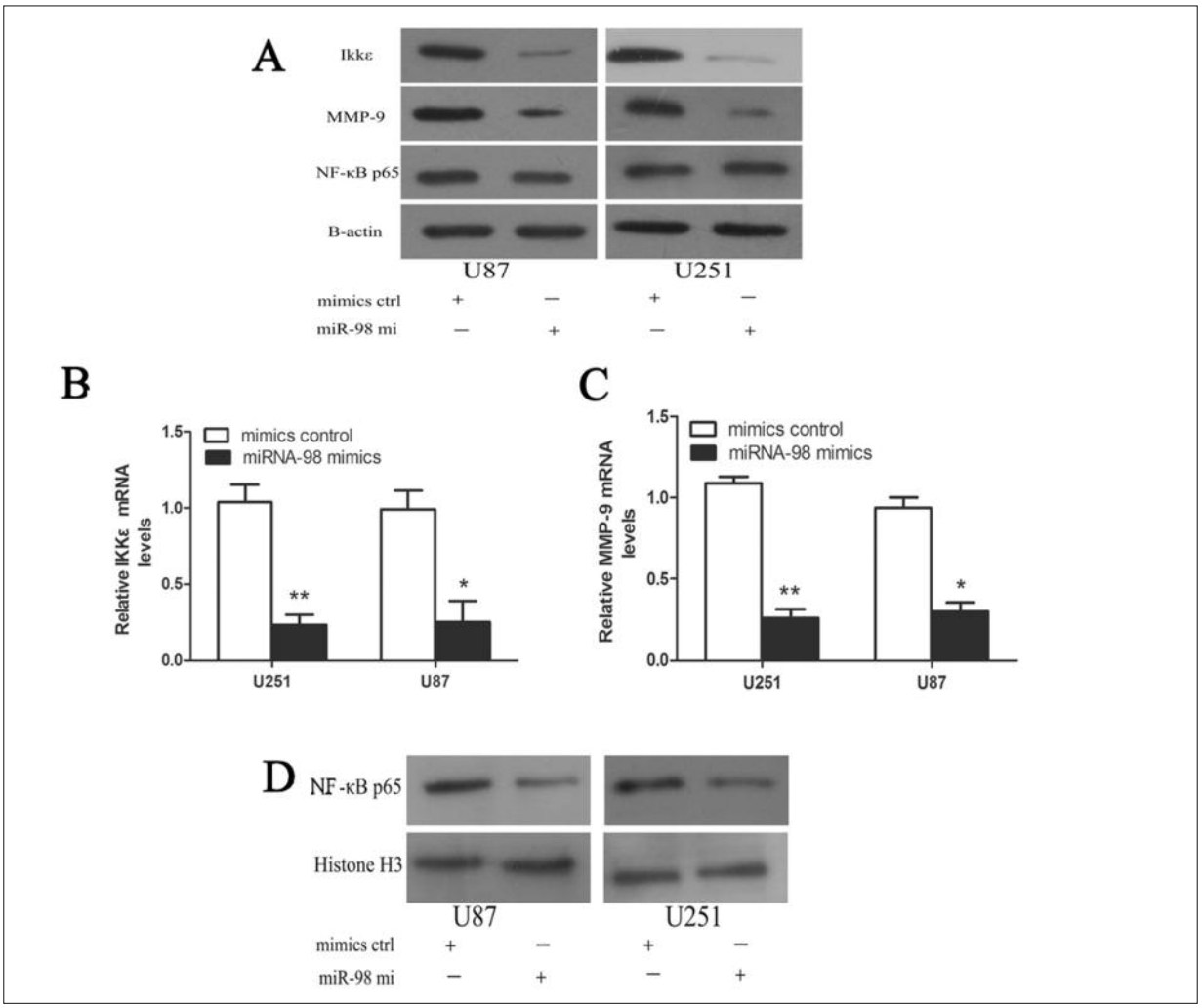

Figure 4. Overexpression of miR-98 expression inhibits IKKε expression and inactivates the nuclear translocation of NF-κB p65 in U87 and U251 cells. ***A,*** The expression levels of IKKε, MMP-9, and NF-κB p65 were evaluated using western blots in U87 and U251 cells transfected with miR-98 mimics or controls. β-actin was used as an internal control. ***B,*** The expression of IKKε was evaluated by quantitative RT-PCR in U87 and U251 cells transfected with miR-98 mimics or controls. ***C,*** The expression of MMP-9 was evaluated by quantitative RT-PCR in U87 and U251 cells transfected with miR-98 mimics or controls. ***D,*** NF-κB p65 expression was analyzed in nuclear extracts of U87 and U251 cells by western blotting. Histone H3 was used as a marker for the nuclear loading control. $*p < 0.05$, $**p < 0.01$.

IKKε Overexpression Rescues Nuclear Translocation of NF-κB and Affects its Downstream Targets

To determine whether miR-98 affects NF-κB activity in glioma cells via IKKε, we analyzed the expression of NF-κB p65 in nuclear extracts of transfected cells by western blot analyses. As shown in Figure 5D, the nuclear translocation of the NF-κB p65 protein was restored in both U87 and U251 cells treated with pcDNA3/IKKε compared with the control. In addition, overexpression of IKKε in U87 and U251 cells upregulated the expression of the NF-κB target gene *MMP-9* at the mRNA (Figure 5A) and protein levels (Figure 5C). Our results demonstrated that IKKε expression activates the NF-κB signaling pathway, which was inhibited by miR-98.

NF-κB p65 Overexpression Rescues Cell Migration and Invasion in U87 and U251 Cells Inhibited by miR-98

To verify that NF-κB p65 is the target of IKKε regulation via miR-98, we performed a rescue experiment by co-transfecting cells with miR-98 mimics and pcDNA3/NF-κB p65. Figure 7A shows that NF-κB p65 was overex-

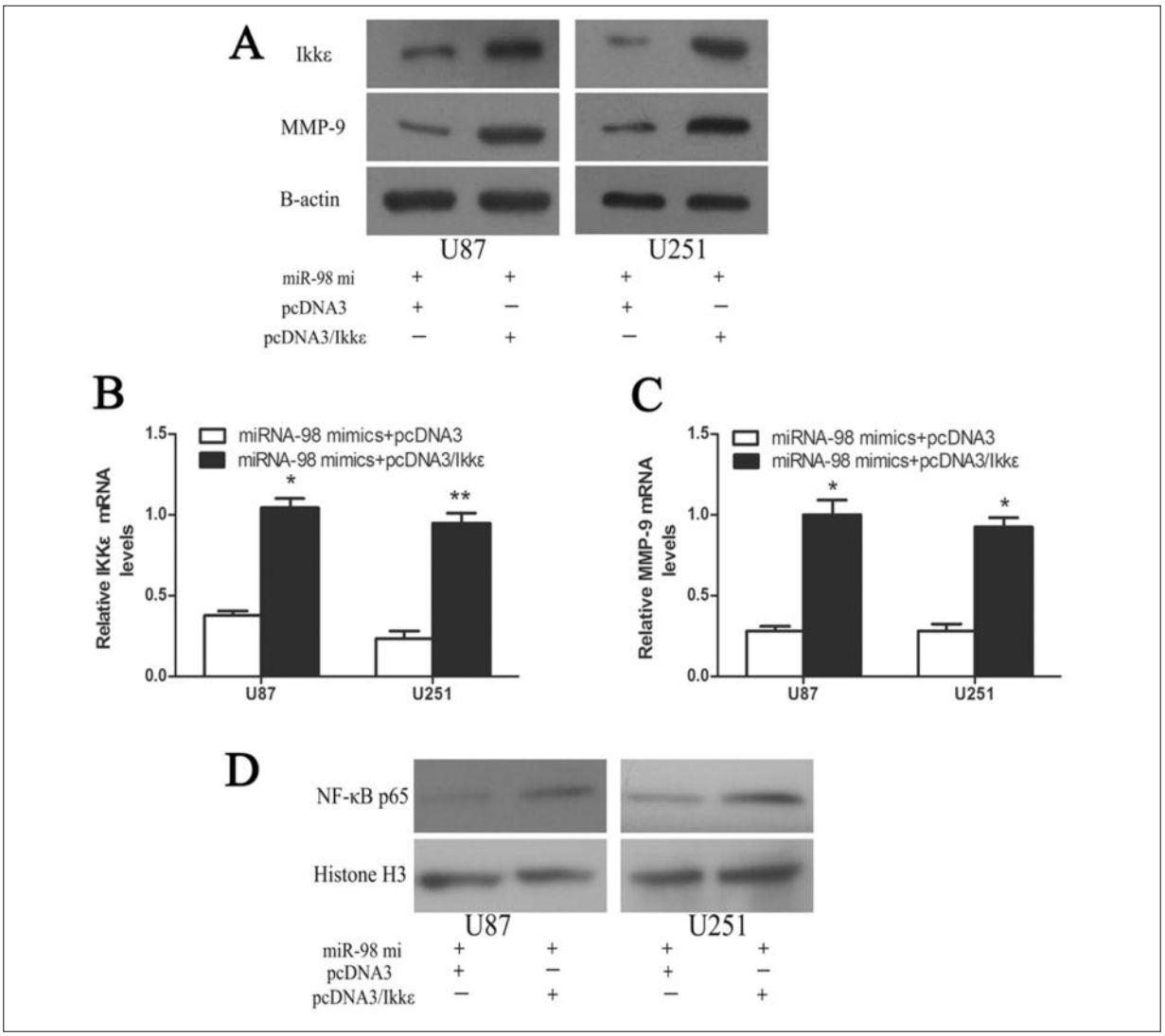

Figure 5. Overexpression of IKKε expression promotes NF-κB signaling in U87 and U251 cells. ***A,*** The expression levels of IKKε and MMP-9 were evaluated by western blot analyses in U87 and U251 cells transfected with co-transfecting miR-98 mimics and pcDNA3/IKKε or controls. β-actin was used as an internal control. ***B,*** The expression of IKKε was evaluated by quantitative RT-PCR in transfected U87 and U251 cells or controls. ***C,*** The expression of MMP-9 was evaluated by quantitative RT-PCR in transfected U87 and U251 cells or controls. ***D,*** NF-κB p65 expression was analyzed in nuclear extracts of U87 and U251 cells by western blotting. Histone H3 was used as a marker for the nuclear loading control. $*p < 0.05$, $**p < 0.01$.

pressed in pcDNA3/NF-κB p65-transfected cells. We found that NF-κB p65 restored cell migration and invasion abilities that were inhibited by miR-98 (Figure 7B-7C). These data supported the notion that miR-98 contributes to the regulation of the NF-κB signaling pathway via IKKε regulation.

Our results demonstrated that miR-98 modulates NF-κB signaling and its target genes via IKKε regulation, which is related to glioma cell migration and invasion.

Discussion

Understanding the molecular mechanisms of cancer development is important for the establishment of effective therapies. Aberrant miRNA expression occurs frequently in human cancers. Therefore, it is crucial to explore the function of deregulated miRNAs in cancers. Previous studies have shown that miR-98 is involved in cancer. Du et al[15] demonstrated that miR-98 is a negative regulator of Fus1 expression in lung cancers and

might play a critical role in the development of cancer by regulating the expression of multiple cancer-related proteins. Hebert et al[16] showed that miR-98 targets HMGA2 in head and neck squamous cell carcinoma cells. miR-98 inhibits breast cancer cell angiogenesis and invasion by targeting activin receptor-like kinase-4 and MMP-11[13]. Huang et al[14] showed that miR-98 overexpression regulates enhancer of zeste homolog 2 and inhibits migration and invasion in human esophageal squamous cell carcinoma. Surely, each miRNA has many targets that differ in expression levels. In this study, we found that miR-98 inhibited cell migration and invasion, functioning as a tumor suppressor in U87 and U251 cells. Furthermore, we determined that IKKε is the target of miR-98 in this process.

Many miRNAs play tumor suppressive or oncogenic roles via binding to the 3′-UTRs of target genes; accordingly, exploring the targets of miR-98 in gliomas is crucial to understand its regulatory mechanism. In this study, we used a bioinformatics approach to predict target genes. Considering the overlap of genes identified by TargetScan, miRanda, and miRwalk, we acquired a few candidates. In addition, we considered the potential functions of each candidate. Based on these analyses, we selected Ikkε as a target for further validation. We found that Ikkε was upregulated in glioma tissues and miR-98 overexpression reduced IKKε expression at both the mRNA and protein levels, suggesting that miR-98 negatively regulates IKKε. We performed an EGFP reporter assay, which is a direct method for target

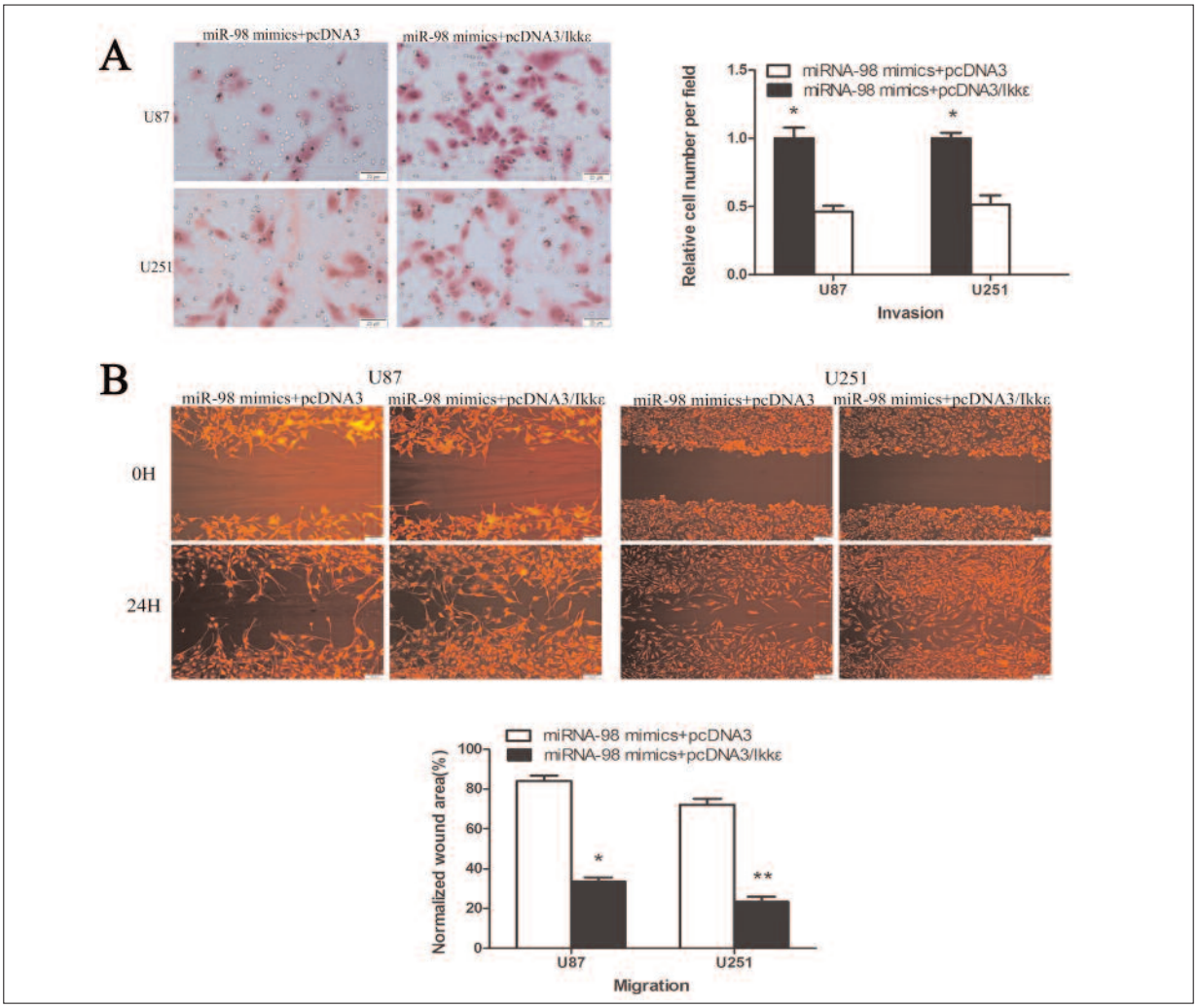

Figure 6. Overexpression of IKKε restores U87 and U251 cell migration and invasion reduced by miR-98. Cells were transfected with pcDNA3/IKKε and controls. ***A, B,*** Cells were co-transfected with miR-98 and IKKε, and then invasion ***(A)*** and migration ***(B)*** assays were performed. *$p < 0.05$, **$p < 0.01$.

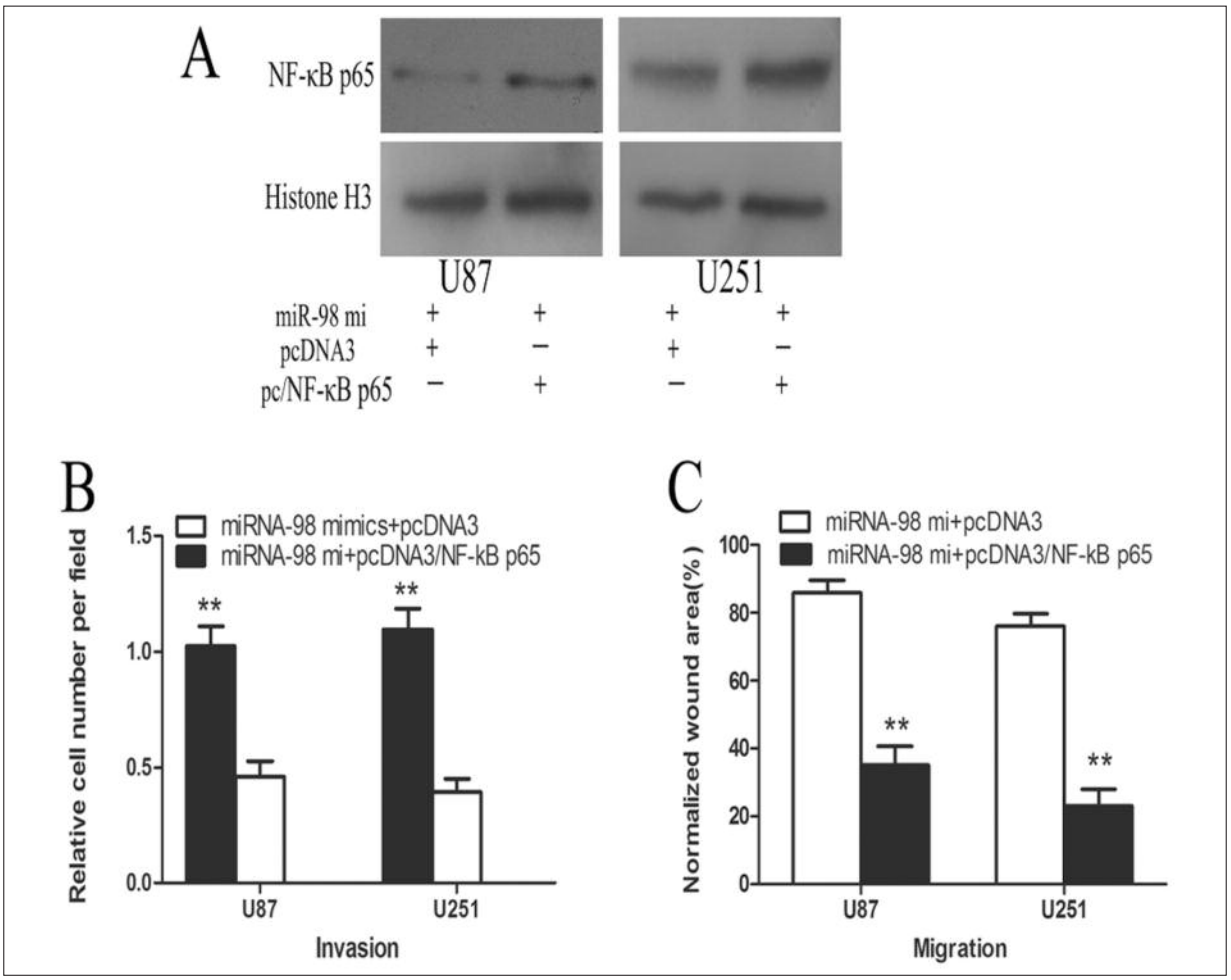

Figure 7. Overexpression of NF-κB p65 restores U87 and U251 cell migration and invasion reduced by miR-98. ***A,*** Cells were transfected with pcDNA3/NF-κB p65 and controls. The NF-κB p65 level was evaluated by a western blot analysis. Histone H3 was used as a marker for the nuclear loading control. (B-C) Cells were co-transfected with miR-98 and NF-κB p65, and invasion ***(B)*** and migration ***(C)*** assays were performed. $^{**}p < 0.01$.

validation. Consistent with our expectations, miR-98 inhibited IKKε 3′-UTR activity, but had no effect on a mutant 3′-UTR. These functional studies indicated that miR-98 overexpression suppressed cell migration and invasion. Importantly, miR-98 inhibits IKKε expression.

Recent studies have implicated IKKε in the pathogenesis of many human cancers; it increases tumor angiogenesis and metastasis and promotes resistance to apoptosis. It has been suggested that IKKε is involved in TNFα- and LPS-induced *MMP-3* and *MMP-13* gene expression via the phosphorylation and activation of the c-JUN pathway. This pathway may be responsible for synovial inflammation and extracellular matrix destruction in rheumatoid arthritis, in addition to its role in tumor invasion and metastasis[17]. Guo et al[18] showed that IKKμ is overexpressed in a significant proportion of ovarian carcinomas, and elevated IKKμ levels serve as a marker for poor prognosis. IKKε is also reported to promote prostate cancer progression via induction of IL-6 secretion, which may act as a positive growth factor in prostate cancer[19]. These data strongly support the role of IKKε in tumorigenesis. Our results were consistent with the above reports and suggested that Ikkε overexpression could restore cell migration and invasion after inhibition by miR-98. These results verified that Ikkε is a direct target of miR-98, and that miR-98 negatively regulates its expression.

NF-κB is a transcription factor that plays a key role in carcinogenesis by controlling the expression of several oncogenes, tumor suppressor genes, growth factors, and cell adhesion molecules[20,21]. NF-κB is comprised of two subunits, most commonly p65 and p50, which are typically restricted to the cytoplasm by IºB inhibitors. IκB activity is controlled by IKK kinases, which respond to cellular stimuli by IκB phosphorylation, resulting in ubiquitin-mediated protein degradation. Subsequently, NF-κB is released and translocated to the nucleus, stimulating an array of target genes that promote cell proliferation and invasion, and preventing apoptosis[22]. Previous studies have shown that overexpression of IKKε leads to IκBα degradation[23,24]. Ectopic expression of IKKε induced p65 phosphorylation, NF-κB activation, and NF-κB-dependent target gene expression. In stimulated T cells, IKKε has also been shown to enhance the activity of p65 via serine 468 phosphorylation[25]. In addition, Superactivation of MMP-9 is associated with the pathogenesis and progression of gliomas[26,27]. NF-κB can induce the expression of MMP-9 by direct promoter regulation[28]. Our studies showed that NF-κB p65 nuclear translocation and MMP-9 are significantly arrested in U87 and U251 cells treated with miR-98 mimics. Moreover, NF-κB p65 nuclear translocation and MMP-9 are restored in cells treated with pcDNA3/IKKε, which were inhibited by miR-98. Importantly, NF-κB p65 overexpression could restore cell migration and invasion, which were inhibited by miR-98. Taken together, these data suggest that miR-98 expression modulates NF-κB signaling via the regulation of IKKε, reducing malignant transformation and invasiveness of glioma cells.

Conclusions

We suggested that miR-98 regulates glioma cell migration and invasion via the suppression of IKKε/NF-κB signaling by directly attenuating NF-κB translocation from the cytoplasm to the nucleus, functioning as a tumor suppressor. Overexpression of miR-98 may act as a novel therapeutic biomarker for glioma patients. Although the application of the miRNA biomarker may be difficult in clinical settings, our results provide a basis for a molecular strategy for disease treatment.

Funding Supports

This study was supported by grants from the Jiangxi Province Natural Science Foundation project (20132BAB205043) and the Jiangxi Province Department of Education Science and Technology Research Project (GJJ14066).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Zhu GY, Shi BZ, Li Y. FoxM1 regulates Sirt1 expression in glioma cells. Eur Rev Med Pharmacol Sci 2014; 18: 205-211.
2) Dong J, Wang XQ, Yao JJ, Li G, Li XG. Decreased CUL4B expression inhibits malignant proliferation of glioma in vitro and in vivo. Eur Rev Med Pharmacol Sci 2015; 19: 1013-1021.
3) Wu L, Yang L, Xiong Y, Guo H, Shen X, Cheng Z, Zhang Y, Gao Z, Zhu X. Annexin A5 promotes invasion and chemoresistance to temozolomide in glioblastoma multiforme cells. Tumour Biol 2014; 35: 12327-12337.
4) Giese A, Bjerkvig R, Berens ME, Westphal M. Cost of migration: invasion of malignant gliomas and implications for treatment. J Clin Oncol 2003; 21: 1624-1636.
5) Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
6) Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. Cold Spring Harb Symp Quant Biol 2006; 71: 513-521.
7) Zhang LY, Liu M, Li X, Tang H. miR-490-3p modulates cell growth and epithelial to mesenchymal transition of hepatocellular carcinoma cells by targeting endoplasmic reticulum-Golgi intermediate compartment protein 3 (ERGIC3). J Biol Chem 2013; 288: 4035-4047.
8) Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A 2004; 101: 2999-3004.
9) Xu HS, Zong HL, Shang M, Ming X, Zhao JP, Ma C, Cao L. MiR-324-5p inhibits proliferation of glioma by target regulation of GLI1. Eur Rev Med Pharmacol Sci 2014; 18: 828-832.
10) Pineau P, Volinia S, McJunkin K, Marchio A, Battiston C, Terris B, Mazzaferro V, Lowe SW, Croce CM, Dejean A. miR-221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci U S A 2010; 107: 264-269.

11) Qu S, Guan J, Liu Y. Identification of microRNAs as novel biomarkers for glioma detection: a meta-analysis based on 11 articles. J Neurol Sci 2015; 348: 181-187.

12) Zhang S, Zhang C, Li Y, Wang P, Yue Z, Xie S. miR-98 regulates cisplatin-induced A549 cell death by inhibiting TP53 pathway. Biomed Pharmacother 2011; 65: 436-442.

13) Siragam V, Rutnam ZJ, Yang W, Fang L, Luo L, Yang X, Li M, Deng Z, Qian J, Peng C, Yang BB. MicroRNA miR-98 inhibits tumor angiogenesis and invasion by targeting activin receptor-like kinase-4 and matrix metalloproteinase-11. Oncotarget 2012; 3: 1370-1385.

14) Huang SD, Yuan Y, Zhuang CW, Li BL, Gong DJ, Wang SG, Zeng ZY, Cheng HZ. MicroRNA-98 and microRNA-214 post-transcriptionally regulate enhancer of zeste homolog 2 and inhibit migration and invasion in human esophageal squamous cell carcinoma. Mol Cancer 2012; 11: 51.

15) Du L, Schageman JJ, Subauste MC, Saber B, Hammond SM, Prudkin L, Wistuba, II, Ji L, Roth JA, Minna JD, Pertsemlidis A. miR-93, miR-98, and miR-197 regulate expression of tumor suppressor gene FUS1. Mol Cancer Res 2009; 7: 1234-1243.

16) Hebert C, Norris K, Scheper MA, Nikitakis N, Sauk JJ. High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. Mol Cancer 2007; 6: 5.

17) Sweeney SE, Hammaker D, Boyle DL, Firestein GS. Regulation of c-Jun phosphorylation by the I kappa B kinase-epsilon complex in fibroblast-like synoviocytes. J Immunol 2005; 174: 6424-6430.

18) Guo JP, Shu SK, He L, Lee YC, Kruk PA, Grenman S, Nicosia SV, Mor G, Schell MJ, Coppola D, Cheng JQ. Deregulation of IKBKE is associated with tumor progression, poor prognosis, and cisplatin resistance in ovarian cancer. Am J Pathol 2009; 175: 324-333.

19) Peant B, Diallo JS, Dufour F, Le Page C, Delvoye N, Saad F, Mes-Masson AM. Over-expression of IkappaB-kinase-epsilon (IKKepsilon/IKKi) induces secretion of inflammatory cytokines in prostate cancer cell lines. Prostate 2009; 69: 706-718.

20) Gilmore TD, Koedood M, Piffat KA, White DW. Rel/NF-kappaB/IkappaB proteins and cancer. Oncogene 1996; 13: 1367-1378.

21) Lee CH, Jeon YT, Kim SH, Song YS. NF-kappaB as a potential molecular target for cancer therapy. Biofactors 2007; 29: 19-35.

22) Naugler WE, Karin M. NF-kappaB and cancer-identifying targets and mechanisms. Curr Opin Genet Dev 2008; 18: 19-26.

23) Eddy SF, Guo S, Demicco EG, Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Sonenshein GE. Inducible IkappaB kinase/IkappaB kinase epsilon expression is induced by CK2 and promotes aberrant nuclear factor-kappaB activation in breast cancer cells. Cancer Res 2005; 65: 11375-11383.

24) Boehm JS, Zhao JJ, Yao J, Kim SY, Firestein R, Dunn IF, Sjostrom SK, Garraway LA, Weremowicz S, Richardson AL, Greulich H, Stewart CJ, Mulvey LA, Shen RR, Ambrogio L, Hirozane-Kishikawa T, Hill DE, Vidal M, Meyerson M, Grenier JK, Hinkle G, Root DE, Roberts TM, Lander ES, Polyak K, Hahn WC. Integrative genomic approaches identify IKBKE as a breast cancer oncogene. Cell 2007; 129: 1065-1079.

25) Mattioli I, Geng H, Sebald A, Hodel M, Bucher C, Kracht M, Schmitz ML. Inducible phosphorylation of NF-kappa B p65 at serine 468 by T cell costimulation is mediated by IKK epsilon. J Biol Chem 2006; 281: 6175-6183.

26) Kondraganti S, Mohanam S, Chintala SK, Kin Y, Jasti SL, Nirmala C, Lakka SS, Adachi Y, Kyritsis AP, Ali-Osman F, Sawaya R, Fuller GN, Rao JS. Selective suppression of matrix metalloproteinase-9 in human glioblastoma cells by antisense gene transfer impairs glioblastoma cell invasion. Cancer Res 2000; 60: 6851-6855.

27) Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 2006; 25: 9-34.

28) Farina AR, Tacconelli A, Vacca A, Maroder M, Gulino A, Mackay AR. Transcriptional up-regulation of matrix metalloproteinase-9 expression during spontaneous epithelial to neuroblast phenotype conversion by SK-N-SH neuroblastoma cells, involved in enhanced invasivity, depends upon GT-box and nuclear factor kappaB elements. Cell Growth Differ 1999; 10: 353-367.