MicroRNA-200a mediates nasopharyngeal carcinoma cell proliferation through the activation of nuclear factor-κB

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Abstract. In nasopharyngeal carcinoma (NPC), the nuclear factor-κB (NF-κB) signaling pathway is highly active. The constitutive activation of NF-κB prompts malignant cell proliferation, and microRNAs are considered an important mediator in regulating the NF-κB signaling pathway. The current study investigated the effect of microRNA-200a (miR-200a) on NF-κB activation. Reverse transcription-quantitative polymerase chain reaction was used to quantify the relative level of miR-200a in NPC tissue samples and CNE2 cells. An MTT assay was used to investigate the effect of miR-200a on cell proliferation. To investigate the activation of NF-κB, western blotting was used to measure the protein levels of NF-κB and its downstream targets. To identify the target genes of miR-200a, a luciferase reporter assay was used. The current study demonstrated that miR-200a was upregulated in NPC tissue samples and cell lines. Overexpression of miR-200a resulted in the proliferation of CNE2 cells. Western blot analysis indicated that the protein levels of p65 increased when CNE2 cells were transfected with miR-200a mimics. Additionally, the downstream targets of miR-200a were upregulated, including vascular cell adhesion molecule, intercellular adhesion molecule and monocyte chemoattractant protein-1. The luciferase assay indicated that IκBα was the target gene of miR-200a. In conclusion, miR-200a was demonstrated to enhance NPC cell proliferation by activating the NF-κB signaling pathway.

Introduction

As a squamous cell carcinoma, nasopharyngeal carcinoma (NPC) is derived from the epithelium of the nasopharynx. Compared with the rest of the world, the incidence of NPC in China is high (1), and therefore is a serious public health problem in China. In regions covered by the cancer registries in 2009, the crude incidence of NPC was 3.61/100,000 (5.08/100,000 in males and 2.10/100,000 in females; 4.19/100,000 in urban areas and 2.42/100,000 in rural areas) (1). Due to the high incidence of early metastasis, the rate of mortality is high in patients with NPC. At present, radiotherapy is the first choice of therapy for patients with NPC. However, despite improvements in the standards of radiotherapy, the five-year survival rate remains to be ~50-60% (2). Therefore, it is important for clinicians and researchers to develop novel effective treatment strategies.

The transcription factor nuclear factor-κB (NF-κB) consists of 5 subunits; Rel (cRel), p65 (RelA, NF-κB3), RelB, p50 (NF-κB1) and p52 (NF-κB2) (3), and the two most common dimers of NF-κB are p65 and p50 (3). In unstimulated cells, inhibitory κB (IκB) is bound to NF-κB, which remains in an inactive form in the cytoplasm (3). When cells are stimulated by extracellular signals, IκB kinase complex (IKK) phosphorylates IκB, exposing the nuclear localization sites of NF-κB (3). Subsequently the free NF-κB translocates into the nucleus where it binds with specific κB sequences, inducing gene transcription (3). Previous histological studies have indicated the importance of local inflammation in NPC tumorigenesis (4). As a key inflammatory signaling pathway, NF-κB has been demonstrated to be constitutively active in NPC tissue by immunohistochemical staining (4). The constitutive activation of NF-κB commonly results in malignant carcinoma cell proliferation in various types of cancer cells and tissues, as the NF-κB signaling pathway regulates a series of target genes involved in cell proliferation, apoptosis, immune responses and transcription (5).

MicroRNAs (miRNAs) are small non-coding RNAs of 20-25 nucleotides. miRNAs negatively regulate gene

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expression by recognizing complementary sequences in the 3'-untranslated regions (UTR) of target mRNAs, resulting in their degradation (6). In previous studies, the aberrant expression of miRNAs has been associated with various types of human cancer (7,8). In addition, as an important mediator, miRNAs are accepted as key modulators and effectors of the NF-κB signaling pathway. For example, miR-146a and miR-146b negatively interact with interleukin-1 receptor-associated kinase 1 and tissue necrosis factor receptor-associated factor 6 protein levels, resulting in the activation of NF-κB (9). Furthermore, miR-199a has been demonstrated to suppress IKKβ, which reduces the activity of NF-κB signaling (10), whilst miR-200a has been reported to regulate various signaling pathways through targeting different genes, including epidermal growth factor receptor, c-Met and ephrin type-A receptor 1 (11,12).

In the current study, the relative expression levels of miR-200a were investigated in human NPC. In addition, the impact of increased miR-200a levels on NPC cell proliferation and migration was explored. The present study aimed to elucidate the effect of miR-200a on NPC cell proliferation and the role of the NF-κB signaling pathway in this process.

Materials and methods

Human samples and cell lines. A total of 40 samples of primary human NPC and 30 normal samples were collected at the People's Liberation Army 113th hospital (Ningbo, China) and written informed consent was obtained from all patients. Clinical information was obtained by reviewing the medical records on radiographic images, by telephone or written correspondence, and by reviewing death certificates. All specimens had confirmed pathological diagnosis and were staged according to the 1992 NPC staging system of China (13). The NPC cell lines (HNE1, CNE1 and CNE2) and an immortalized nasopharyngeal epithelial cell line (NP69) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Eagle's minimum essential medium (MEM; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum(FBS; GE Healthcare Life Sciences). The HEK293T cells were cultured in 2 ml Dulbecco's modified Eagle's medium (GE Healthcare Life Sciences) supplemented with 100 U/ml penicillin (Beijing SolarBio Science & Technology Co., Ltd., Beijing, China, Beijing, China), 100 U/ml streptomycin and 10% FBS.

Tumor necrosis factor-α (TNF-α) treatment. CNE2 cells were seeded in the six-well plate at the concentration of $1x10^6$ cells/well. After 24 h, the CNE2 cells were treated with 10 ng/ml TNFα for 48 h. The relative levels of miR-200a were then determined.

Transient transfection procedures. Shortly prior to transfection, $1.5x10^5$ cells were seeded per well in a 6-well plate in 2 ml Dulbecco's modified Eagle's medium containing serum and supplemented with 100 U/ml penicillin and 100 U/ml streptomycin. Prior to transfection, the cells were incubated under normal growth conditions (typically 37°C and 5% CO_2). Subsequently, miR-200a mimics, miR-200a inhibitor or the miR negative control (Shanghai Genepharma Co., Ltd.,

Shanghai, China) were pre-incubated with HiPerFect transfection reagent (Qiagen China Co., Ltd., Shanghai, China) with the final concentration of microRNA analogues at 100 nmol/l. The sequence of miR-200a was as follows: 5'-CAGUGCAAU AGUAUUGUCAAAGC-3'.

siRNA transfection. Specific siRNA targeting p65 was purchased from Shanghai Jima Co. (Shanghai, China). The siRNAs were transfected into the cells using Vigofect transfection reagent (Vigorous Biotechnology Beijing Co., Ltd., Beijing, China).

RNA extraction. Total RNA (2 μg) was extracted from cell lines and human samples (5 mg) with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to detect and quantify mature miRNA-200a, a TaqMan MicroRNA Reverse Transcription kit and a TaqMan MicroRNA Assay were used according to the manufacturer's instructions (Applied Biosystems Life Technologies, Foster City, CA, USA). U6 RNA was used for normalization. To quantify miRNA levels, 10 ng total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems Life Technologies) with specific primers for miR-200a and U6. Subsequently, the PCR amplifications were performed in 20 μl reaction volume containing 10 μg TaqMan 2X Universal PCR Master Mix, 1 μl 20X TaqMan MicroRNA Assay mix (Applied Biosystems Life Technologies) and 1.33 μl template cDNA in the same system used for mRNA quantification. The thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Relative miRNA expression of miR-200a was normalized against the endogenous control, U6 RNA, using the comparative $2^{-\Delta\Delta Ct}$ method (14). CFX Manager™ software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for quantification analysis for mRNA and miRNA. For reverse transcription, the specific primers are listed, as follows: (5'-3'): miR-200a, CUGGAUUUCCCA GCUUGACUCUAACACUGUCUGGUAACGAUGUUCAAA GGUGACCCGC; U6, GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGACAAATATG. The primers used for qPCR were as follows (5'-3'): miR-200a forward, GCAAAGTGCATCCATTTTGTTTGT; U6 forward, GCGCGTCGTGAAGCGTTC; universal reverse primer, GTGCAGGGTCCGAGGT.

Protein extraction, western blotting and antibodies. Cellular proteins were extracted using RIPA buffer [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS; Beijing SolarBio Science & Technology Co., Ltd.) containing 1% (v/v) phenylmethanesulfonylfluoride (Beijing SolarBio Science & Technology Co., Ltd.), 0.3% (v/v) protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% (v/v) phosphorylated proteinase inhibitor (Sigma-Aldrich). Lysates were centrifuged at 13,000 g at 4°C for 15 min and the supernatant was collected for total protein analysis. A bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) was used to determine the protein concentration. Equal

amounts of protein (15 µg) were separated on an SDS-PAGE gel [10% (v/v) polyacrylamide; Beijing SolarBio Science & Technology Co., Ltd.] and transferred onto a polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany). Nonspecific binding was blocked using 8% (w/v) milk in Tris-buffered saline-Tween 20 (TBST) for 2 h at room temperature. The membranes were incubated with primary antibodies against β-actin (8H10D10; mouse monoclonal; cat. no. 3700; 1:3,000, Cell Signaling Technology, Inc., Danvers, MA, USA), NF-κB p65 (D14E12) XP® Rabbit mAb (cat. no. 8242; 1:1,000; Cell Signaling Technology, Inc.), NF-κB1 p105/p50 antibody (cat. no. 3035; 1:1,000; Cell Signaling Technology, Inc.), Cox2 (D5H5) XP® rabbit mAb (cat. no. 12282; 1:1,000; Cell Signaling Technology, Inc.), MMP-2 (D8N9Y) rabbit mAb (cat. no. 13132; 1:1,000; Cell Signaling Technology, Inc.), human vascular endothelial growth factor-165 (cat. no. 8065; 1:1,000; Cell Signaling Technology, Inc.) Phosphorylated (p)-NF-κB p65 (Ser536; 93H1) rabbit mAb (cat. no. 3033; 1:1,000; Cell Signaling Technology, Inc.), ICAM-2 (D7P2Q) rabbit mAb (cat. no. 13355; 1:1,000, Cell Signaling Technology, Inc.), MCP-1 antibody (cat. no. 2027; 1:1,000; Cell Signaling Technology, Inc.) and IκBα (44D4) rabbit mAb (cat. no. 4812; 1:1,000; Cell Signaling Technology, Inc.) overnight at 4°C. Following three washes with TBST, the membranes were incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit (cat. no. ZB-2307; 1:5,000; Zhongshan Gold Bridge Biotechnology Co., Ltd., Beijing, China) and anti-mouse (cat. no. ZB-2305 1:5,000; Zhongshan Gold Bridge Biotechnology, Co., Ltd.) or HRP-conjugated mouse anti-goat antibodies (cat. no. ZB-5305; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China; 1:5,000) for 2 h at room temperature and then washed with TBST three times (5 min each). The target proteins were subsequently visualized using enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions and quantified using density analysis with ImageJ 4.0 software (National Institutes of Health, Bethesda, MA, USA) normalized against β-actin, and expressed as the fold-change, compared with the control.

Luciferase target assay. TargetScan (http://www.targetscan.org/) was used to determine the potential binding sites of miR-200a on the 3'UTR of IκBα. For the luciferase assay, the 3'UTR of IκBα, including the binding site for miR-200a, was amplified from CNE2 cells using the following primers: IκBα-F, 5'-AAGGAGGAGGGCAGAATCAT-3'; IκBα-R, 5'-ATCTGCATGGTGATGTTGGA-3'.

The PCR product was digested with *Xba*I (New England Biolabs, Beverly, MA, USA) and cloned into the reporter plasmid pGL3 (Promega Corporation, Madison, WI, USA) downstream of the luciferase reporter gene. The modified firefly luciferase vector (500 ng/µl; Promega Corporation) was transfected into HEK293 cells (2x10^{5}cells/ml; as previously described). Firefly and Renilla luciferase activities were measured 48 h following transfection with the Dual-Luciferase Reporter Assay System (Promega Corporation). Firefly activity was normalized to *Renilla* activity to control the transfection efficiency.

Dihydroethidium (DHE) staining. The cells were cultured in six-well chamber slides at a density of 1x10^6 cells/well, washed with PBS three times (5 min/wash) and incubated with ROS Fluorescent Probe-DHE (10 µM; Vigorous Biotechnology Beijing Co., Ltd.) in serum-free DMEM F-12 medium for 30 min at 37°C in the dark. Following incubation, the slides were fixed in 4% paraformaldehyde for 30 min at room temperature, were washed again and mounted. Immunofluorescence images were captured using fluorescence microscopy (Leica CM3000; Leica Microsystems GmbH, Wetzlar, Germany).

Dimethyl thiazolyl diphenyl tetrazolium (MTT) assay. To evaluate the effect of miR-200a on cell proliferation, cells were seeded 5,000 cells/well in 100 µl MEM in 96-well plates and transfected with miR-200a mimics (50 nM) and negative control-miRNA mimics (50 nM), as described above. At 24, 48 and 72 h post-transfection, 20 µl MTT reagent (Beijing SolarBio Science & Technology Co., Ltd.) was added to the wells, which were then incubated for 4 h at 37°C. Following removal of the medium, 200 µl dimethyl sulfoxide was added to dissolve the formazan and the absorbance was measured at 550 nm. Wells containing only CNE2 cells, which served as blanks.

Statistical analysis. Data are presented as the mean ± standard error from 3 independent experiments. Statistical analysis was conducted with Student's t-test using GraphPad Prism 5 software (National Institutes of Health). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-200a is upregulated in human NPC cells and tissue samples. The relative levels of miR-200a in human NPC cells and tissue samples were measured in human NPC cells and patient tissue samples using RT-qPCR. Compared with NP69 immortalized nasopharyngeal epithelial cells, miR-200a levels were increased by greater than 2.38-fold in HNE1, CNE1 and CNE2 human NPC cells, and miR-200a expression levels were normalized to U6 (P<0.05; Fig. 1A). The expression levels of miR-200a were measured in 40 human NPC cancer specimens compared with 30 normal tissue samples. Compared with the normal tissue, the mean expression level of miR-200a was increased by greater than 3-fold (P<0.05; Fig. 1B). This indicated that miR-200a was significantly increased in the human NPC cells and tumor tissue.

Downregulation of miR-200a increases CNE2 cell viability. In order to investigate the effect of miR-200a on CNE2 cell viability, CNE2 cells were transfected with miR-200a mimics, miR-200a inhibitors or the negative control for 24, 48 and 72 h. In the current study, the mimics were analogues that enhanced miR-200a expression levels while inhibitors were analogues that reduced the expression of miR-200a. The MTT assay indicated that when miR-200a mimics were transfected into CNE2 human NPC cells, cell viability was significantly decreased by 23 and 35% at 48 and 72 h, respectively (Fig. 2A). However, when miR-200a expression was inhibited, cell viability was enhanced by 17 and 36% at 48 and 72 h, respectively (Fig. 2B). These results indicated that miR-200a may increase CNE2 cell viability.
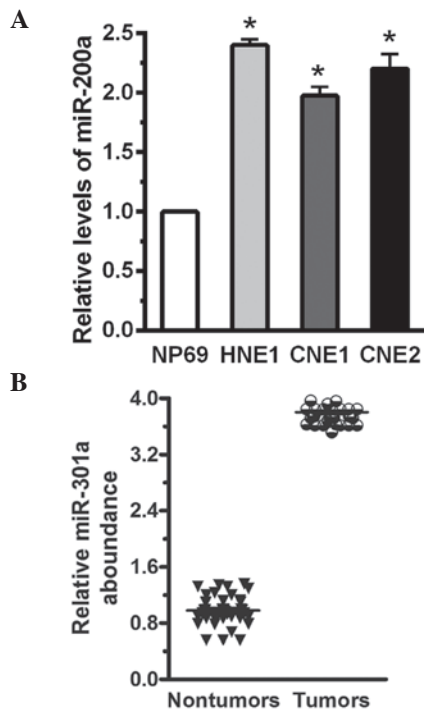
Figure 1. Expression levels of miR-200a in human NPC cells and tissue samples. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-200a expression in HNE1, CNE1 and CNE2 human NPC cells and NP69 immortalized nasopharyngeal epithelial cells. (B) Analysis of miR-200a expression in 40 human NPC tissue samples (Tumors) and paired nontumor tissue samples (Nontumors). U6 was used as an endogenous control. *P<0.05, vs. NP69. miR-200a, microRNA-200a, NPC, nasopharyngeal carcinoma.
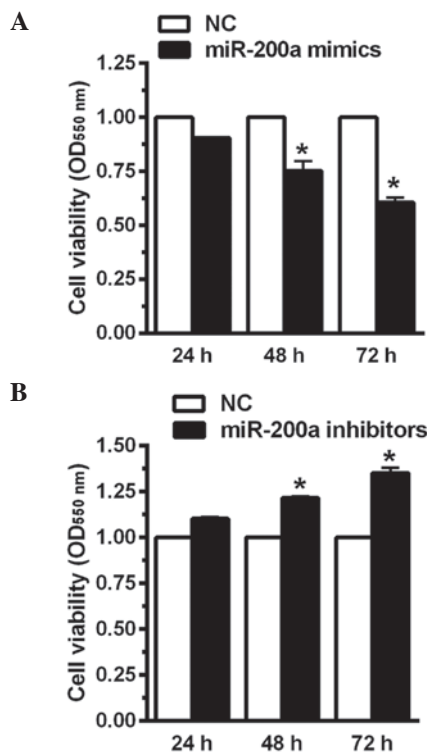

Figure 2. CNE2 human NPC cell viability was altered by miR-200a. CNE2 cells were transfected with (A) miR-200a mimics, (B) miR-200a inhibitors or the negative control for 24, 48 and 72 h. Cell viability was measured using an MTT assay. Data represent the mean ± standard error, n=6 independent experiments. *P<0.05 vs. NC. NPC, nasopharyngeal carcinoma; miR-200a, microRNA-200a; NC, negative control; OD, optical density.

miR-200a activates the NF-κB signaling pathway. In NPC, the NF-κB signaling pathway is constitutively activated (15). In the current study, CNE2 cells were treated with 10 ng/µl TNF-α for 48 h, following which the relative levels of miR-200a were measured. As presented in Fig. 3A, the relative level of miR-200a was increased by greater than 4.8-fold with TNF-α treatment. To address whether miR-200a contributes to NF-κB activation, CNE2 cells were transfected with miR-200a mimics. Western blot analysis indicated that when miR-200a was overexpressed, NF-κB was significantly activated. As presented in Fig. 3, compared with the negative control, the p-NF-κB/NF-κB ratio was increased by greater than 2.36-fold (Fig. 3B). Vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM) and monocyte chemoattractant protein-1 (MCP-1) are important adhesion molecules that are aberrantly increased in various types of cancer (16). To further investigate the alterations in NF-κB activation, the protein levels of VCAM, ICAM and MCP-1 were measured. As presented in Fig. 3B, the relative levels of VCAM, ICAM and MCP-1 were significantly increased. Together, these data indicate that miR-200a contributed to TNF-α-stimulated NF-κB activation. To investigate the effect of NF-κB on cell proliferation, a small interfering RNA (siRNA) targeting NF-κB was used. Following treatment with the siRNA against NF-κB, the protein levels of NF-κB were reduced, as were the expression levels of VCAM, ICAM and MCP-1. Additionally, this effect was observed in the cells transfected with miR-200a mimics (Fig. 3C). VCAM was increased by 2.1-fold following the addition of mimics, although the reduction in MCP-1 was less marked. Furthermore, as presented in Fig. 3D, with NF-κB knockdown, cell viability was significantly reduced even in the cells transfected with miR-200a mimics. These data suggest that miR-200a enhances cell proliferation through the activation of the NF-κB signaling pathway.

IκBα is the host gene of miR-200a. To further investigate whether NF-κB was activated by miR-200a overexpression, immunofluorescence was used. As presented in Fig. 4A, increased NF-κB (p65) protein levels were observed when CNE2 cells were transfected with miR-200a mimics. In previous studies, numerous miRNAs have been reported to activate NF-κB, for instance, miR-200a targeted NF-κB-repressing factor and correlated with altered NF-κB activation (17,18). In the current study, the target gene of miR-200a was identified using a bio-informatics database. TargetScan predicted miR-200a to target position 356-362 in the 3'UTR in IκBα (Fig. 4B). IκB is an NF-κB inhibitory protein, which in unstimulated cells is bound to p65 and P50, resulting in the inactivation of NF-κB in the cytoplasm (19). Following the activation of IKK, IκBα is degraded and the two subunits of NF-κB translocate from the cytoplasm to the nucleus, thereby inducing the downstream signaling pathway (20). Therefore, the effect of miR-200a on IκBα expression was investigated. When CNE2 cells were transfected with miR-200a mimics for 4 h, the expression levels of IκBα were significantly reduced compared with the negative control (Fig. 4B). Furthermore, 48 h following transfection with miR-200a in CNE2 cells, the expression level of IκBα was reduced by 56%. However, when miR-200a was inhibited in CNE2 cells, the expression level of IκBα was increased by almost 1-fold (Fig. 4C). A luciferase reporter assay was used to investigate the effect of miR-200a on the 3'-UTR
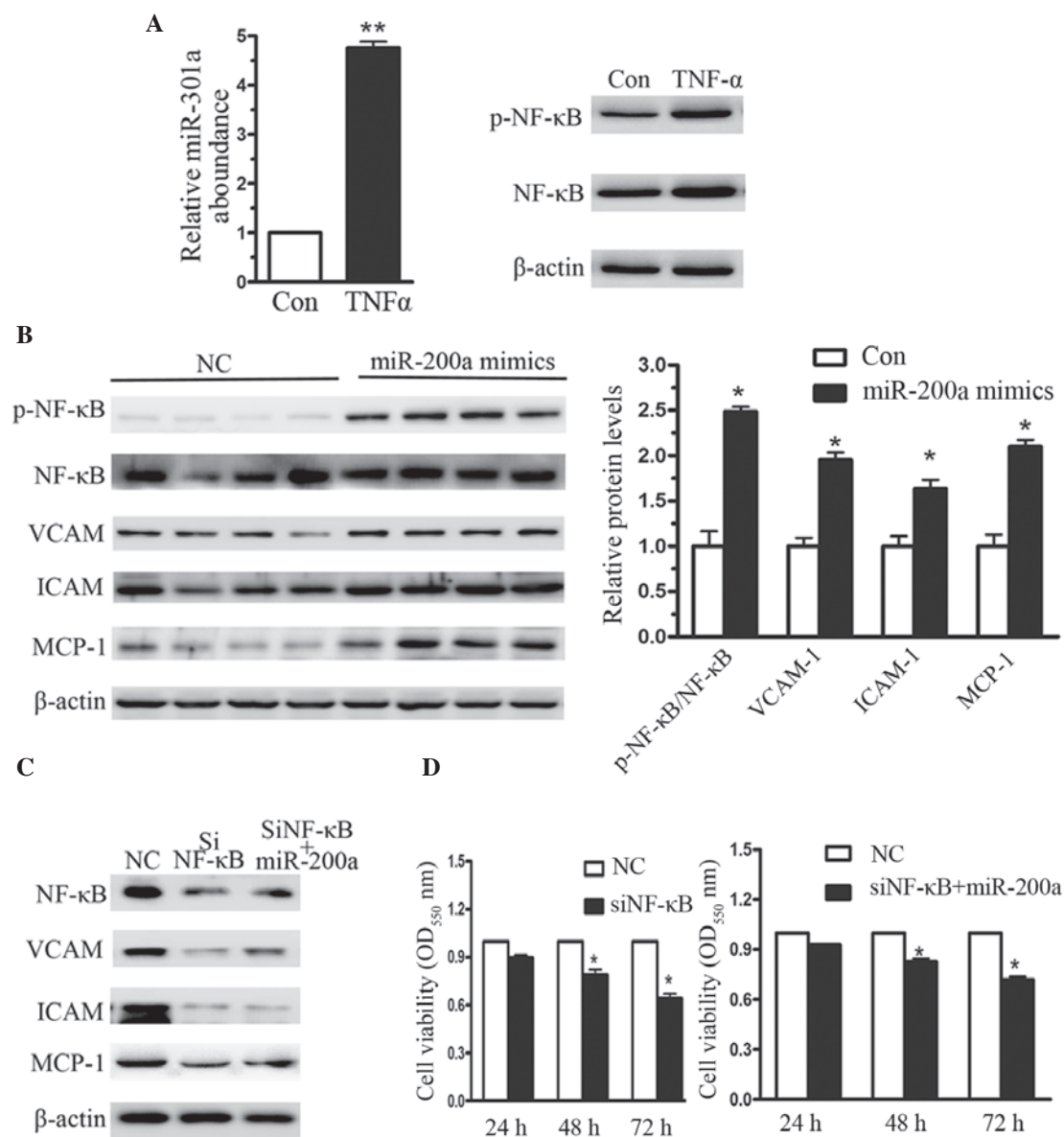
Figure 3. NF-κB signaling pathway activation with overexpression of miR-200a in CNE2 cells. (A) Reverse transcription-quantitative polymerase chain reaction was used to measure the relative levels of miR-200a when CNE2 cells were treated with 10 ng/μl TNFα for 48 h. (B) Western blot analysis of NF-κB activation and its downstream regulators following miR-200a overexpression. (C) Western blot analysis of an short interfering RNA targeting NF-κB. (D) MTT assay indicated a reduced rate of cell proliferation in cells cotransfected with si-NF-κB and miR-200a mimics. Data represent the mean ± standard error, n=3 independent experiments. *P<0.05 vs. control. NF-κB, nuclear factor-κB; miR-200a, microRNA-200a; TNFα, tissue necrosis factor α; si-NF-κB, short interfering NF-κB; Con, control; p-NF-κB, phosphorylated NF-κB; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; MCP-1, monocyte chemoattractant protein-1.

of IκBα, and indicated that miR-200a significantly reduced IκBα-3'-UTR-luciferase reporter activity (Fig. 4D). These data indicated that miR-200a induced NF-κB activation, predominantly by targeting IκBα in the human NPC cells.

Discussion

MicroRNAs have been widely demonstrated to regulate various cellular processes, in particular cancer development and progression (21). NPC is common in men and women and several studies have indicated abnormal miRNA levels in NPC (22,23). The current study reported that miR-200a was upregulated in CNE2 human NPC cells and suggests an oncogenic role for miR-200a in NPC progression.

In order to investigate the association between miR-200a and human NPC, the relative levels of miR-200a were measured in human NPC cells and tissue samples. This indicated that miR-200a was upregulated in human NPC. Furthermore, the MTT assay demonstrated that miR-200a is able to activate CNE2 cell proliferation. TNF-α treatment was observed to induce increased expression of miR-200a, with a 4-fold increase in CNE2 cells. In addition, NF-κB was activated when CNE2 cells were transfected with miR-200a mimics for 48 h, and upregulation of the downstream regulators of NF-κB signaling was observed, including that of VCAM, ICAM and MCP-1. The activation of the NF-κB signaling pathway was further validated via the investigation of the relative levels of IκBα using western blotting and a luciferase reporter assay.
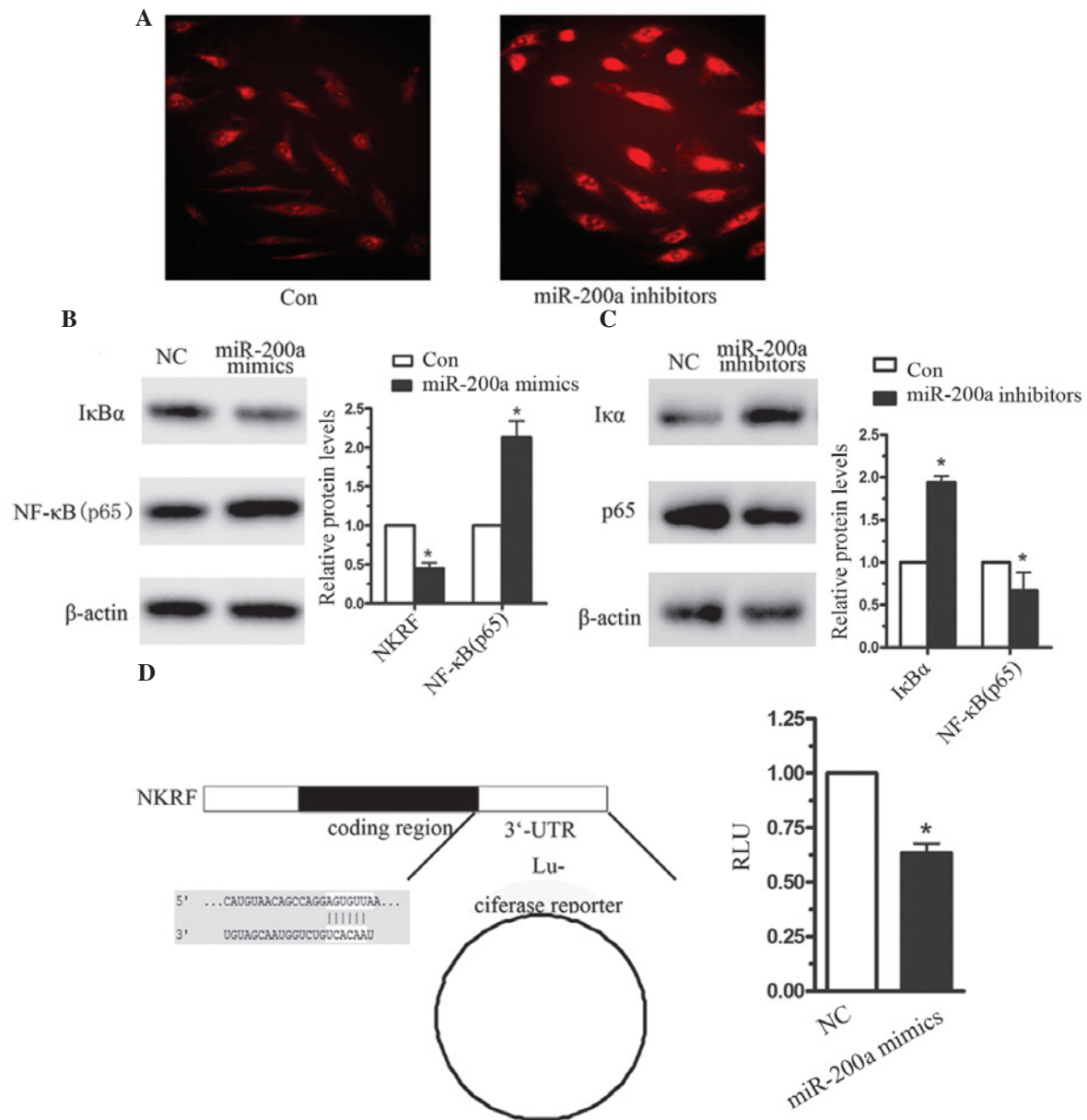
Figure 4. miR-200a targets IκBα in CNE2 cells. (A) Immunofluorescence analysis of NF-κB expression in CNE2 cells following transfection with miR-200a inhibitors or the negative control. (B) Western blot analysis of IκBα, following transfection of CNE2 cells with (B) miR-200a mimics, (C) miR-200a inhibitors and the negative control. (D) A luciferase reporter assay was used to investigate the effect of miR-200a on IκBα-3'-UTR. Data are presented as the mean ± standard error, n=3 independent experiments. *P<0.05 vs. control. miR-200a, microRNA-200a; IκB, inhibitory κB; NF-κB, nuclear factor-κB; UTR, untranslated region; NC, negative control, NKRF, NF-κB repressing factor.

Together, these data indicated that miR-200a induced NF-κB activation through the targeting IκBα.

Dysregulation of the NF-κB signaling pathway is well characterized in cancer cell proliferation, angiogenesis, migration and invasion (24,25); NF-κB signaling has been observed to be significantly activated in glioma, and the NF-κB pathway has been reported to significantly induce cancer cell proliferation and invasion in thyroid cancer. The current study provided further evidence of NF-κB activation in human NPC and investigated the association with miR-200a. These data indicated that NF-κB and its downstream regulator proteins were positively regulated by miR-200a. In addition, the current study demonstrated that IκBα is a target gene for miR-200a. IκBα has been demonstrated to repress NF-κB

translation through binding with specific negative regulatory elements (26).

In conclusion, increased levels of miR-200a were observed in the current study in human NPC tissue samples and cell lines. In addition, miR-200a was demonstrated to enhance the proliferation of CNE2 cells. Furthermore, miR-200a activated the NF-κB signaling pathway through targeting IκBα, a negative regulator of NF-κB.

Acknowledgements

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