Original Article
miR-106b promoted growth and inhibited apoptosis of nasopharyngeal carcinoma cells by suppressing the tumor suppressor PTEN

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Abstract: The cell proliferation and apoptosis are crucial to cancer progression. Although multiple miRNAs have been known to regulate the proliferation and apoptosis in nasopharyngeal carcinoma (NPC), the role of miR-106b in cancer progression remains unknown. In the present study, we have discovered the involvement of miR-106b in the cell proliferation and apoptosis of NPC cells. Initially, we observed that miR-106b was highly expressed in tumor tissues and lymph node metastasis NPC samples. Subsequently, we demonstrated that miR-106b enhanced cell proliferation and apoptosis in *vitro*, and cell proliferation *in vivo*. Furthermore, mechanistic studies revealed that miR-106b targeted a major human tumor suppressor PTEN, modulating PI3K/Akt signaling, which are responsible for cell proliferation and apoptosis. Thus, this study shows a mechanism by which miR-106b modulated the cell proliferation and apoptosis of NPC cells, and a clinical implication of miR-106b as a potential biomarker or therapeutic target.

Keywords: miR-106b, PTEN, proliferation, apoptosis, NPC

Introduction

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma derived from epithelial cells located in the nasopharynx, which is highly malignant with local invasion and early distant metastasis [1]. And, NPC is a rare malignancy in most parts of the world but is common in southern China [2]. MicroRNAs (miRNAs) are a family of small non-coding RNA molecules of about 20-23 nucleotides in length, which negatively regulate protein-coding genes at post-transcriptional level including NPC [3, 4].

Some miRNAs play key roles in tumorigenesis, progression, invasion or metastasis of NPC. miR-200a-mediated downregulation of ZEB2 and CTNNB1 differentially inhibits NPC cell growth, migration and invasion [5]. MiR-634 sensitizes nasopharyngeal carcinoma cells to paclitaxel and inhibits cell growth both in vitro and in vivo [6]. miR-141 is involved in a nasopharyngeal carcinoma-related genes network [7]. miR-29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins [8]. miR-26a inhibits cell growth and tumorigenesis of nasopharyngeal carcinoma through repression of EZH2 [1]. Furthermore, a large number of microRNAs (miRNAs) have been described for Epstein-Barr virus (EBV), and their expression may vary between the different latency states found in normal and malignant tissue [9]. EBV-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5 [10]. The EBV BART microRNAs target the pro-apoptotic protein Bim [11]. EBV-encoded miR-BART3* microRNA targets the DICE1 tumor suppressor in nasopharyngeal carcinoma [12].

To date, there is no evidence that miR-106b has a functional role in NPC tumorigenesis. In this study, we have characterized miR-106b in NPC and found upregulation of miR-106b promotes malignant progression of NPC cells by targeting crucial tumor suppressor gene PTEN.

Materials and methods

Clinical tissues and cell culture

We made a collection of tumor specimens from 32 patients with NPC and 16 patients without NPC from the Linyi People's Hospital (Linyi, Shandong, China). Written informed consents were obtained from the patients, in accordance with the institutional guidelines. And the study was approved by the Committees for the Ethical Review of Research at the Linyi City People's Hospital. Fresh-frozen samples were used for experiment. The human nasopharyngeal carcinoma cell lines CNE1, CNE2, SUNE1, and NP-69 (Xingu biological technology, shanghai, china) were cultured in DMEM Medium (Life Technologies, Carlsbad, USA) supplemented with 10% FBS (Life Technologies, Carlsbad, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 incubator at 37°C.

Plasmids and transfection

Sequences with wild type or mutant putative target site of the PTEN 3'-UTR were subcloned into the luciferase reporter plasmid pEZX-MT01 vector (Gene Copoeia, Maryland, USA). All sequences were verified by sequencing. Cells were transfected by using Lipofectamine2000 kit (Invitrogen, Carlsbad, USA) with miR-106b mimics or inhibitors and their controls (GenePharma, Shanghai, China) according to the manufacturer's instructions.

RNA isolation and real-time PCR

Total RNAs were isolated from tissues or cells by using TRIzol (Invitrogen, Carlsbad, USA) and microRNAs enrichment were conducted by using a miRVana miRNA isolation kit (Ambion, Austin, USA), according to the manufacturer's instructions. microRNAs were reverse transcribed to cDNAs using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, San Diego, USA) according to the manufacturer's protocol. Then cDNAs were used as template for the real-time PCR by using Step-OnePlus™ real-time PCR instruments (Applied Biosystems, San Diego, USA). The miR-106b primers were purchased from Taqman (Applied Biosystems, San Diego, USA). Real-time PCR of PTEN mRNA examination was used SYBR Green (Takara, Japan) according to the manufactur-

er's instructions. The primers for PTEN were shown as below: Forward primer: 5'-TGGATTC-GACTTAGACTTGACCT-3', Reverse primer: 5'-GG-TGGGTTATGGTCTTCAAAAGG-3'.

Western blot assay

Total proteins were extracted from the cultured cells or tissues and quantified using a BCA Protein Assay Kit (Beyotime, Jiangsu, China) with BSA as a standard. Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% BSA (5% w/v in PBS + 0.1% Tween 20) and incubated with primary antibodies at room temperature. The antibodies which are against PTEN, p-AKT, and GAPDH were used according to the manufacturer's instructions, and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After using the secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) at 1:2,000 (v/v) dilutions in PBS + 0.1% Tween 20, the signals were detected by Super Signal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, USA) according to manufacturer's instructions.

Luciferase assay

CNE1 and CNE2 cells were co-transfected with luciferase reporter plasmid pEZX-MT01 (Gene-Copoeia, Maryland, USA), and the miR-106b mimics and controls. Twenty-four hours after transfection, Renilla luciferase activities were measured using a Luc-Pair™ miR Luciferase Assay Kit (GeneCopoeia, Maryland, USA). Each transfection was performed in triplicate and repeated three times.

Cell proliferation and apoptosis assay

Cell proliferation was performed with Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan) according to the instructions. Cell Counting Kit-8 reagent was added at 0, 24, 48, and 72 h respectively after seeding 5×10^3 cells per well in a 96-well plate, and incubated at 37°C for 2 h. The OD (optical density) 450 nm value was detected by using a microplate reader (Bio-Rad, Richmond, CA, USA). Apoptosis was evaluated with Annexin V FITC assay kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. The flow cytometry was performed with FACS Calibur (BD Bioscience, USA).
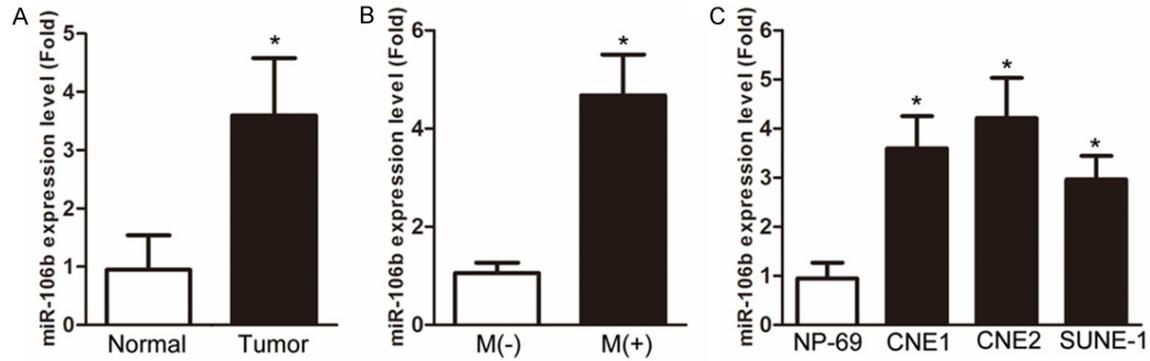
Figure 1. Expression of miR-106b in NPC tissues and cell lines. A. Relative expression of miR-106b in non-tumor tissues (n = 16) and NPC tissues (n = 32). Real-time PCR was used to determine the expression of miR-106b and normalized to U6 expression. Normal, non-tumor tissues; Tumor, tumor tissues. B. The relative expression of miR-106b in patients without and with lymph node metastasis. Data are means ± SEM. *, $P < 0.05$. M, lymphnode metastasis. C. The relative expression of miR-106b in normal nasopharyngeal epithelial cell line (NP-69) and NPC cell lines (CNE1, CNE2, and SUNE-1). Data are means ± SEM. *, $P < 0.05$. (n = 3).

Nude mice model

Nude mice were purchased from the Vital River Laboratories (Beijing, China). All animals were used in accordance with institutional guidelines and the current experiments were approved by the Use Committee for Animal Care from Shandong University. The harvested cell suspension was washed twice by centrifugation in medium containing serum at room temperature and then resuspended in medium without serum at 4°C immediately prior to i.p. injection. 10^5 cells were suspended in 0.1 ml PBS. The animals were sacrificed 3 weeks after injection.

Statistical analysis

All results were expressed as the mean ± SEM. The Student's t-tests were used to analyze significant differences between samples. All the histogram was evaluated by performing Graph-Pad Prism, version 4.0 (GraphPad Software, USA). $P < 0.05$ indicated statistically significant.

Results

miR-106b was overexpressed in tissues and cell lines from NPC patients

To explore the expression of miR-106b, real-time PCR was performed. Our results showed that the expression of miR-106b intissues from NPC patients was significantly higher than that in tissues from normal group (**Figure 1A**). The expression of miR-106b was enhanced in the

advance of clinical tumor-node-metastasis stage (**Figure 1B**). Additionally, miR-106b expression in NPC cell lines CNE1, CNE2, and SUNE-1 was higher compared with that in normal nasopharyngeal epithelial cell line NP-69 (**Figure 1C**). These data shown that miR-106b was overexpressed in tissues and NPC cell lines.

miR-106b stimulated NPC cell growth and inhibited apoptosis in vitro

To investigate the effect of miR-106b on the cell growth and apoptosis of NPC cells, we transfected miR-106b mimics and inhibitors in CNE1 and CNE2 cell lines. After transfection, the miR-106b expression was increased with miR-106b mimics treatment and reduced miR-106b levels with miR-106b inhibitors in CNE1 and CNE2 cells (**Figure 2A**). The results of CCK-8 assay showed that miR-106b mimics led to an increase growth and proliferation in CNE1 and CNE2 cell. By contrast, miR-106b inhibitors decreased CNE1 and CNE2 cell growth and proliferation (**Figure 2B**). Flow cytometry data indicated that overexpression of miR-106b significantly inhibited cell apoptosis compared with the control in CNE1 and CNE2 cells. Conversely, cell apoptosis increased in CNE1 and CNE2 cells with miR-106b inhibitors treatment (**Figure 2C**). These results demonstrated that miR-106b stimulated NPC cell growth and inhibited apoptosis *in vitro*.

miR-106b promotes tumor growth in vivo

The equal numbers (10^5) of CNE1 cells treated with miR-106b mimics or the control were sub-
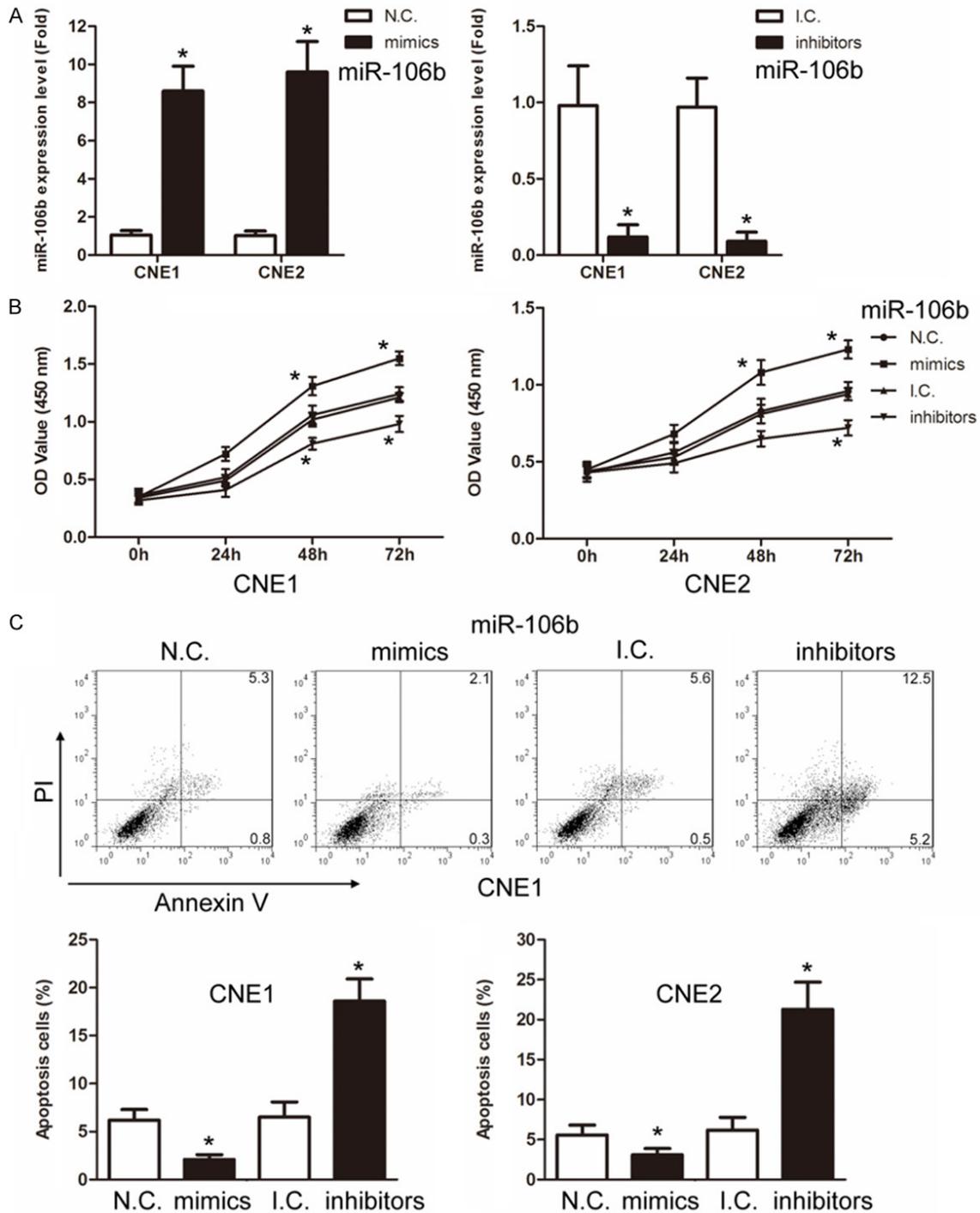
Figure 2. The effect of miR-106b on NPC cell growth and apoptosis *in vitro*. A. Relative expression of miR-106b in CNE1 and CNE2 cells transfected by negative control (N.C.), miR-106b mimics, inhibitor control (I.C.), or miR-106b inhibitors detected by real-time PCR. The results were normalized to U6 expression and corresponding negative controls. Data are means ± SEM. *, $P < 0.05$. (n = 3). B. Proliferation of CNE1 and CNE2 cells transfected by negative control (N.C.), miR-106b mimics, inhibitor control (I.C.), or miR-106b inhibitors. Data are means ± SEM. *, $P < 0.05$. (n = 3). C. The apoptosis of CNE1 and CNE2 cells transfected with negative control (N.C.), miR-106b mimics, inhibitor control (I.C.), or miR-106b inhibitors. Data are means ± SEM. *, $P < 0.05$. (n = 3).

cutaneously injected into nude mice. The results of tumor volume revealed that miR-106b treatment led to larger tumors in CNE1 cells treated with miR-106b mimics (**Figure 3A**
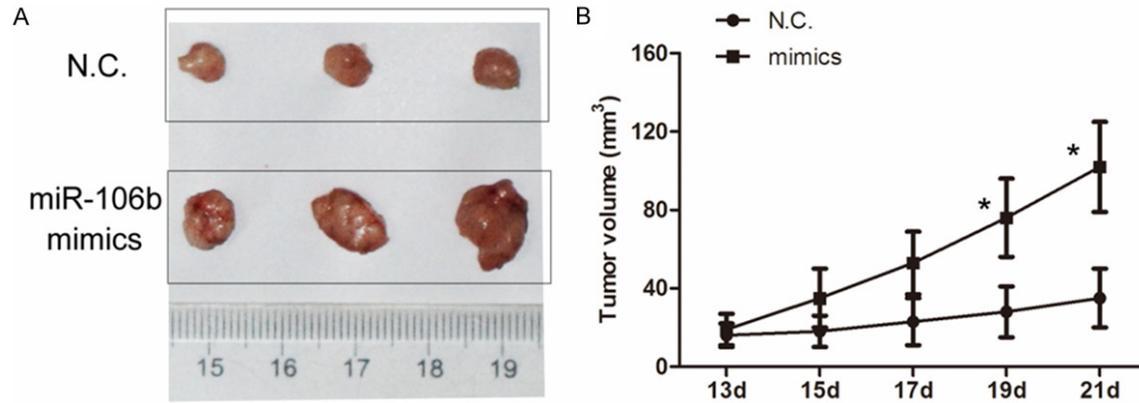
Figure 3. The effect of miR-106b on CNE1 cell growth in nude mice. A. CNE1 cells (10^5 cells/tumor), treated with negative control (N.C.) and miR-106b mimics with transfection reagent Lipofectamine2000, were injected subcutaneously into the right abdomen of nude mice. Photographs of tumors from miR-106b mimics and control groups. B. Growth curves for CNE1 cells treated with negative control (n = 6) and miR-106b mimics (n = 6) obtained from nude mice. *, $P < 0.05$.

and **3B**) than CNE1 control cells in nude mice. These results demonstrated that miR-106b promoted tumor growth *in vivo*.

miR-106b regulates the PI3K/Akt signaling via targeting the PTEN tumor suppressor gene

We further explored how miR-106b could promote cell growth and inhibited apoptosis. Potential target genes of miR-106b were predicted from TargetScan and miRanda. Phosphatase and tensin homolog (PTEN) was one of the potential candidates, which is an important tumor suppressors associated with the growth and apoptosis in various cancers including NPC. Bioinformatics analysis showed that the 3'-UTR of PTEN was well matched with the sequence of miR-106b (**Figure 4A**). Then, we cloned a sequence with the predicted target sites of miR-106b or a mutated sequence with the predicted target sites to the pEZX-MT01luciferase reporter vector. Furthermore, we employed Luciferease assay and Western blotting assay to confirm whether miR-106b directly target the 3'-UTR of PTEN mRNA. After cells were transfected with miR-106b mimics, the luciferase activity of WT vector was significantly decreased compared with mutant vector. However, there was no significant difference between WT and mutant vector when transfected with negative control of miR-106b (**Figure 4B**). Furthermore, cells transfected with miR-106b inhibitors increased the levels of PTEN mRNA and protein expression (**Figure 4C** and

4D). And, the level of phosphorylated AKT was decreased when cells were transfected with miR-106b inhibitors (**Figure 4D**). These data suggested that miR-106b regulated PTEN expression at the post-transcriptional level and influenced the phosphorylation of AKT.

Discussion

The diagnosis and treatment of nasopharyngeal carcinoma (NPC) have grown markedly during the past decade with the advent of molecular markers [13, 14]. Specific miRNAs have important roles in NPC carcinogenesis [15], and the documentation of tumor-related miRNAs is critical for understanding the function of miRNAs in NPC development, and may reveal novel prognostic and therapeutic targets for NPC treatment [16]. Here, we discovered that miR-106b stimulated NPC cell growth and inhibited apoptosis via targeting the PTEN gene to regulate the PI3K/Akt signaling.

The phosphatase and tensin homolog (PTEN) gene functions as a negative regulator of the PI3K/Akt pathway via dephosphorylation of PI(3,4,5)P3 [17], ultimately participating in regulation of the cell cycle, proliferation, apoptosis, cell adhesion, and EMT during embryonic development and cancer progression [18, 19]. The tumor suppressor PTEN could inactivate the PI3K/AKT/GSK3β pathway in NPC, leading to NPC tumorigenesis, progression, invasion or metastasis [20]. miR-21 regulates expression of the PTEN tumor suppressor gene in human
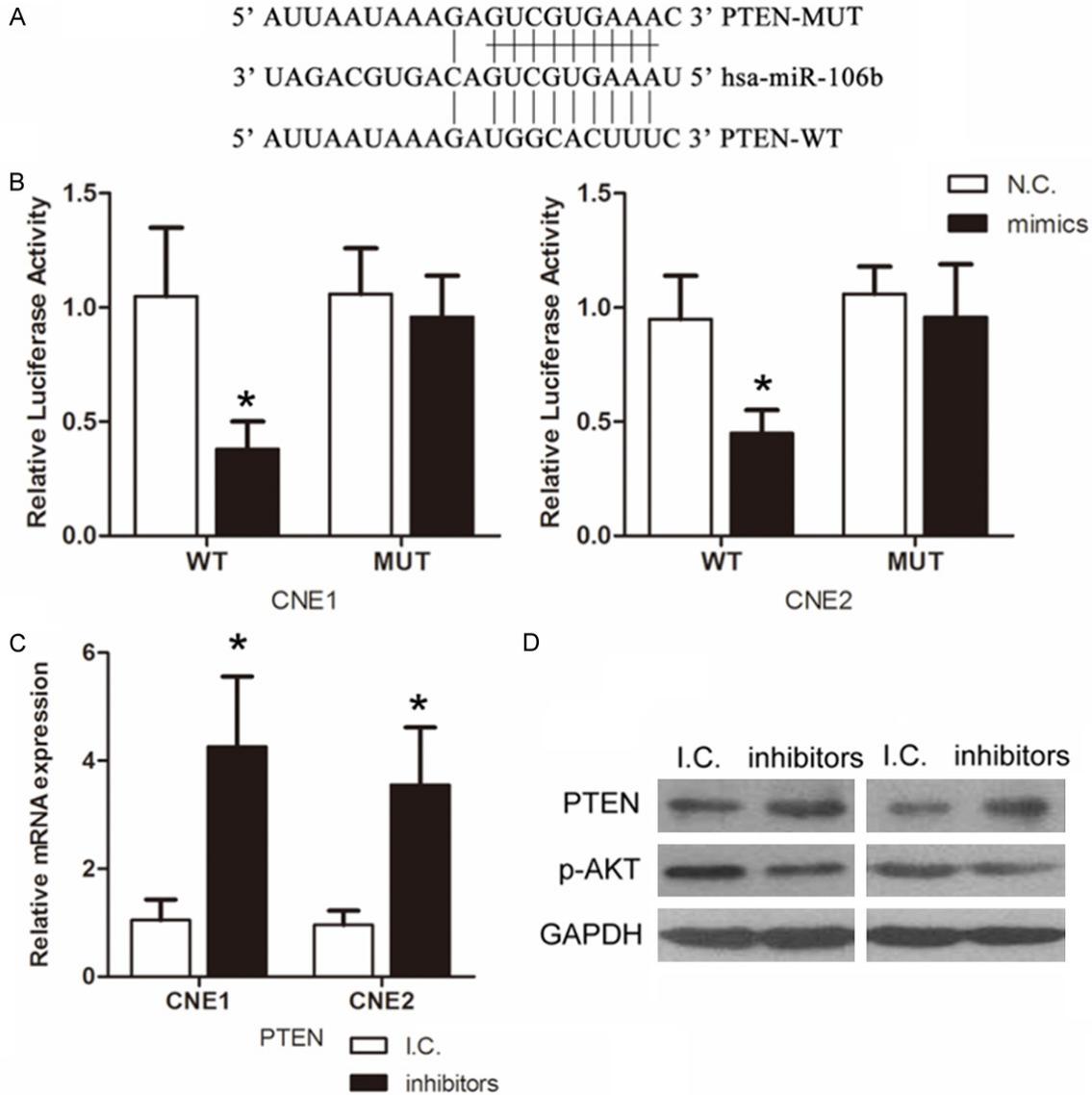
Figure 4. miR-106b regulated PTEN expression at the post-transcriptional level and influenced the phosphorylation of AKT. A. miR-106b and its putative binding sequences in the 3'-UTR of PTEN. Mutation was generated in the complementary site that binds to the seed region of miR-106b. B. Relative luciferase activity of wild type (WT) or mutant (MUT) reporter plasmid co-transfected into CNE1 and CNE2 cells with negative control (N.C.) and miR-106b mimics. Luciferase activity was normalized to that of the control group to obtain relative luciferase activity. Data are means ± SEM. *, $P < 0.05$. (n = 3). C. Expression of PTEN mRNA examined by real-time PCR in CNE1 and CNE2 after treatment with inhibitor control (I.C.) or miR-106b inhibitors. Data are means ± SEM. *, $P < 0.05$. (n = 3). D. Expression of PTEN and p-AKT examined by Western blot in CNE1 and CNE2 after treatment with inhibitor control (I.C.) or miR-106b inhibitors. The results were normalized to GAPDH protein expression and expressed as fold change relative to the corresponding negative control. Data are means ± SEM. *, $P < 0.05$. (n = 3).

hepatocellular cancer [21]. miR-205 regulates A549 cells proliferation by targeting PTEN [22]. miR-106a promotes growth and metastasis of non-small cell lung cancer by targeting PTEN [23]. However, to date, only a few miRNAs have been implicated in NPC progression. miR-205 determines the radioresistance of human naso-

pharyngeal carcinoma by directly targeting PTEN [24]. Some miRNAs have been found to target PTEN in NPC. miR-144 promotes cell proliferation, migration and invasion in nasopharyngeal carcinoma through repression of PTEN [25]. Activation of miR-21 by STAT3 induces proliferation and suppresses apoptosis in naso-

pharyngeal carcinoma by targeting PTEN gene [26]. EBV-miR-BART7-3p promotes the EMT and metastasis of nasopharyngeal carcinoma cells by suppressing the tumor suppressor PTEN [27]. Epstein-Barr virus-encoded microRNA BART1 induces tumour metastasis by regulating PTEN-dependent pathways in nasopharyngeal carcinoma [28]. Combining the research data with ours, it can be supposed that several miRNAs including miR-106b targeted PTEN gene to enhance NPC cell growth and inhibited apoptosis; as a result, they deserve to be fully explored in the future.

miR-106b is an oncogenic miRNA which is overexpressed in various types of cancer. miR-106b regulates p21/CDKN1A and promote cell cycle progression [29]. Expression of miR-106b in conventional renal cell carcinoma is a potential marker for prediction of early metastasis after nephrectomy [30]. miR-106b aberrantly expressed in a double transgenic mouse model for Alzheimer's disease targets TGF-beta type II receptor [31]. miR-106b promotes cell proliferation via targeting RB in laryngeal carcinoma [32]. Down-regualtion of miR-106b induces epithelial-mesenchymal transition but suppresses metastatic colonization by targeting Prrx1 in colorectal cancer [33]. miR-106b impairs cholesterol efflux and increases A beta levels by repressing ABCA1 expression [34]. miR-106b and miR-15b modulate apoptosis and angiogenesis in myocardial infarction [35]. miR-106b downregulates adenomatous polyposis coli and promotes cell proliferation in human hepatocellular carcinoma [36]. miR-106b regulates the tumor suppressor RUNX3 in laryngeal carcinoma cells [37]. Over-expression of miR-106b promotes cell migration and metastasis in hepatocellular carcinoma by activating epithelial-mesenchymal transition process [38]. Down-regulation of miR-106b suppresses the growth of human glioma cells [39]. miR-106b modulates epithelial-mesenchymal transition by targeting TWIST1 in invasive endometrial cancer cell lines [40]. miR-106b-5p boosts glioma tumorigensis by targeting multiple tumor suppressor genes [41]. The expression of miR-106b in colonic cancer was increased in the in situ hybridization analysis [42]. miR-106b-mediated down-regulation of C1orf24 expression induces apoptosis and suppresses invasion of thyroid cancer [43]. In the present study, we found that miR-106b upregulated in NPC cells,

and it had a key role in determining whether PTEN promoted or inhibited cell proliferation.

The data herein provide new mechanistic insights into the oncogenic functions of miR-106b in NPC tumorigenesis. Moreover, miR-106b has important roles in the NPC cell growth and apoptosis by directly suppressing the tumor suppressor PTEN, which provides a molecular basis for the regulation of PI3K/Akt and a better understanding of the PTEN in NPC.

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Disclosure of conflict of interest

None.

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