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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-