MicroRNA-23b regulates nasopharyngeal carcinoma cell proliferation and metastasis by targeting E-cadherin

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Abstract. MicroRNA-23b (miR-23b) is important in tumor proliferation and metastasis. In this study, it was suggested that the levels of miR-23b were increased in nasopharyngeal carcinoma (NPC) tissues compared with the adjacent normal tissues. The present study aimed to explore the role of miR-23b upregulation in NPC. Functional studies demonstrated that inhibition of miR-23b could significantly suppress NPC cell proliferation, migration and invasion. An in vitro reporter assay suggested that E-cadherin is a direct target gene of miR-23b. Furthermore, the expression of miR-23b in NPC tissues was inversely correlated with that of E-cadherin. These findings provide evidence that miR-23b is key in promoting NPC cell proliferation, migration and invasion through targeting E-cadherin, and strongly suggests that an exogenous miR-23b inhibitor may have therapeutic value in treating NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is the most common type of cancer originating in the nasopharynx, and has a higher incidence in certain regions of East Asia and Africa than in other parts of the world. NPC is caused by a combination of factors: Viral, environmental influences and heredity (1). It has been shown that NPC is sensitive to radiotherapy and chemotherapy, with a cure rate of ~70% (2,3). The viral influence is associated with infection with Epstein-Barr virus (EBV) (4). EBV-encoded RNA signals are present in all NPC cells, and early diagnosis of the disease is possible through the detection of raised antibodies against EBV. However, a number of genes are reported to contribute to the risk of NPC according to studies regarding genetic linkage and association (5,6).

Therefore, separate efforts are required to investigate the underlying molecular mechanisms of carcinogenesis.

In recent years, a class of novel non-coding RNAs termed microRNAs (miRNAs) have been identified in plants and animals. MicroRNAs (miRNAs) are 18-26 nucleotides long and post-transcriptionally regulate gene expression in multicellular organisms by affecting the stability and translation of mRNAs. In the process of tumor formation, the abnormal expression or the loss of the dynamic balance between onco-genes and tumor suppressor genes leads to tumorigenesis and cancer development. miRNAs, important regulatory factors of gene expression, are also involved in tumor formation and progression. Considerable evidence has demonstrated critical functions of miRNAs in diverse biological processes, such as proliferation (7-15), apoptosis (16-23), angiogenesis (24-30), cell differentiation (31-33), adhesion and metastasis (34) of tumor cells. Therefore, downregulation of the expression of certain miRNAs may result in the development of cancer. Previous studies have confirmed the presence of cancer-specific miRNAs in numerous types of cancers, such as breast cancer (35), lung cancer (36) and hepatocellular carcinoma (37).

The incidence of NPC involves changes in the expression of oncogenes and tumor suppressor genes. The present study aimed to determine the effects of miR-23b on the phenotypes of NPC cells as well as identify its target genes, in order to investigate the molecular mechanisms underlying the involvement of miR-23b in the initiation and progression of NPC.

Materials and methods

Patient and samples. Samples were obtained from 17 patients (3 men and 14 women) with NPC who underwent complete resection at the Huai’an First People’s Hospital from April 2010 to January 2014. NPC tissue biopsies were obtained at the time of diagnosis prior to any therapy, and the cancerous tissue sections were immediately frozen at -80°C following removal from the patients. Regarding the primary tumor stage, 5 patients had a T1-2 stage and 12 patients had a T3-4 stage according to the 2008 Chinese staging system (38). In terms of clinical stage, 7 patients were in stage I-II and 7 patients in stage III-IV (information regarding the clinical stage of the remaining patients was unavailable). The clinicopathological characteristics of the patients with NPC are summarized in Table I. The present study was approved by the ethics
committee of Huai'an First People's Hospital (Huai'an, China), and written-informed consent was obtained from all the study participants.

miRNA target prediction. TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and miRBase (http://www.mirbase.org/) were used to predict miRNA targets.

Cell culture and transfection. CNE1 and CNE2z cells (1x10⁶/ml; obtained from the department of ENT, Huai'an First People's Hospital) were cultured in RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL), 100 IU penicillin/ml (Gibco-BRL) and 0.1 mg streptomycin/ml (Gibco-BRL) in a humidified 5% (v/v) atmosphere of CO₂ at 37°C. The cells were transfected with Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. Briefly, the cells were seeded (1x10⁶) to 90% confluence at transfection, and 4 µl of Lipofectamine 2000 were diluted in 250 µl Opti-Medium Essential Medium (MEM; Invitrogen Life Technologies). miR23b mimics, miR23b control, antisense oligonucleotides (ASO) miR23b, and ASO control (2 µg of each; Guangzhou RiboBio Co., Ltd., Guangzhou, China) were then diluted in 250 µl Opti-MEM, and the diluted DNA was further diluted with Lipofectamine 2000 (1:1 ratio). The solution was incubated for 5 min at room temperature, and the DNA-lipid complex was added to the cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To detect the relative level of transcription, RT-qPCR was performed. Briefly, cDNA was generated through reverse transcription using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) with 2 µg large RNA extracted from the cells. Total RNA was separated into large and small RNAs, which were isolated using the mirVana miRNA Isolation kit (Invitrogen Life Technologies). cDNA (1 µg) was used for the amplification of E-cadherin and β-actin, which was used as an endogenous control for the PCR reaction. PCR was performed under the following conditions: 94°C for 4 min followed by 40 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, using an iQ5 Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative fold-change in the transcripts was calculated with the 2⁻ΔΔCt method (39). The primers used were as follows: E-cadherin forward, 5'-CAATCTCAAGCCTCATGG-3'; reverse, 5'-CCATCTGTTAGTACTGC-3'; β-actin forward, 5'-ATGCCCAACACAGTGTCTCTTG-3'; and reverse, 5'-TACCTCTGTTGCTGATCCACAT-3'; miR-23b forward, 5'-CGCGCCGTCGATTATATTGT-3'; and reverse, 5'-CAC ATTTAAAAACATA-3'; and U6 forward, 5'-TGCTCGCGTCCGACC-3'; and reverse, 5'-CCAGTGCAG GTTCGGAGGT-3'.

Western blotting. Cultured cells were lysed in radioimmuno-precipitation assay buffer (containing 0.1% SDS, 1% Triton X-100, 1 mM MgCl₂ and 10 mM Tris-HCl; pH 7.4; Invitrogen Life Technologies) at 4°C for 25 min. The lysates were collected and cleared by centrifugation at 10,000 g for 10 min, and protein concentration was determined. Briefly, total cell lysates (50 µg) were fractionated by 10% SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Nonspecific binding sites of membranes were saturated with 5% skimmed milk in Tris-buffered saline with Tween-20 solution (TBST; 100 mMol/1 Tris-Cl, pH 7.5; 150 mMol/1 NaCl and 0.1% Tween-20) and incubated for 2 h with antibodies at room temperature. The following antibodies were used: Monoclonal mouse anti-human E-cadherin (1:100;
cat. no. ab1416; Abcam, Cambridge, UK) and monoclonal mouse anti-human GAPDH (1:1,000; cat. no. ab8245; Abcam). After four washes with TBST, the filters were incubated with polyclonal goat anti-mouse peroxidase-conjugated secondary antibody (Sigma-Aldrich, Carlsbad, CA, USA) in 5% skimmed milk in TBST solution for 1 h at room temperature. Reactions were developed using enhanced chemiluminescence (Perkin Elmer, Waltham, MA, USA).

**Cell proliferation assay.** CNE1 cells were seeded in a 96-well plate at 8,000 cells per well the day prior to transfection. The cells were transfected with 0.2 µg/well miR-23b, anti-miR-23b or control vector (Gene Pharma, Shanghai, China). After four washes with TBST, the filters were incubated with polyclonal goat anti-mouse peroxidase-conjugated secondary antibody (Sigma-Aldrich, Carlsbad, CA, USA) in 5% skimmed milk in TBST solution for 1 h at room temperature. Reactions were developed using enhanced chemiluminescence (Perkin Elmer, Waltham, MA, USA).

**Colony formation assay.** After transfection, CNE1 cells were counted and seeded in 6-well plates (in triplicate) at 50, 60 and 75 cells per well. Fresh culture medium was provided every three days. Colonies were counted only if they contained >50 cells, and the number of colonies was counted from the 6th day after seeding. The number of colonies was counted from the 6th day after seeding. The colony formation rate was calculated and was shown (P<0.05).

**Enhanced green fluorescent protein (EGFP) reporter assay.** Cells were cotransfected with miR-23b mimics or miR-23b control, together with a pcDNA3/EGFP-E-cadherin 3'-untranslated region (UTR) or a mutant UTR with a 4 base mutation in the complementary reporter vector seed sequence (all Guangzhou RiboBio Co., Ltd.). The pDsRed2-N1 red fluorescent protein (RFP) expression vector (Clontech Laboratories, Inc., Mountain View, CA, USA) was used as an internal control. A total of 48 h post-transfection, the cells were lysed with radioimmunoprecipitation assay lysis buffer (150 mM...
miR-23b directly targets the E-cadherin 3'-untranslated region (UTR) in NPC cells. To determine the target gene mediating the function of miR-23b, bioinformatics methods were used to predict potential target genes. It was identified that the 3'UTR of E-cadherin mRNA contains miR-23b complementary binding sites (Fig. 4A). To validate that E-cadherin can be directly targeted by miR-23b, an EGFP reporter assay was performed using engineered EGFP reporter vectors that had either the wild-type 3'UTR of E-cadherin or a mutant UTR with a 4-base mutation in the complementary seed sequence (Fig. 4A). pDsRed2-NI was also cotransfected for normalization. After CNE1 cells were cotransfected with pGL3-E-cadherin-WT and miR-23b mimics or miR-control, overexpression of miR-23b significantly repressed EGFP expression, compared with the control group (Fig. 4B). By contrast, EGFP expression levels by mutants of E-cadherin 3'UTR binding sites were not influenced by overexpression of miR-23b (Fig. 4B), indicating that miR-23b could bind to the specific sites of the E-cadherin mRNA 3'UTR and negatively regulate the expression of the E-cadherin gene.
miR-23b exhibits a negative regulatory role at the E-cadherin posttranscriptional level. miRNAs regulate target genes at the post-transcriptional level by binding their target genes 3'UTR to silence the gene function (40). CNE1 cells were transfected with miR-23b in order to examine whether miR-23b depresses endogenous E-cadherin expression through translational repression, the expression of E-cadherin protein was examined by western blotting. The results showed that overexpression of miR-23b resulted in a decrease in the expression level of E-cadherin protein (Fig. 4C), suggesting that miR-23b negatively regulates endogenous E-cadherin protein expression through a translational repression mechanism. Furthermore, a high expression level of miR-23b in CNE1 cells also decreased the endogenous E-cadherin mRNA level (Fig. 4D). In the 17 pairs of NPC tissues, the expression level of E-cadherin in NPC tissues was identified to be significantly lower than that in the matched adjacent normal tissues (Fig. 5A). The expression level of E-cadherin was negatively correlated with miR-23b expression (Fig. 5B). These data suggest that miR-23b negatively regulates the expression of E-cadherin through mRNA cleavage at the post-transcriptional level.

Discussion

Transformation of malignant tumors is regulated by the synergy of multiple genes, including overexpression of oncogenes and
low expression or even loss of function of tumor suppressor genes. Recent studies have demonstrated that the regulation of oncogenes and tumor suppressor genes not only occurred at the transcriptional level, but also at the post-transcriptional level. miRNAs, as important regulatory factors, are involved in the altered gene expression that occurs in human carcinogenesis. In recent years, miRNA-mediated post-transcriptional gene silencing and its relevance in tumor formation have become the focus of attention in miRNA research. Tumor cells and normal cells have significantly different in miRNA expression profiles. The majority of miRNA genes are located in chromosomal regions that frequently display amplification, deletion or translocation in human cancer. In cancer, miRNA genes that appear in high frequency are closely associated with variability of the genome, suggesting that miRNAs may participate in cancer development and progression. Detection of differential expression of miRNAs in human NPC may determine the role of miRNAs in cancer and function of their target genes, and provide a novel direction for the diagnosis and treatment of human NPC.

The present study aimed to identify a novel miRNA that regulates the expression of E-cadherin, and evaluate its effects on cell phenotype using NPC cells. Initially, RT-qPCR was conducted and demonstrated that miR-23b was significantly upregulated in human NPC tissue, compared with the adjacent normal tissue. The results suggested that miR-23b may be important in the development of human NPC. Therefore, it was hypothesized that miR-23b was a positive factor in carcinogenesis in human NPC cells due to high expression levels of miR-23b in human NPC tissue. Cell growth viability was determined using the MTT assay to detect the correlation between miR-23b and the growth capacity of CNE1 NPC cells. The cell growth viability of CNE1 cells transfected with anti-miR-23b was significantly decreased when compared with the control group (Fig. 2B). Moreover, overexpression of miR-23b increased cell growth and viability when compared with the control group (Fig. 2B). It was also demonstrated that low expression of miR-23b significantly reduced the migration and invasion capacity of NPC cells (Fig. 3A and B).

Secondly, bioinformatics analyses predicted an miR-23b binding site on the E-cadherin transcript (Fig. 4A). Experimental evidence indicated that E-cadherin was a target of miR-23b. The ability of miR-23b to regulate E-cadherin expression was likely direct as it bound to the 3'UTR of E-cadherin mRNA complementarily to the miR-23b seed region. The EGFP fluorescence intensity of pGL3-E-cadherin-WT was specifically responsive to miR-23b overexpression (Fig. 4B). However, mutation of the miR-23b binding site abolished the effect of miR-23b on the regulation of EGFP fluorescence intensity (Fig. 4B). Conversely, endogenous E-cadherin protein expression was decreased in CNE1 cells transfected with miR-23b, while it was increased in CNE1 cells transfected with anti-miR-23b (Fig. 4C). In addition, it was observed that the change in miR-23b expression effected the E-cadherin mRNA level. These results suggested that miR-23b regulated E-cadherin protein expression.

E-cadherin is a classical member of the cadherin superfamily. Cadherins comprise of a family of calcium-dependent adhesion glycoproteins that mediate cell–cell binding to maintain differentiated tissue structure and morphogenesis. Loss of E-cadherin function or expression has been implicated in cancer progression and metastasis (41). Downregulation of E-cadherin decreases the strength of cellular adhesion within a tissue, resulting in an increase in cellular motility. This in turn may allow cancer cells to cross the basement membrane and invade surrounding tissues.

In conclusion, it was demonstrated that miR-23b is important in the regulation of E-cadherin gene expression. The effect of miRNAs on NPC cell expression occurred at the mRNA and transcriptional levels, and at least in part through targeting E-cadherin. However, miR-23b may be capable of controlling tumor-specific gene(s), consequently favoring cell growth and migration. Therefore, this study suggests that targeting miR-23b may provide a promising strategy for inhibiting tumor proliferation and metastasis.

References

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