miR-93 enhances cell proliferation by directly targeting CDKN1A in nasopharyngeal carcinoma

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Abstract. Nasopharyngeal carcinoma (NPC) is an epithelial malignancy of the head and neck with the highest incidence rate in southern China. The aim of the present study was to understand the molecular mechanisms that underlie the progression of NPC. The relative expression of miR-93 and CDKN1A was detected by the reverse-transcription quantitative PCR. Western blot analysis was applied to detect the protein levels of genes. Luciferase activity report was applied to verify the target of miRNA. Cell growth was assayed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. miR-93 was upregulated in NPC tissues and cell lines compared with normal samples. Re-expression of miR-93 promoted cell growth in vitro as determined by the MTT assay. CDKN1A was identified by luciferase reporter as a direct target of miR-93. Its expression was downregulated by miR-93. Furthermore, the results showed that the expression of miR-93 was inversely correlated with the expression of CDKN1A protein. miR-93 enhanced cell proliferation in NPC by directly targeting CDKN1A. It is suggested that miR-93/CDKN1A axis may present a new target for the treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy of the head and neck with the highest incidence rate in southern China, with a high prevalence of 20-50 cases per 100,000 individuals (1). Several factors are involved in the progression and development of NPC, including Epstein-Barr virus (EBV) infection, genetic components, environmental factors and interactions between these factors (2). Although the application of intensity-modulated radiotherapy and chemoradiotherapy has increased the 5-year overall survival rate to approximately 70%, the prognosis remains poor because of distant metastasis and recurrence (3,4). Thus, it is critical to understand the molecular mechanisms that underlie the progression of NPC in order to promote the development of novel therapeutic strategy.

MicroRNAs (miRNAs) are small non-coding RNA sequences, 19-25 nucleotides long, which negatively regulate gene expression by targeting the 3'-untranslated region (3'-UTR) of their target mRNA transcripts at the post-transcriptional level (5,6). Increasing evidence showed that miRNAs regulate a wide range of cell functions, including proliferation, migration, differentiation and apoptosis (5). Emerging evidence indicates that miRNAs are abnormally expressed in human disease, especially cancer, and they function as either tumor suppressors or oncogenes in the progression of tumors (7,8). Multiple miRNAs have been found to be dysregulated in NPC, including miR-124 (9), miR-29c (10), miR-34c (11) and miR-16 (12). These findings indicated that the abnormally expressed miRNAs may contribute to the progression and development of NPC. miR-93, derived from a paralogue (miR-106b-25), is dysregulated in several cancer types, such as epithelial ovarian carcinoma (13), ischemic heart disease (14) and bone cancer pain mouse model (15). The evidence indicates that miR-93 plays a key role in cancer progression.

CDKN1A, also known as p21, is a cyclin-dependent kinase inhibitor that inhibits the complexes of CDK2 and CDK1, and regulates the cell cycle progression at G1 and S phase (16). Its expression is tightly controlled by the tumor suppressor protein p53, through which CDKN1A mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli (17). p21 can also interact with proliferating cell nuclear antigen (PCNA) and plays a regulatory role in DNA damage repair and S-phase DNA replication (18).

In the present study, we demonstrated that miR-93 was upregulated in NPC tissues and cells. Re-expression of miR-93 promoted cell growth in vitro. CDKN1A was downregulated by miR-93 by directly targeting its 3'-UTR. The newly identified miR-93/CDKN1A axis may provide new evidence to understand the molecular mechanism of NPC and present a new target for the treatment of NPC.
Materials and methods

Tissue specimens and cell lines. A collection of 23 freshly frozen NPC biopsy specimens and 13 normal nasopharyngeal epithelial tissue specimens were obtained from the Yidu Central Hospital. The specimens were confirmed by histopathological examination. None of the patients with NPC had received chemotherapy or radiotherapy before biopsy. Written informed consent was obtained from the patients. This study was approved by the Ethics Review Committee of the Yidu Central Hospital (Shandong, China).

The human NPC cell line SUNE-1 and the human immortalized nasopharyngeal epithelial cell line NP69 were used in this study. All cells were cultured in a humidified atmosphere at 37°C with 5% CO₂.

Oligonucleotide and plasmid transfection. Cells were cultured on 6-well plates 24 h prior to transfection. The oligonucleotide was transfected into NPC cells using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA). The transfected cells were incubated at 37°C in complete medium and harvested at the indicated time-points.

Quantitative real-time PCR. qRT-PCR was used to detect miR-93 and CDKN1A expression levels. Total RNA was extracted from cells or tissues by using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. qRT-PCR was performed using agents from Takara Bio (Dalian, China) with the Stratagene Mx3000P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). For miR-93 detection, U6 was used as an internal control. For CDKN1A detection, U6 was used as an endogenous control. The PCR primers for CDKN1A were as follows: 5'-GTGGGGTTATCTCTGGTTAGG-3' and 5'-CCCTGTCCATAGCACTGC-3'. The primers for GAPDH were as follows: 5'-CCAATACGACCAAATCCGTT-3' and 5'-CCCATGCTTCGACAGTCAGC-3'. The relative expression was calculated with the 2^(-ΔΔCT). All reactions were performed in triplicate.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay was used to detect the proliferation of NPC cells. After transfection, cells were plated in 96-well plates at 1,000 cells/well and cultured for 1-5 days. Cell viability was examined once daily. On the indicated days, the MTT dye were added to the well and incubated for 4 h at 37°C. Then the medium were removed and dimethyl sulfoxide (DMSO) at 150 µl/well (Sigma Corp., Ronkonkoma, NY, USA) was added to dissolve the formazan crystals. The absorbance at 490 nm was measured on a spectrophotometer (Perkin-Elmer, Waltham, MA, USA).

Luciferase reporter assay. 293 cells were used for luciferase reporter assay. 3' UTR mutation of CDKN1A were cloned from mRNA and inserted into the downstream of luciferase expression gene in psiCHECK-2 plasmids (psi-CDKN1A-3' UTR-MUT). Cells were co-transfected with miR-93 mimic or control, and psi-CDKN1A-3'-UTR-WT or psi-CDKN1A-3'-UTR-MUT using Lipofectamine 2000 (Invitrogen). The mutation (MUT) of 3'-UTR was generated using QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies). After 24-h incubation, cells were collected and assayed for luciferase activity using the Dual-Luciferase reporter assay system (Promega Bio Systems, Sunnyvale, CA, USA). The Renilla luciferase activity was used as internal control.

Western blotting. Cells were cultured, lysed and processed for western blotting according to the standard protocols. The primary antibodies include anti-CDKN1 (1:1,000; Cell Signaling Technology, Danvers, MA, USA), anti-GAPDH (1:1,000; Bioworld Technology, Inc., Nanjing, China). Then the membrane was incubated with appropriate secondary antibody at room temperature for 1 h. Protein bands were detected using enhanced chemiluminescence system (GE Healthcare, Chicago, IL, USA).

Statistical analysis. All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). All data are presented as mean ± SD. Differences between groups were assessed using the Student’s t-test or Tukey’s post hoc test after one-way analysis of variance in SPSS. Differences were considered significant at P<0.05.

Results

miR-93 is upregulated in NPC tissues and cell lines. To study the expression level of miR-93 in NPC, 23 freshly frozen NPC biopsy specimens and 13 normal nasopharyngeal epithelial...
tissue specimens were analyzed by qRT-PCR. The expression of miR-93 was increased by ~2-fold in 23 NPC tissues compared with 13 normal tissues (Fig. 1A). Furthermore, we detected the expression of miR-93 in SUNE-1 NPC cell lines. The qRT-PCR results revealed that miR-93 was significantly upregulated in SUNE-1 cell line compared with the normal nasopharyngeal epithelial cell line NP69 (Fig. 1B). Taken together, miR-93 was aberrantly upregulated in NPC tissues and cell lines.

**miR-93 promotes NPC cell proliferation in vitro.** To further understand the biological functions of miR-93 in the development of NPC, we re-expressed miR-93 or inhibited miR-93 expression separately. The successful re-expression or inhibition of miR-93 was confirmed by qRT-PCR (Fig. 2A). MTT assay showed that re-expression of miR-93 significantly promoted cell proliferation and miR-93 inhibitor significantly decreased cell growth. *P<0.05, **P<0.01, ***P<0.001.

**CDKN1A is a direct target of miR-93.** To explore the downstream of miR-93, we performed bioinformatics analysis by using two online algorithms, TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do) to predict its putative mRNA targets. CDKN1A was identified as a direct target, and a highly conserved putative binding site was found at 468-474 bp of CDKN1A 3'-UTR (Fig. 3A). Thus, we further performed Dual-Luciferase reporter assay to confirm the prediction. In cells co-transfected with psi-CDKN1A-3'UTR-WT and miR-93 mimic, luciferase activity was dramatically decreased compared with cells co-transfected with miR-control and miR-93 inhibitor increased CDKN1A expression. *P<0.05, **P<0.01.

![Figure 2. Re-expression of miR-93 promotes NPC cell growth in vitro.](image)

(A) The successful re-expression of miR-93 and inhibition of miR-93 in SUNE-1 cells were confirmed by qRT-PCR. (B) MTT assay showed that re-expression of miR-93 significantly promoted cell growth and miR-93 inhibitor significantly decreased cell growth. *P<0.05, **P<0.01, ***P<0.001.

![Figure 3. CDKN1A is a direct target of miR-93.](image)

(A) The putative binding site of miR-93 was at 468-474 bp of CDKN1A 3'-UTR. (B) Luciferase activity assay. Firefly luciferase activity was decreased in 293T cells co-transfected with miR-93 mimic and WT 3'-UTR reporter. The inhibition of miR-93 was abolished in cells cotransfected with miR-93 mimic and MUT 3'-UTR reporter. Luciferase activity was normalized to Renilla. (C and D) CDKN1A expression fold change was detected by qRT-PCR and western blot analysis. Re-expression of miR-93 decreased CDKN1A expression and miR-93 inhibitor increased CDKN1A expression. *P<0.05, **P<0.01.
Dysregulation of miRNAs has been reported in many types of cancer. Increasing evidence indicate that miRNAs function as oncopgenes or tumor suppressors. Although the tumorigenesis of NPC is a multistage process relying on the control of the gene expression, several dysregulated miRNA expression profiles and dysregulated genes of NPC have been identified in some studies (19-21). Several miRNAs are identified upregulated, including miR-206, miR-99a, miR-30a and some are down-regulated, such as let-7i, miR-7 and miR-222 (21). The capability to regulate proliferation, invasion and migration is considered as an important determinant in the process of tumor progression. In the present study, we demonstrated that the expression of miR-93 was significantly increased in NPC cell lines and tumor tissues. The evidence indicates that miR-93 was upregulated in NPC, which is consistent with the results obtained from bone cancer (15), myocardial ischemia/reperfusion (I/R) injury (14) and polycystic ovarian syndrome (22). The enhanced proliferation of NPC cell lines indicated that miR-93 was an oncogene in NPC. To date, the ectopic expression of miR-93 is shown as a frequent epigenetic tumor promoter in various types of cancer (23-25). Furthermore, aberrant expression of miR-93 has been demonstrated to regulate tumor migration and invasion (26). The evidence indicates that miR-93 exerts pivotal biological and pathological functions, which may contribute to the development and progression of NPC.

miRNAs exert their function by regulating their target genes by binding to the 3′-UTR. Several studies have identified the target genes of miR-93, including RAB11 family interacting protein 1 (27), PTEN (14), PDCD4 (24), Sirt7 and Tbx3 (28). In the present study, we identified CDKN1A as a direct target of miR-93 by bioinformatics analysis and luciferase activity report, which is consistent with the findings in polycystic ovarian syndrome (22). Re-expression of miR-93 significantly reduced CDKN1A expression and silencing of miR-93 increased CDKN1A expression. Furthermore, we demonstrated that the expression of miR-93 was inversely correlated with the expression of CDKN1A by directly targeting its 3′-UTR.

The expression of miR-93 is inversely correlated with the expression of CDKN1A. To further investigate the expression of CDKN1A, 23 freshly frozen NPC tissues and 13 normal nasopharyngeal epithelial tissue specimens were detected to analyze the clinicopathological significance. The relative expression of CDKN1A in NPC tissues were significantly decreased compared with that in normal tissues as assayed by qRT-PCR (Fig. 4A). Furthermore, we detected the relationship between the endogenous expression levels of CDKN1A and miR-93 in the same NPC tissues. The results showed a significant inverse correlation between the expression of miR-93 and CDKN1A in NPC tissues (r=-0.62; P<0.05) (Fig. 4B).

**Discussion**

Dysregulation of miRNAs has been reported in many types of cancer. Increasing evidence indicate that miRNAs function as

Figure 4. The expression of miR-93 is inversely correlated with the expression of CDKN1A. (A) CDKN1A expression was significantly downregulated in NPC tissues compared with normal samples. (B) The expression of miR-93 was inversely correlated with the expression of CDKN1A. **P<0.01.**

psi-CDKN1A-3′UTR-WT. However, the inhibition of luciferase activity was abolished when cells were co-transfected with miR-93 mimic and psi-CDKN1A-3′UTR-MT (Fig. 3B).

Besides, re-expression of miR-93 in SUNE-1 cells could reduce the expression of CDKN1A at both mRNA and protein levels and silencing of miR-93 could increase endogenous CDKN1A at both mRNA and protein levels in SUNE-1 cells (Fig. 3C and D). The evidence indicated that miR-93 downregulated CDKN1A by directly targeting its 3′-UTR.

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