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miR-218 inhibits the invasive ability of glioma cells by direct downregulation of IKK-β

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ABSTRACT

Aberrant activation of nuclear factor-kappa B (NF-κB) pathway has been proven to play important roles in the development and progression of cancers. Activation of NF-κB via the classical pathway is modulated by IκBs kinase (IKK-β). However, the mechanism underlying the epigenetic regulation of IKK-β/NF-κB pathway remains largely unknown. In this study, we found that the expression level of miR-218 was markedly downregulated in glioma cell lines and in human primary glioma tissues. Upregulation of miR-218 dramatically reduced the migratory speed and invasive ability of glioma cells. Furthermore, we showed that ectopically expressing miR-218 in glioma cells resulted in downregulation of matrix metalloproteinase-9 (MMP-9) and reduction in NF-κB transactivity at a transcriptional level, but inhibition of miR-218 enhanced the expression of MMP-9 and transcriptional activity of NF-κB. Moreover, we showed that miR-218 inactivated the NF-κB pathway through downregulating IKK-β expression by directly targeting the 3′-untranslated region (3′-UTR) of IKK-β. Taken together, our results suggest that miR-218 plays an important role in preventing the invasiveness of glioma cells, and our results present a novel mechanism of miRNA-mediated direct suppression of IKK-β/NF-κB pathway in gliomas.

1. Introduction

Nuclear factor-kappa B (NF-κB) is a transcription factor with pleiotropic activity owing to the central roles it plays in various biological processes [1,2]. Numerous studies have shown that aberrant activation of NF-κB pathway is strongly associated with the migration and invasion of cancer cells [3–5]. NF-κB can upregulate and activate the expression of matrix metalloproteinases (MMPs), which is mechanistically and clinically associated with the invasive ability of cancer cells, either by targeting their promoters or by regulating their activity via posttranslational modifications, respectively [6,7]. It has been also demonstrated that NF-κB plays important roles in epithelial–mesenchymal transition (EMT), which is an early step in the metastatic process, via upregulation of the expression of Snail and Twist, thereby leading to an increase in the invasive and metastatic capacity of tumor cells [8,9]. Ectopic expression of NF-κB RelB induces invasiveness in estrogen receptor (ER)-negative breast cancer cells [9]. Belguise et al. reported that coexpression of NF-κB c-Rel and protein kinase CK2 induced an invasive phenotype in immortalized mouse mammary epithelial cell line NMuMG through upregulation of Slug [10]. Consistent with these results, inhibition of NF-κB activity drastically reduced metastasis of Ras-transformed epithelial cells (EpRas cells) to the lung and inhibited the invasive ability of mammary tumor cells [11].

NF-κB signaling pathway has been found to be frequently constitutively activated in various cancer types [1,2]. In the classical pathway, IκBs kinase IKK-β activates NF-κB through phosphorylation of inhibitors of NF-κB (IκBs), which results in the translocation of cytoplasmic NF-κB into the nucleus [1,2,12]. However, the molecular mechanisms that regulate the IKK-β pathway in cancer remain largely unknown.

MicroRNAs (miRNAs), a class of small regulatory RNA molecules, negatively regulate the expression of target genes by binding to complementary sequences of the 3′-untranslated region (3′-UTR) of mRNAs [13,14]. Deregulation of miRNAs is involved in the multiple oncogenic activities, such as cellular differentiation, proliferation, oncogenesis, angiogenesis, invasion, and metastasis [15–17].

In the present study, we found that miR-218 expression in glioma cells and clinical glioma tissues was substantially downreg-

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ulated, compared to miR-218 expression in normal human astrocytes (NHA) and normal brain tissues. Ectopic expression of miR-218 reduced the migratory and invasive abilities of glioma cells, whereas inhibition of miR-218 increased these abilities. Furthermore, we demonstrated that miR-218 could inactivate NF-κB/MMP-9 signaling by directly targeting the 3′-UTR of the IKK-β. Taken together, our results suggest that downregulation of miR-218 plays an important role in the progression and pathogenesis of human glioma.

2. Materials and methods

2.1. Cell lines

Primary normal human astrocytes (NHA) were purchased from Sciencell Research Laboratories (Carlsbad, CA, in 2006) and cultured under the condition as manufacturer suggested. Glioma cell lines, including U118MG, U373MG, U87MG, D247MG, SNB19, LN464, LN428, T98G, and LN444 were grown in the DMEM supplemented with 10% FBS (HyClone, Logan, UT).

2.2. Patient information and tissue specimens

A total of 12 human primary glioma tissues, were collected for this study, which had been histopathologically and clinically diagnosed at the Sun Yat-sen University-Affiliated First Hospital. Three normal brain tissues were obtained by donation from individuals who died in traffic accident and confirmed to be free of any prior pathologically detectable conditions. For the use of these clinical materials for research purposes, prior patient's consents and approvals from the Institutional Research Ethics Committee were obtained.

2.3. Western blotting

Western blotting was performed according to standard methods as described previously [18], using anti-IKK-β, anti-IκBα, and anti-p-IκBα antibodies (Cell Signaling, Danvers, MA). The membranes were stripped and re-probed with an anti-α-tubulin Ab (Sigma, Saint Louis, MI) as a loading control.

2.4. Plasmids and transfection

pNF-κB-luc and the control plasmid (Clontech, Mountain View, CA) were used to quantitatively examine NF-κB activity. The region of human IKK-β-3′UTR, from 1108 to 1468, generated by PCR amplification from NHA, were cloned into the SacII/PstI sites of the pGL3-basic luciferase reporter plasmid (Promega, Madison, WI). The primers selected are as the following: IKK-β-3′UTR-wt-up: 5′-AACCCGCGGTCTTTTATATAAAGGCAAG AGCA CAAA-3′; IKK-β-3′UTR-wt-dn: 5′-CCGCTGCAGCCAAAATTGTGCTTTATTAAATG C-3′; IKK-β-3′UTR-mu-up: 5′-AACCCGCGGTCTTTTATATAAAGGCAAGAG CGG AAA-3′; IKK-β-3′UTR-mu-dn: 5′-CCGCTGCAGCCAAAATTCCG CTTTATTAAATGC-3′. The miR-218 mimics, negative control, and miR-218 inhibitor were purchased from RiboBio (RiboBio Co. Ltd., Guangzhou, Guangdong). Transfection of microRNA or microRNA inhibitor was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction.

2.5. RNA extraction, reverse transcription (RT) and real-time PCR

Total miRNA from cultured cells and fresh surgical glioma tissues was extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The expression level of miR-218 was quantified using miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems) and defined based on the threshold cycle (Ct), and relative expression levels were calculated as $2^{-[(\text{Ct of miR-218}) - (\text{Ct of U6})]}$ after normalization with reference to expression of U6 small nuclear RNA. Total RNA from cultured cells was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) as the manufacturer instructed. cDNAs were amplified and quantified in ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) . The primers were selected as the following: MMP-9, forward: 5′-TTGGTCCAC CTGGTTCAACT-3′, and reverse: 5′-ACGACGTCTTCCAGTA CCGA-3′; BcL-xL, forward: 5′-TCCTTGTCTACGCTTTCCACG-3′, and reverse: 5′-GGT CGCATTGTGGCCTTT-3′. TNF-α, forward: 5′-CCAGGCAGTC AGATCATCTTCTC-3′, and reverse: 5′-AGCTGGTTATCTCTCAGCTCC AC-3′. MYC, forward: 5′-TCAAGAGGC GAACACACAAC-3′, and reverse: 5′-GGCCTTTTCATTGTTTTCCA-3′. CCND1, forward: 5′-AACTA CCTGGACCGCTTCCT-3′, and reverse: 5′-CCACTTGAGCTTGTTCACC A-3′. IL-6, forward: 5′-TCTCCACAAGCGCCTTCG-3′, and reverse: 5′-CTCAGGGCTGAGAT GCCG-3′. IL-8, forward: 5′-TGCCAAGGAGTG CTAAAG-3′, and reverse: 5′-CTCCACA ACCCTCTGCAC-3′. Expression data were normalized to the geometric mean of housekeeping gene β-actin (forward: 5′-GCACAGAGCCTCGCCTT-3′, and reverse: 5′-GT TGTCGACGA CGAGCG-3′) to control the variability in expression levels and calculated as $2^{-[(\text{Ct of gene}) - (\text{Ct of β-actin})]}$, where Ct represents the threshold cycle for each transcript.

2.6. Transwell assay and Transwell matrix penetration assay

Cells (1×10^4) were plated on the top side of polycarbonate Transwell filter (without Matrigel for Transwell assay) or plated on the top side of polycarbonate Transwell filter coated with Matrigel (for Transwell matrix penetration assay) in the upper chamber of the BioCoat™ Invasion Chambers (BD, Bedford, MA) and incubated at 37 °C for 22 h, followed by removal of cells inside the upper chamber with cotton swabs. Migrated and invaded cells on the lower membrane surface were fixed in 1% paraformaldehyde, stained with hematoxylin, and counted (Ten random 100× fields per well). Cell counts were expressed as the mean number of cells per field of view. Three independent experiments were performed and the data are presented as means ± standard deviation (SD).

2.7. Three-dimension spheroid invasion assay

Cells (1×10^4) were trypsinized and seeded in 24-well plates coated with Matrigel (2%, BD Biosciences), and medium was changed every other day. Pictures were taken under microscope at 2 days intervals for 8 days.

2.8. ELASA

The concentration of MMP9 in the cell conditioned medium was determined by a commercially available MMP-9 ELISA Kit (Calbiochem/Oncogene, Cambridge, MA). ELISAs were performed according to the instructions of the manufacturer. Briefly, the condition medium collected as the Gelatin zymography assay was added to a well coated with MMP-9 polyclonal antibody, and then immunosorbented by biotinylated monoclonal anti-human MMP-9 antibody at room temperature for 2 h. The color development catalyzed by horseradish peroxidase was terminated with 2.5 M sulfuric acid and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

2.9. Luciferase assay

Cells (3.5×10^4) were seeded in triplicates in 48-well plates and allowed to settle for 24 h. One hundred nanogram of luciferase re-

porter plasmids of IKK-β-3′UTR (wt/mu), or pNF-κB-luc plasmid, or the control-luciferase plasmid, plus 1 ng of pRL-TK renilla plasmid (Promega, Madison, WI), were transfected into astrocytoma cells using the Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's recommendation. Luciferase and renilla signals were measured 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) according to a protocol provided by the manufacturer. Three independent experiments were performed and the data are presented as means ± SD.

2.10. Statistical analysis

Data are shown as mean ± SD. Statistical significance was determined by Student's *t* test, with *P*-value of <0.05 considered to be statistically significant.

3. Results

3.1. Upregulation of miR-218 inhibits the invasive ability of glioma cells

Real-time PCR analysis revealed that the expression of miR-218 was significantly lower in the examined 9 glioma cell lines than that in NHA (Fig. 1A). Furthermore, miR-218 expression in 12 freshly-frozen glioma tissues of varying WHO grades was dramatically downregulated as compared to that in three human normal brain tissues (Supplementary Fig. 1). Taken together, our results suggest that miR-218 is downregulated in glioma cell lines and glioma tissues.

Since invasiveness is one of the pathophysiological features of human malignant gliomas, we were promoted to ask whether miR-218 was associated with the invasiveness of gliomas. Transwell assay (without Matrigel) showed that the migratory speed of U87MG and SNB19 glioma cells that ectopically expressing miR-218 was markedly slower than that of control cells (Fig. 1B). Furthermore, Transwell matrix penetration (coated with Matrigel) assay and three-dimensional spheroid invasion assay showed that the upregulation of miR-218 dramatically reduced the invasiveness of both U87MG and SNB19 glioma cells (Fig. 1C and D); this finding suggests that the upregulation of miR-218 inhibits the invasive ability of glioma cells *in vitro*.

3.2. Upregulation of miR-218 reduces MMP-9 expression via inactivation of NF-κB signaling

Expression and activation of MMP-9 are known to be associated with the invasive ability of glioma cells [19,20]. Results of enzyme-linked immunosorbent assay (ELISA) showed that MMP-9 activities in glioma cells transfected with miR-218 mimic were lower than those in control cells (Fig. 2A). Real-time PCR analysis revealed that upregulation of miR-218 drastically repressed the expression level of MMP-9 mRNA in U87MG and SNB19 glioma cells (Fig. 2B), which indicated that miR-218 mediated the downregulation of MMP-9 at the transcriptional level. Since MMP-9 has been demonstrated to be transcriptionally upregulated by transcription factor NF-κB, the effect of miR-218 on the NF-κB activity was further examined. Luciferase activity assay indicated that ectopic expression of miR-218 in glioma cells significantly inhibited the transac-

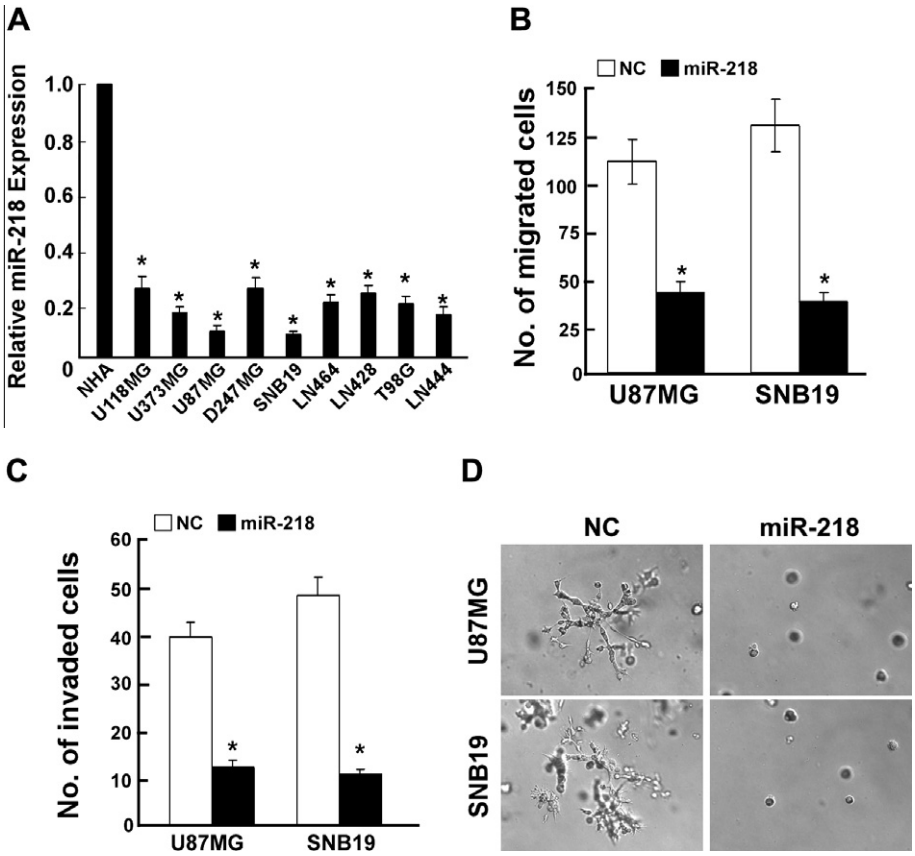

Fig. 1. Upregulation of miR-218 inhibits the invasive ability of glioma cells. (A) Real-time PCR analysis of miR-218 expression in normal human astrocytes (NHA) and glioma cell lines, including U118MG, U373MG, U87MG, D247MG, SNB19, LN464, LN428, T98G and LN444. *U6* was used as a loading control. (B) The number of migrated cells was determined using Transwell assay (without Matrigel). (C) The number of invaded cells was determined using transwell matrix penetration assay (with Matrigel). (D) Representative micrographs of the indicated cultured cells after 8-day culture in three-dimensional spheroid invasion assays. Error bars represent mean (standard deviation, SD) of 3 independent experiments with similar results. $^{*}P < 0.05$.

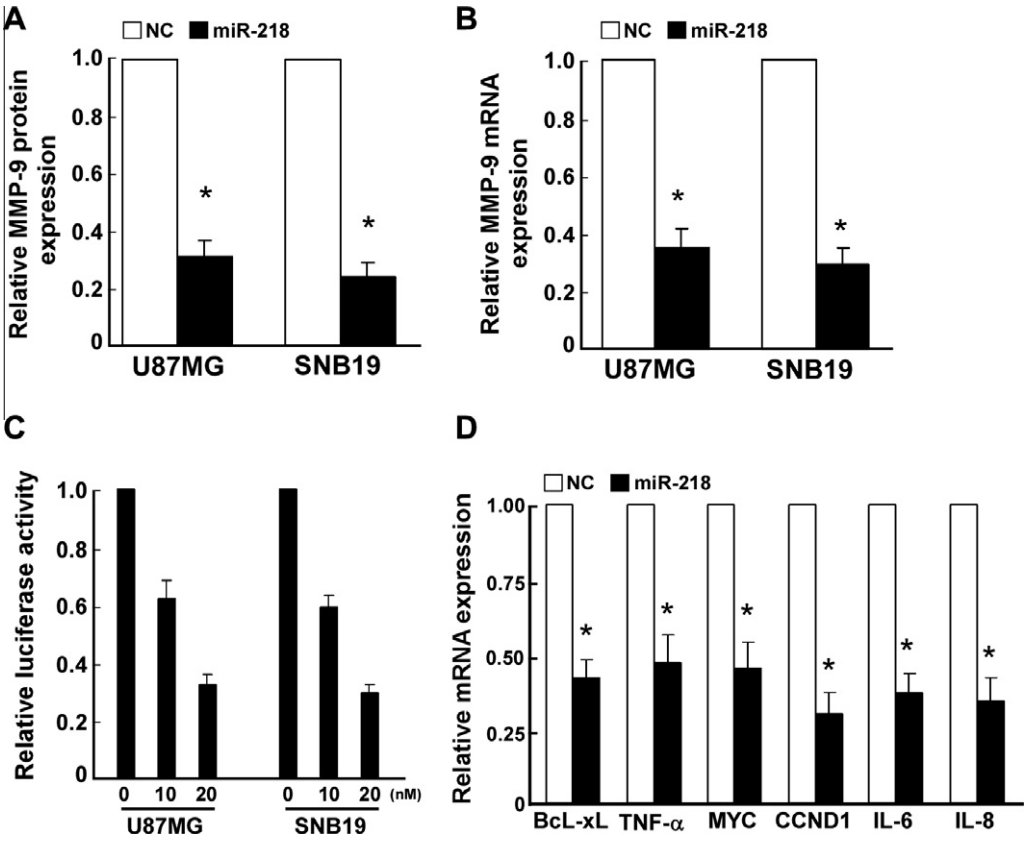

Fig. 2. Upregulation of miR-218 reduces matrix metalloproteinase-9 expression and inhibits nuclear factor kappa B transactivity. (A) Matrix metalloproteinase- 9 (MMP-9) protein levels in the supernatants of the indicated cells were determined using enzyme-linked immunosorbent assay. (B) The changes in the levels of MMP-9 mRNA in control cells and miR-218 mimic-transfected cells were measured. β-Actin was used as a loading control. (C) Luciferase activity assay indicated that upregulation of miR-218 decreased NF-κB transactivity in glioma cells. (D) Real-time PCR was performed to determine changes in the expression levels of mRNA of NF-κB-regulated genes in U87MG cells. β-Actin was used as a loading control. Error bars represent mean (standard deviation, SD) of 3 independent experiments with similar results. $^{*}P < 0.05$.

tivity of NF-κB in a dose-dependent manner (Fig. 2C). Moreover, the mRNA expression levels of 6 classical NF-κB target genes, including *BcL-xL*, *TNF-α*, *MYC*, *CCND1*, *IL-6*, and *IL-8*, were found to be repressed in miR-218 transfected-glioma cells (Fig. 2D and Supplementary Fig. 2). Taken together, our results suggest that upregulation of miR-218 reduces MMP-9 expression and inactivates NF-κB signaling.

3.3. miR-218 directly targets the IKK-β in glioma cells

To further investigate the mechanism of miR-218-mediated reduction in the invasiveness and inhibition of NF-κB pathway, we next sought to identify the molecular targets of miR-218. Analysis by using three publicly available algorithms (TargetScan, Pictar, and miRANDA) indicated that IKK-β, which is involved in the activation of NF-κB pathway, is theoretically the target gene of miR-218 (Fig. 3A). As predicted, overexpression of miR-218 significantly decreased the expression of IKK-β in both glioma cells (Fig. 3B). Change in IKK-β downregulation was simultaneously associated with change in the phosphorylation level of IκBα, a downstream target protein of IKK-β, which further supports the notion that miR-218 may contribute to the regulation of NF-κB signaling. Moreover, a fragment of the IKK-β 3′-UTR containing the 2 miR-218 binding sites (REs) was subcloned into the pGL3 dual luciferase reporter vector. We observed a consistent and dose-dependent reduction of luciferase activity in both cell lines upon miR-218 transfection (Fig. 3C). However, point mutations of IKK-β 3′-UTR in miR-218-binding seed region did not show the miR-218-mediated suppressive effect (Fig. 3C). Taken together, our results demonstrate that IKK-β is a *bona fide* target of miR-218.

3.4. Inhibition of miR-218 enhances IKK-β expression and invasiveness of glioma cells

Furthermore, we examined the effect of inhibition of miR-218 on the invasiveness of glioma cells and activation of NF-κB pathway. As expected, inhibition of miR-218 restored the luciferase activity of pGL3-IKK-β-3′-UTR (Fig. 3D) and upregulated the expression levels of IKK-β and p-IκBα in U87MG and SNB19 glioma cells (Fig. 4A). The NF-κB transactivities and the MMP-9 expression level in both glioma cells dramatically increased upon miR-218 inhibitor transfection (Fig. 4B and C). Moreover, Transwell matrix penetration assay showed that the inhibition of miR-218 dramatically enhanced the invasive ability of both glioma cells (Fig. 4D), which suggested that suppression of miR-218 induces invasiveness in glioma cells by activation of IKK-β/NF-κB/MMP-9 pathway.

4. Discussion

The key finding of our study is that miR-218 expression is drastically downregulated in glioma cells and clinical glioma tissues as compared to that in NHA and normal brain tissues. The migratory speed and invasive ability of glioma cells were reduced with ectopic expression of miR-218, whereas they increased with inhibition of miR-218. Furthermore, we found that miR-218 was involved in modulation of the NF-κB/MMP-9 signaling pathway and downregulation of IKK-β expression by directly targeting the IKK-β 3′-untranslated region (3′-UTR). Taken together, our results suggest that downregulation of miR-218 plays an important role in the invasiveness of gliomas.

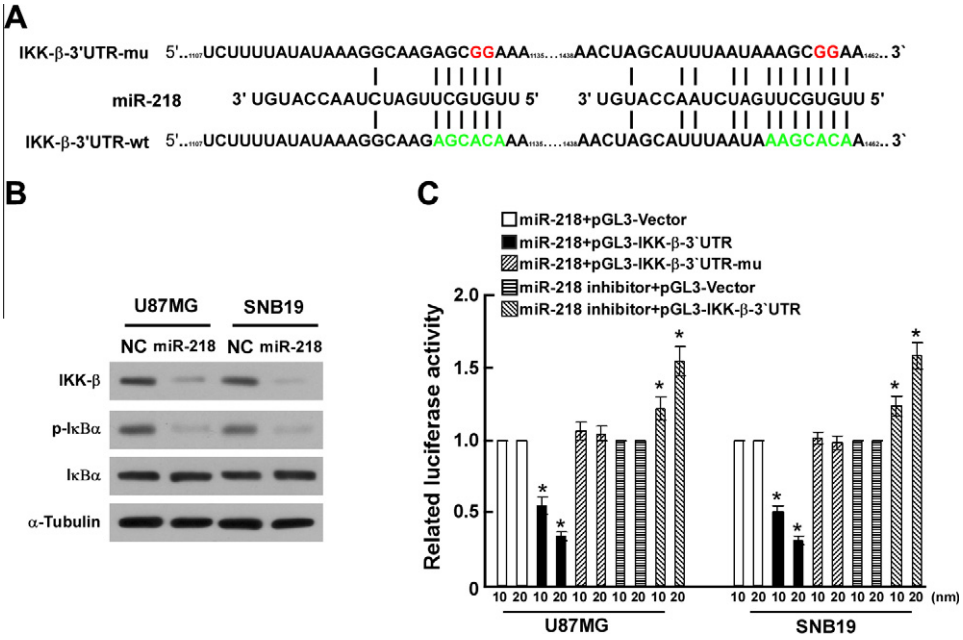

Fig. 3. miR-218 directly targets the inhibitor of kappa kinase-β in glioma cells. (A) Predicted miR-218 target sequences in 3′-untranslated region (3′-UTR) of IKK-β (IKK-β-wt) and mutant containing 2 mutated nucleotides in 3′-UTR of IKK-β (IKK-β-mu). (B) Western blot analysis of the expression levels of IKK-β, p-IκBα, and total IκBα in indicated cells. (C) Luciferase activity assay of indicated cells transfected with pGL3-IKK-β-3′-UTR-wt reporter or pGL3-IKK-β-3′UTR-mu reporter with increasing amounts (10 and 20 nM) of miR-218 oligonucleotides or miR-218 inhibitor oligonucleotides. Error bars represent mean (standard deviation, SD) of 3 independent experiments. $^{*}P < 0.05$.

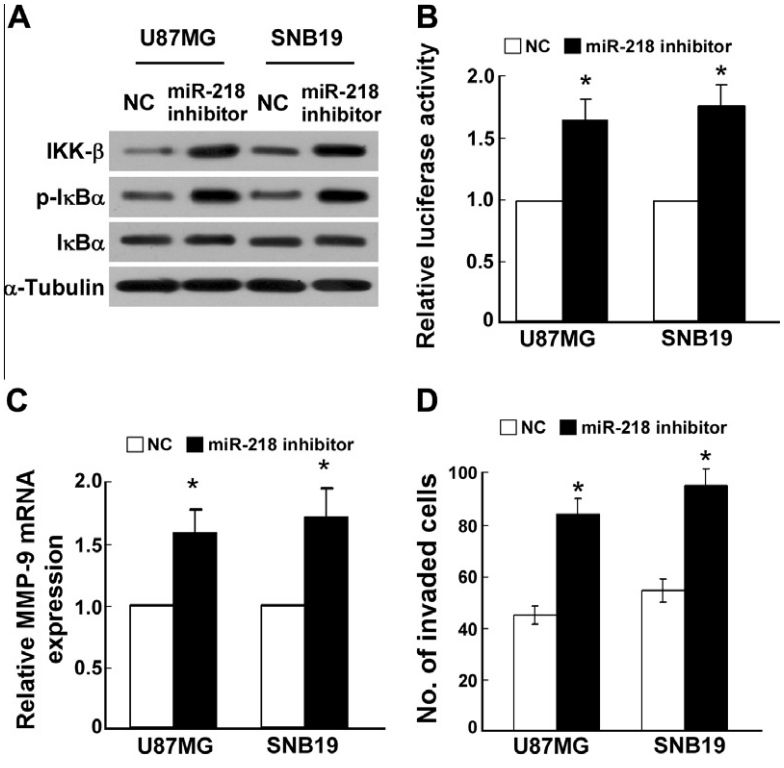

Fig. 4. Inhibition of miR-218 enhances inhibitor of kappa kinase-β expression and invasive ability of glioma cells. (A) The expression levels of IKK-β, p-IκBα, and total IκBα in indicated cells were analyzed by Western blotting. (B) Relative activities of NF-κB reporter in the indicated cell lines. (C) ELISA analysis of MMP-9 protein levels in the supernatants of the indicated cells. (D) The number of invaded cells was analyzed using Transwell matrix penetration assay (with Matrigel). Error bars represent mean (standard deviation, SD) of 3 independent experiments. $^{*}P < 0.05$.

Glioma, the most aggressive type of tumor arising in the central nervous system (CNS), is considered to be one of the deadliest of human cancers. The 1-year overall survival rate was less than 30%, and the median survival time of patients with high-grade gliomas was only approximately 15 months [21,22]. Gliomas have a poor prognosis mainly because of the high invasive ability of glioma cells, which show extensive migration and diffuse invasion into the surrounding normal brain tissue and thus result in only partial surgical resection [23,24]. More than 90% of recurrent gliomas develop from infiltrated glioma cells and occur within few

centimeters of the resected region or adjacent to resected margin [25,26]. Thus, understanding the molecular mechanism underlying the invasiveness of gliomas is of a great clinical value. In this study, we found that the expression of miR-218 in glioma cells lines and glioma tissues was significantly downregulated as compared to that in NHA and normal human brain tissues, suggesting that downregulation of miR-218 may be associated with the development and progression of gliomas. Consistent with our results, the expression level of miR-218 is frequently downregulated in several human cancers, including gastric cancer, lung squamous cell carcinoma, malignant astrocytomas, and medulloblastomas, which suggest that miR-218 may function as a tumor suppressor [27–30]. Indeed, overexpression of miR-218 in gastric cancer cells and bladder cancer cells has been reported to increase apoptosis and inhibit cell proliferation [27,31]. Further, the expression of miR-218 dramatically decreased in metastatic prostate cancer and upregulation of miR-218 inhibited the invasion and metastasis of gastric cancer [32,27]. Our results showed that upregulation of miR-218 drastically reduced the migration and invasion of glioma cells, while suppression of miR-218 enhanced the invasive ability of glioma cells. Taken together, downregulation of miR-218 may play an important role in cancer invasion and metastasis.

Upregulation or superactivation of MMP-9 is associated with the pathogenesis and progression of gliomas [19,20]. The transcriptional factor NF-κB can induce the expression of MMP-9 by directly targeting its promoter [6]. In this study, we showed that upregulation of miR-218 inhibited NF-κB/MMP-9 signaling pathway. Furthermore, on the basis of bioinformatics analysis we predicted that IKK-β, the upstream regulator of NF-κB, to be a theoretical target gene of miR-218. Western blotting showed that the upregulation of miR-218 decreased IKK-β expression, and the luciferase activity assay showed that the IKK-β downregulation mediated by miR-218 was through the 3′-UTR of IKK-β, which showed that IKK-β is a *bona fide* target of miR-218. We are currently investigating the biological role of miR-218 in glioma cells in inducing apoptosis and inhibiting cell survival, as related to IKK-β/NF-κB signaling, in our laboratory.

5. Conclusion

We have shown, for the first time, an important link between the downregulation of miR-218-mediated glioma cell invasiveness and inactivation of IKK-β/NF-κB/MMP signaling. Our results suggest that miR-218 plays a critical role in the inhibition of development and progression of gliomas and understanding its role may provide important insights in the treatment of gliomas.

Conflict of interest

No conflicts of interest were declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.003.

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