MicroRNA-152 inhibits cell proliferation, migration and invasion by directly targeting MAFB in nasopharyngeal carcinoma

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Abstract. Aberrant expression of microRNAs (miRs) has been reported to be involved in nasopharyngeal carcinoma (NPC) carcinogenesis and development. The expression and functions of miR-152 have previously been studied in several types of cancer. However, to the best of our knowledge, no previous studies have investigated the effects of miR-152 on NPC. The present study aimed to explore the expression, functions and molecular mechanisms of miR-152 in NPC. The expression levels of miR-152 were detected in NPC tissues and cell lines using quantitative polymerase chain reaction (qPCR). Cell proliferation, migration and invasion were measured by MTT, cell migration and invasion assays, respectively. Dual-luciferase reporter assay was used to determine whether V-maf avian musculosarcomatous fibrosarcoma oncogene homolog B (MAFB) was a direct target gene of miR-152. qPCR and western blotting were used to detect the mRNA and protein expression levels of MAFB. In addition, functional assays were performed to explore the effects of endogenous MAFB on NPC. The results of the present study demonstrated that miR-152 was significantly downregulated in NPC tissues and cell lines. Furthermore, ectopic expression of miR-152 suppressed cell proliferation, migration and invasion of NPC cells. Dual-luciferase reporter assay demonstrated that MAFB was a direct target gene of miR-152, and qPCR and western blotting indicated that miR-152 negatively regulated MAFB expression at the mRNA and protein level. Knockdown of MAFB expression markedly suppressed NPC cell proliferation, migration and invasion. These findings suggested that miR-152 may target MAFB to regulate NPC initiation and progression; therefore, it may be investigated as a target for the treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a specific type of head and neck squamous cell carcinoma, which is derived from epithelial cells in the nasopharynx (1). It is a relatively common malignancy in Southeast Asia compared with other regions, and is particularly common in the Cantonese region of Guangdong, China (2). More than 84,000 novel cases of NPC are diagnosed each year, according to global cancer statistics reported by the International Agency for Research on Cancer. Among these cases, 80% are located in Asia and 5% are located in Europe (3). There are various factors that are associated with the initiation and progression of NPC, including genetic susceptibility, environmental factors and Epstein-Barr virus latent infection (4). At present, the main therapeutic strategies for the treatment of patients with NPC are radiotherapy and comprehensive chemotherapy (5). However, despite advances in the development of therapeutic strategies for the treatment of patients with primary NPC, the 5-year survival rate increased from 50% in the 1980s to 70% in the 1990s (6). Distant metastasis is the predominant cause of treatment failure, and 30-60% of patients with NPC will eventually develop distant metastasis and succumb to disseminated disease (7). Therefore, a better understanding regarding the molecular mechanisms underlying NPC metastasis is essential for determining novel therapeutic targets for the suppression of cancer metastasis.

MicroRNAs (miRNAs/miRs) belong to a group of highly conserved, endogenous, non-protein-coding small RNA molecules, which are 19-25 nucleotides in length. miRNAs negatively regulate gene expression at the post-transcriptional level by binding to the 3'-untranslated region (3'-UTR) of target mRNAs, thus resulting in mRNA degradation or translational inhibition (8-10). Previous studies have demonstrated that miRNAs serve important functions in various biological processes, including cell cycle progression, cell proliferation, migration, invasion, apoptosis, differentiation and development (11,12).
Therefore, aberrant expression of miRNAs may be involved in the pathogenesis of a wide range of human diseases, including cancer (13). It has previously been indicated that miRNAs can function as oncogenes by suppressing the expression of target tumor suppressor genes, or as tumor suppressors by inhibiting the expression of target oncogenes in tumor progression (14). Therefore, targeting miRNAs may be investigated as a novel therapeutic strategy for the treatment of patients with NPC.

The expression and functions of miR-152 have been studied in several types of cancer; however, to the best of our knowledge, no studies have elucidated the expression, functions and mechanisms of miR-152 in NPC. The present study demonstrated that miR-152 was significantly downregulated in NPC tissue samples and cell lines. The present study further explored the functions of miR-152 in cell proliferation, migration and invasion. In addition, V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) was identified as a direct target gene of miR-152. The newly identified miR-152/MAFB pathway provided information regarding the functions of miRNAs in NPC initiation and progression, and may provide a novel therapeutic strategy for the treatment of NPC.

Materials and methods

Clinical specimens. A total of 17 primary NPC specimens were obtained from patients who had undergone surgery at the Affiliated Sixth People's Hospital of Shanghai Jiao Tong University (Shanghai, China) between January 2012 and November 2014 (8 male and 9 female; age range, 15-63 years). A total of 8 normal nasopharyngeal epithelial specimens were obtained from biopsy-negative cases at the Affiliated Sixth People's Hospital of Shanghai Jiao Tong University (6 male and 2 female; age range, 18-45 years). Tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C. None of the patients with NPC had received radiotherapy or chemotherapy prior to surgery. The present study was approved by the Affiliated Sixth People's Hospital's Protection of Human Subjects Committee. Written informed consent was obtained from each patient involved in the present study.

Cell culture. Four human NPC cell lines: CNE1, SUNE1, 5-8F and CNE2, and the NP69 normal nasopharyngeal epithelial cell line were purchased from American Type Culture Collection (Manassas, VA, USA). The human embryonic kidney (HEK)293T cell line was purchased from the Chinese Academy of Sciences (Shanghai, China). NP69 cells were cultured in keratinocyte-serum-free medium (KSF, Sigma-Aldrich; Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.). CNE1, CNE2, 5-8F and SUNE1 cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) and 2% streptomycin. The cells were incubated at 37°C until further assessment.

Cell transfection. mir-152 mimics, mir-152 inhibitor, negative control (NC) mimics, NC inhibitor, MAFB small interfering (si) RNA, NC siRNA and luciferase reporter plasmid were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences were as follows: mir-152 mimics, 5'-UCAGUGCAU GACAGACGUUG-3'; mir-152 inhibitor, 5'-CAACAAGUUCU GUCAUGCAGUCA-3'; NC, 5'-UUCUCGGACUUGACUGC UTF-3'; and NC inhibitor, 5'-CAUACUUUUGUUGAUA CAA-3'. Cell transfection and co-transfection were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Post-transfection, the cells were incubated at 37°C until further assessment.

Cell proliferation assay. Cell proliferation was determined using the MTT assay (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). A total of 24 h post-transfection, cells were harvested and seeded into 96-well plates at a density of 3,000 cells/well and were incubated at 37°C. The MTT assay was performed every 24 h for 4 days, according to the manufacturer's protocol. Briefly, 20 µl MTT solution (5 mg/ml) was added to each well. Following a 4 h incubation, cell culture medium was carefully removed and the formazan precipitates were dissolved in 200 µl dimethyl sulfoxide. Absorbance was measured at 490 nm using an automatic multi-well spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were performed in triplicate.

Cell migration and invasion assays. Cell migration and invasion assays were performed using Transwell apparatus (Costar; Corning Incorporated, Corning, NY, USA); pore diameter, 8 µm. For the invasion assay, the Transwell apparatus was
pre-coated with Matrigel (BD Biosciences) according to the manufacturer's protocol. A total of 48 h post-transfection, cells were harvested and resuspended as single-cell solutions in serum-free medium. A total of 1x10⁶ cells in 200 µl serum-free RPMI-1640 medium were added to the upper chamber of the Transwell apparatus. RPMI-1640 medium (500 µl) containing 20% FBS was added to the lower chamber, thus acting as a chemoattractant. Following a 24 h incubation at 37°C, the cells were fixed in 100% methanol for 5 min and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) for 5 min. Subsequently, the cells that had not migrated or invaded through the pores were carefully removed with a cotton swab. Values for migration and invasion were obtained by counting five fields per membrane under a microscope (CKX41; Olympus Corporation, Tokyo, Japan).

Western blotting. A total of 48 h post-transfection, cells were washed with ice-cold PBS and lysed with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). Bicinchoninic Acid Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to measure the protein concentration, according to the manufacturer's protocol. Equal amounts of protein (20 µg) were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk at room temperature for 1 h. The membranes were then incubated with mouse anti-human monoclonal MAFB (1:500 dilution; cat. no. sc-376387; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit anti-human polyclonal anti-β-actin (1:1,000 dilution; cat. no. sc-1616-R; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Following washing, the bands were visualized using an enhanced chemiluminescence solution (Pierce; Thermo Fisher Scientific, Inc.). β-actin was used as a loading control. AlphaEase FC software, version 4.0.1 (ProteinSimple, San Jose, CA, USA) was used to analyze the western blotting results.

Dual-luciferase report assay. HEK293T cells were cultured in a 12-well plate until they reached 80-90% confluence. Luciferase reporter plasmids, including pGL3-MAFB-3'UTR wild-type (Wt) and pGL3-MAFB-3'UTR mutant-type (Mut), were synthesized and confirmed by Shanghai GenePharma Co., Ltd. HEK293T cells were co-transfected with miR-152 mimics or NC mimics and pGL3-MAFB-3'-UTR Wt or PGL3-MAFB-3'-UTR Mut using Lipofectamine® 2000, according to the manufacturer's protocol. A total of 48 h post-transfection, luciferase activity was detected using the Dual-Luciferase Reporter Assay system according to the manufacturer's instructions (Promega Corporation). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. Each assay was replicated three times.

Statistical analysis. Each assay was repeated a minimum of 3 times. Data are presented as the mean ± standard deviation, and were analyzed using Student's t-test with SPSS 17 software (SPSS, Inc., Chicago, IL, USA). Two-tailed P<0.05 was considered to indicate a statistically significant difference.

Results

miR-152 expression in NPC tissues and cell lines. In the present study, the expression levels of miR-152 were detected in 17 NPC biopsy specimens and eight normal nasopharyngeal epithelial specimens using RT-qPCR. As shown in Fig. 1A, miR-152 was downregulated in NPC tissues compared with normal nasopharyngeal epithelial specimens (P<0.05).

To further explore the functions of miR-152 in NPC carcinogenesis and progression, the expression levels of miR-152 were detected in NPC cell lines and the NP69 normal nasopharyngeal epithelial cell line. Compared with in NP69 cells, miR-152 was significantly downregulated in CNE1, SUNE1, 5-8F and CNE2 cells (Fig. 1B). Among these NPC cell lines, miR-152 expression was lowest in CNE1 and SUNE1 cells compared with 5-8F and CNE2 cells. Therefore, CNE1 and SUNE1 cells were used to perform further cell function experiments.

To investigate the functions of miR-152 in CNE1 and SUNE1 cells, miR-152 mimics, NC mimics, miR-152 inhibitor and NC inhibitor were transfected into CNE1 and SUNE1 cells. A total of 48 h post-transfection, RT-qPCR was performed to detect the expression levels of miR-152. As shown in Fig. 1C, miR-152 expression was significantly upregulated in CNE1 and SUNE1 cells transfected with miR-152 mimics, whereas miR-152 was downregulated in CNE1 and SUNE1 cells transfected with the miR-152 inhibitor (P<0.05).

miR-152 inhibits NPC cell proliferation. An MTT assay was performed to determine the effects of miR-152 on cell proliferation. Transfection with miR-152 mimics inhibited NPC cell proliferation, whereas miR-152 inhibitor transfection enhanced NPC cell proliferation (Fig. 2; P<0.05). These results indicate that miR-152 may function as a tumor growth suppressor in NPC.

miR-152 inhibits NPC cell migration and invasion. Transwell chambers were used to determine whether miR-152 inhibited cell migration and invasion. The number of migrated CNE1 and SUNE1 cells was significantly decreased following transfection with miR-152 mimics, as compared with cells transfected with NC mimics. Conversely, transfection with the miR-152 inhibitor had the opposite effect on migration (Fig. 3A; P<0.05). Furthermore, transfection with miR-152 mimics decreased the invasive ability of NPC cells compared with those transfected with NC mimics. Conversely, transfection with the miR-152 inhibitor enhanced cell invasive ability, as compared with cells transfected with the NC inhibitor (Fig. 3B; P<0.05). These results suggest that overexpression of miR-152 may inhibit NPC cell migration and invasion.

MAFB is a direct target gene of miR-152. TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org/microrna/) were used to predict a set of target genes for miR-152. Among these predicted targets, MAFB was selected since it was predicted by the two bioinformatic analyses. As shown in Fig. 4A, MAFB was predicted to be a direct target gene of miR-152.
To investigate whether MAFB was a direct target gene of miR-152, a dual-luciferase reporter assay was performed. As shown in Fig. 4B, miR-152 significantly inhibited luciferase activity in PGL3-MAFB-3’UTR Wt-transfected HEK293T cells, but not in PGL3-MAFB-3’UTR Mut-transfected HEK293T cells (P<0.05). These results indicate that MAFB is a direct target gene of miR-152.

miR-152 negatively regulates MAFB expression at the mRNA and protein level. To determine the association between miR-152 and MAFB expression, RT-qPCR and western blotting were performed. As presented in Fig. 5A, transfection with miR-152 mimics decreased the mRNA expression levels of MAFB, whereas the miR-152 inhibitor increased MAFB mRNA expression (P<0.05). Western blotting revealed that compared with NC mimics-transfected cells, the protein expression levels of MAFB were also significantly downregulated in CNE1 and SUNE1 cells transfected with miR-152 mimics, whereas transfection with the miR-152 inhibitor enhanced MAFB expression at the protein level (Fig. 5B; P<0.05).

MAFB is associated with the effects of miR-152 on NPC cells. The present study explored the endogenous expression of MAFB in CNE1, SUNE1 and NP69 cells by western blotting. The expression levels of MAFB were significantly upregulated in CNE1 and SUNE1 cells compared with in NP69 cells (Fig. 6A; P<0.05). It is in accord with the down-regulation of miR-152 in NPC.

To determine whether MAFB functions as an important mediator of the effects of miR-152 in NPC cells, MAFB siRNA

Figure 1. Expression of miR-152 in NPC tissues and cell lines. (A) Relative expression levels of miR-152 were decreased in NPC tissues compared with normal nasopharyngeal epithelial tissues. (B) Relative miR-152 expression levels were downregulated in NPC cell lines compared with in the NP69 normal nasopharyngeal epithelial cell line. (C) miR-152 was markedly upregulated in CNE-1 and SUNE-1 cell lines post-transfection with miR-152 mimics, whereas miR-152 was downregulated in CNE-1 and SUNE-1 cell lines post-transfection with a miR-152 inhibitor. *P<0.05 vs. respective controls. miR, microRNA; NPC, nasopharyngeal carcinoma; NC, negative control.

Figure 2. Cell proliferation was determined using the MTT assay. Transfection with miR-152 mimics significantly suppressed NPC cell growth, whereas miR-152 inhibitor transfection enhanced NPC cell growth. *P<0.05 vs. respective controls. miR, microRNA; NPC, nasopharyngeal carcinoma; NC, negative control.
Figure 3. miR-152 inhibited cell migration and invasion. (A) Cell migration assay indicated that miR-152 mimics significantly decreased cell migration, whereas the miR-152 inhibitor exerted the opposite effects. (B) Cell invasion assay indicated that miR-152 mimics-transfected NPC cells exhibited decreased invasion, whereas miR-152 inhibitor-transfected cells exhibited enhanced cell invasion. Magnification, x200. *P<0.05 vs. respective controls. miR, microRNA; NPC, nasopharyngeal carcinoma; NC, negative control.
Figure 4. MAFB was identified as a direct target gene of miR‑152 in vitro. (A) miR‑152 binding site in the 3'‑UTR of MAFB, and the MAFB 3'‑UTR Mut sequence. (B) Dual‑luciferase reporter assay demonstrated that miR‑152 decreased luciferase activity in PGL3‑MAFB‑3'UTR Wt‑transfected HEK293T cells, but not in PGL3‑MAFB‑3'UTR Mut‑transfected HEK293T cells. *P<0.05 vs. respective controls. miR, microRNA; MAFB, V‑maf avian musculoaponeurotic fibrosarcoma oncogene homolog B; 3'‑UTR, 3'-untranslated region; Wt, wild‑type; Mut, mutant; HEK293T, human embryonic kidney 293T; NC, negative control.

Figure 5. miR‑152 regulated MAFB expression at the mRNA and protein level. (A) Transfection with miR‑152 mimics decreased MAFB expression at the mRNA level, whereas the miR‑152 inhibitor increased MAFB mRNA expression. (B) miR‑152 mimics downregulated the protein expression levels of MAFB, whereas the miR152 inhibitor upregulated MAFB protein expression. *P<0.05 vs. respective controls. miR, microRNA; MAFB, V‑maf avian musculoaponeurotic fibrosarcoma oncogene homolog B; NC, negative control.
Figure 6. Effects of MAFB on CNE1 and SUNE1 cells. (A) Western blotting revealed that MAFB was upregulated in CNE1 and SUNE1 cells compared with NP69 cells. (B) Western blotting revealed that the protein expression levels of MAFB were downregulated in CNE1 and SUNE1 cells following transfection with MAFB siRNA. (C) CNE1 and SUNE1 cells transfected with MAFB siRNA exhibited significantly decreased cell proliferation. (D) Cell migration assay indicated that transfection with MAFB siRNA inhibited CNE1 and SUNE1 cell migration; magnification, x200. *P<0.05 vs. respective controls. miR, microRNA; MAFB, V‑maf avian musculoaponeurotic fibrosarcoma oncogene homolog B; siRNA, small interfering RNA; NC, negative control.
and NC siRNA were transfected into NPC cells. Western blotting was performed to explore transfection efficiency post-transfection with the siRNAs. As shown in Fig. 6B, MAFB was downregulated in CNE1 and SUNE1 cells following transfection with MAFB siRNA (P<0.05).

The results of an MTT assay demonstrated that transfection with MAFB siRNA significantly inhibited cell growth, as compared with the NC siRNA group (Fig. 6C; P<0.05). Furthermore, migration and invasion assays demonstrated that transfection with MAFB siRNA inhibited the cell migration and invasion abilities of NPC cells compared with cells transfected with NC siRNA (Fig. 6D and E; P<0.05). These results suggest that the functions of MAFB siRNA were similar to those induced by miR-152 mimics in NPC cells, thus indicating that MAFB is a functional target of miR-152 in vitro.

Discussion

miR-152 belongs to the miR-148/152 family, which also includes miR-148a, miR-148b and miR-152 (16). miR-152 is located at chromosomal region 17q21.32 (17). Aberrant expression of miR-152 has been detected in various types of human tumor, including non-small cell lung cancer (18), glioblastoma (12), gastric cancer (19), hepatocellular carcinoma (20), prostate cancer (21) and ovarian cancer (22). However, to the best of our knowledge, there have been no studies regarding the expression levels of miR-152 in NPC. The present study demonstrated that miR‑152 was significantly downregulated in NPC tissues and cell lines. This finding indicated that miR-152 may exhibit a tumor-suppressive capacity in the carcinogenesis and progression of NPC.

Aberrant expression of miR-152 is thought to contribute to the malignant phenotype of several types of tumor. For example, in non-small cell lung cancer, miR-152 was downregulated in tumor tissues and serum. The low expression levels of miR-152 were significantly associated with more aggressive tumors (18,23). Furthermore, the results of a functional assay indicated that upregulation of miR‑152 inhibited proliferation, colony formation, migration and invasion of non-small cell lung cancer cells by targeting ADAM metallopeptidase domain 17 and fibroblast growth factor 2 (16,24).

Zheng et al reported that the expression levels of miR-152 were decreased in glioma tissues. Ectopic expression of miR-152 suppressed cell invasion and angiogenesis by targeting neuropilin 2 and matrix metalloproteinase-3 (25). In gastric cancer, reduced expression of miR-152 has been observed in tumor tissues and cell lines, and restoration of miR-152 expression reduced cell growth, migration and invasion via blockade of cluster of differentiation 151 (19). Furthermore, Dang et al demonstrated that the expression levels of miR-152 were markedly downregulated in hepatocellular carcinoma, and were significantly correlated with advanced clinical stage, larger tumor size and positive hepatitis B infection. In addition, transfection with miR-152 mimics suppressed cell proliferation, migration and invasion, and enhanced caspase activity and apoptosis by directly targeting the tumor necrosis factor receptor superfamily member 6b gene (20). These findings indicated that miR-152 may serve important roles in these types of cancer, and may function as a potential therapeutic target for the treatment of specific cancers.

The present study demonstrated that miR-152 significantly inhibited NPC cell growth, migration and invasion. Since miR-152 contributed to NPC carcinogenesis and development, the present study aimed to determine the potential molecular mechanism underlying miR-152-induced inhibition of NPC proliferation, migration and invasion. Subsequently, an important molecular association between miR-152 and MAFB was detected in NPC. Initially, TargetScan and miRanda databases predicted that MAFB contained a miR‑152 seed match at position 1619‑1626 of the MAFB 3’-UTR. Secondly, a dual-luciferase reporter assay demonstrated that miR-152 directly targeted the MAFB 3’-UTR. Thirdly, RT-qPCR and western blotting revealed that miR-152 negatively regulated MAFB expression at the mRNA and protein level. Finally, knockdown of MAFB decreased NPC cell proliferation, migration and invasion, similar to the effects of miR-152. These results indicated that miR-152 may target MAFB to inhibit NPC cell growth, migration and invasion. Identification of miR-152 target genes is
essential for understanding its role in NPC carcinogenesis and progression. In addition, it is important for developing novel targeted therapies for the treatment of NPC.

The Maf oncogene was initially identified in an acutely oncogenic avian retrovirus AS42 and was isolated from a spontaneous chicken tumor (26). The Maf family encompasses three small and four large Maf proteins. These Maf proteins are divided into two groups: Large MAF proteins, including c-MAF, MAFB and MAFA; and small MAF proteins, including MAFK, MAFF and MAFG (27-29). MAFB, which is a member of the Maf protein family, is a transcription factor that shares a conserved basic region and leucine zipper motif (30). Previous studies have demonstrated that MAFB serves an important role in early tissue specification and terminal differentiation (31). The present study demonstrated that MAFB was upregulated in NPC cell lines compared with in the NP69 normal nasopharyngeal epithelial cell line. The results indicated that miR-152 may function as a tumor suppressor through the downregulation of MAFB in NPC. Therefore, the upregulation of MAFB detected in NPC may be due to the downregulation of miR-152. In addition, knockdown of MAFB significantly inhibited NPC cell proliferation, migration and invasion. The present study revealed that miR-152 negatively regulates MAFB expression to decrease NPC cell growth, migration and invasion; therefore, it may be beneficial to investigate a novel targeted therapy against MAFB in NPC.

In conclusion, the present study demonstrated that miR-152 was downregulated in NPC tissues and cell lines. In addition, miR-152 expression and MAFB knockdown inhibited cell proliferation, migration and invasion, and miR-152 suppressed the expression of MAFB at the mRNA and protein levels. These results indicated that miR-152 targets MAFB, which may be associated with NPC carcinogenesis and progression. Therefore, miR-152 and MAFB may be investigated as therapeutic targets for the treatment of NPC. Future work is required to address whether the potential of miR-152 may be fully realized in NPC treatment.

References