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microRNA-222 promotes tumor growth and confers radioresistance in nasopharyngeal carcinoma by targeting PTEN

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Abstract. MicroRNA-222 (miR-222) has been reported to be involved in the initiation, development and metastasis of tumors, as well as conferring resistance to chemotherapeutic drugs or radiotherapy in various types of cancer. However, the role and the underlying molecular mechanism of miR-222 specifically in nasopharyngeal carcinoma (NPC) remains unclear. Thus, the biological function and underlying mechanism of in miR-222 was investigated in NPC tissue specimens and cell lines. miR-222 was upregulated in NPC tissues and malignant cell lines compared with adjacent normal samples and cell lines. miR-222 upregulation significantly increased NPC cell proliferation, colony formation and cell apoptosis. Furthermore, miR-222 upregulation conferred radioresistance. It was also confirmed that phosphatase and tensin homolog (PTEN) was a direct target for miR-222 in NPC cells. Alteration of miR-222 expression was demonstrated to regulate the phosphoinositide 3-kinase/protein kinase B pathway in NPC cells. These results suggest that miR-222 may act as an oncomir in NPC by targeting PTEN, and has potential as a therapeutic target in NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant cancer derived from epithelial cells located in the nasopharynx (1). Radiotherapy is one of the most common treatment options for patients with NPC (2). However, due to distant metastases and local recurrence in some patients with NPC (3), radioresistance can be a serious obstacle to therapy success. Therefore, there is an urgent medical need to understand the molecular mechanisms of NPC progression and radioresistance, to develop novel diagnostic and therapeutic strategies that can potentially enhance tumor cell radiosensitivity.

MicroRNAs (miRNAs) are a class of endogenous single stranded non-coding RNAs of 18-25 nucleotides in length that regulate gene expression by binding to the 3'untranslated regions (3'UTRs) of target mRNAs (4,5). Accumulating evidence has demonstrated that miRNAs regulate numerous physiological processes, including cell proliferation, cell cycle stage, apoptosis, migration, invasion and differentiation (6,7). Growing evidence supports the critical role of miRNAs in the progression of human cancers, where they function as either oncogenic miRNAs (oncomirs) or tumor suppressors through the regulation of cellular proliferation, differentiation and apoptosis (8-10). A number of studies have demonstrated that miRNAs are involved in cellular ionizing radiation (IR) responses through cell cycle regulation and the apoptosis signal pathway in several types of cancer, including NPC (11-13).

miRNA-222 (miR-222) has been demonstrated to function as an oncomir in various types of human cancer, with effects on cell growth, oncogenesis, invasion, migration and drug resistance of tumor cells (14-18). A recent report revealed that miR-222 confers radioresistance in glioblastoma cells through the activation of the protein kinase B (AKT) signaling pathway, independent of phosphatase and tensin homolog (PTEN) status (17). However, the detailed function of miR-222 in NPC remains unclear. The present study investigated the role of miR-222 in NPC carcinogenesis, particularly in NPC radioresistance.

Materials and methods

Cell culture and tissue samples. The three human NPC cell lines (HONE-1, C666-1 and TWO3) and the NP69 human immortalized nasopharyngeal epithelial cell line were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) and 100 mg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2.

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Human NPC and adjacent normal tissue samples (n=30 each) were harvested at The Tumor Hospital of Jilin Province (Changchun, China) between July 2014 and July 2015. All tissue samples were immediately snap-frozen in liquid nitrogen following surgery, and stored in liquid nitrogen until use. The study was approved by the Ethic Committee of the Tumor Hospital of Jilin Province (Changchun, China) and written informed consent was obtained from every patient

Cell transfection. miR-222 mimic, 5'-AGCUACAUCGGC UACUGGGUUU-3' and the corresponding negative control, miR-NC, UUCUCCGAACGUGUGUCACGUTT, miR-222 inhibitors, anti-miR-222, 5'-AGCUACAUCUGGCUACUG GGU-3' and the corresponding miRNA negative control, anti-miR-NC, 5'-UCUACUCUUUCUAGGAGGUUGUGA-3', were obtained from Qiagen Sciences, Inc. (Frederick, MD, USA). A PTEN overexpression plasmid was obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Plasmids (100 ng) and miRNAs (100 nM) were transiently transfected into C666-1 cells using Lipofectamine 3000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfected cells were cultured for 1-3 days until subsequent analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of cell lines was extracted with TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the purity and concentration of the RNA was determined by a dual-beam ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). The total RNA was then reverse transcribed into cDNA using a Universal cDNA synthesis kit (Exiqon, Inc., Woburn, MA, USA) according to the manufacturer's protocol. cDNA was amplified and quantified using the Strotatagene Mx3005P real-time PCR System (Agilent Technologies, Inc., Santa Clara, CA, USA) with the Taqman Universal PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Specific primer sequences were: Mature miR-222 forward, ACACTCCAGCTGGGAGCT ACATCTGGCTACTG and reverse, CTCAACTGGTGTCGT GGA) and U6 (control) forward, CTCGCTTCGGCAGCA CA and reverse, AACGCTTCACGAATTTGCGT (Applied Biosystems; Thermo Fisher Scientific, Inc. PTEN, forward, TTGTGGTCTGCCAGCTAAA and reverse, CGCTCTATA CTGCAAATGCT and GAPDH forward, GCACCGTCAAGG CTGAGAAC and reverse-TGGTGAAGACGCCAGTGGA. Primers were used in this study as described previously (17). qPCR was performed in triplicate consisting of 40 cycles of a denaturation step at 95˚C for 10 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 40 sec following a cycle of a pre-denaturation step at 95˚C for 40 sec. U6 and GAPDH were used as endogenous controls for the detection of miR-222 and PTEN, respectively. For data analysis, the $2^{-\Delta\Delta Cq}$ method (19) was used to calculate fold change using the Rotor-Gene 6000 Series Software 1.7 (Qiagen Sciences, Inc.).

Cell viability assay. For the cell viability assay, transfected cells were seeded in 96-well plates at a density of $2x10^3$ cells/well. Cells were continually cultured for 72 h prior to the addition of 10 μl 0.5 mg/ml MTT to each well. Cells were incubated for 4 h at 37˚C. The medium was subsequently removed and 100 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well. Following 20 min of agitation, absorbance was detected at 490 nm with an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Colony formation assay. Transfected cells ($1x10^3$ per well) were seeded in 6-well plates and cultured for 10 days. Colonies were washed with phosphate-buffered saline (pH, 7.2) and subsequently fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) at room temperature for 10 min and stained with 1% crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 5 min. Colony numbers were counted under an IX71 inverted light microscope (Olympus Corporation, Tokyo, Japan).

Cell apoptosis assay. Cell apoptosis was examined using flow cytometry in transfected cells. At 48 h post-transfection, cells were harvested and the apoptosis assay was performed using an Annexin V/Propidium Iodide Detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) in a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. The apoptosis ratio was calculated using CellQuest software 3.4 (BD Biosciences).

In vitro radiosensitivity assay. Cells transfected with the miR-222 mimic were treated with 0, 1, 2, 4, 6 and 8 Gy X-ray radiation, using a Faxitron RX-650 (Faxitron Bioptics, Lincolnshire, IL, USA) with 100 kVp. The dose was administered at a rate of 3 Gy/min at room temperature. A colony formation assay was then performed on the irradiated cells., survival curve parameters were determined using a Kaplan-Meier plot. Additionally, cells transfected with miR-222 mimic or miR-NC were exposed to 4 Gy of X-ray radiation. Cells were harvested at 48 h after IR and a cell apoptosis assay was subsequently performed as described above.

Vector construction and luciferase activity assay. The 3'UTR of PTEN containing the putative miR-222 binding site was synthesized (GenePharma Co., Ltd) and inserted into the pGL3 control vector (Promega Corporation, Madison, WI, USA). Mutations in the miR-222 binding site of the PTEN 3'UTR were introduced with the QuikChange Site-Directed Mutagenesis kit (Stratagene; Aligent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol.

For the luciferase reporter assay, C666-1 cells were cultured in 96-well plates and co-transfected with 100 ng pGL3 with wild type PTEN-3'UTR (Wt-PTEN-3'UTR) or pGL3 with mutant PTEN-3'UTR (Mut-PTEN-3'UTR). Additionally, 80 ng luciferase co-reporter vector pRL-SV40 was added, alone or in combination with miR-NC (100 nM) or miR-222 mimic (100 nM), using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) 48 h post-transfection. Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well.

Western blotting. Protein extracts were obtained from cultured cells using radioimmunoprecipitation lysis buffer (Beyotime

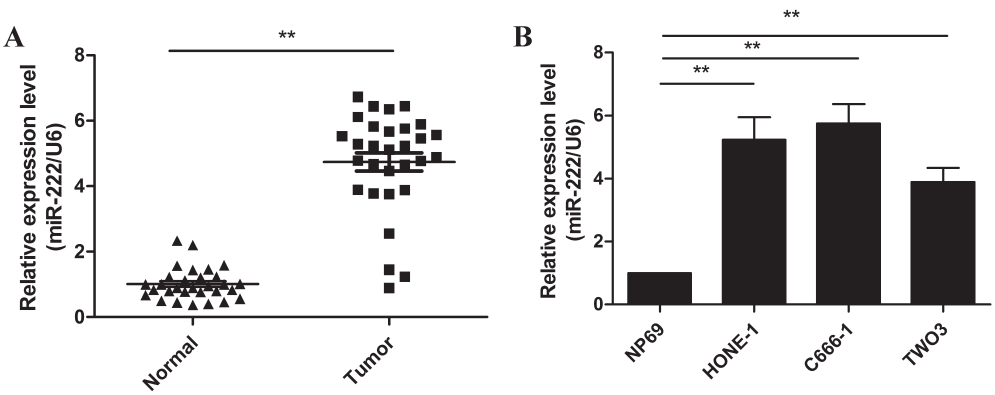

Figure 1. (A) Relative expression of miR-222 in NPC tissues (n=30 NPC and adjacent normal tissues). (B) Relative expression of miR-222 in three different NPC cell lines (C666-1, HONE-1 and TWO3), and a normal human nasopharyngeal epithelial cell line (NP69). Results were determined by reverse transcription-quantitative polymerase chain reaction. **P<0.01. miR-222, microRNA-222; NPC, nasopharangeal carcinoma.

Institute of Biotechnology, Beijing, China). The total concentration of protein was measured using a bicinchoninic acid protein assay kit (Boster Biological Technology, Pleasanton, CA, USA). Protein lysates (30 μg per lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, USA), prior to transfer onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% non-fat milk in Tris-buffered saline for 1 h at room temperature and incubated with the following antibodies overnight at 4˚C: Anti-PTEN (1:1,000; sc-133197; Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-phosphoinositide 3-kinase (PI3K; 1:1,000; sc-293172; Santa Cruz), anti-AKT (1:500; sc-8312; Santa Cruz Biotechnology Inc.) and anti-phosphorylated-AKT (p-AKT; Ser473; sc-271966; 1:500; Santa Cruz Biotechnology Inc.). Anti-GAPDH (1:1,000; sc-47724; Santa Cruz Biotechnology Inc.) was used as an internal control for protein loading. The membrane was subsequently incubated with horseradish peroxidase conjugated goat anti-mouse immunoglobulin G (IgG; 1:5,000; sc-516102; Santa Cruz Biotechnology Inc.) or goat anti-rabbit IgG (1:5,000; sc-2040; Santa Cruz Biotechnology Inc.) secondary antibodies for 2 h at room temperature. Proteins were visualized using a chemiluminescent detection system (Thermo Fisher Scientific, Inc.) and exposed on X-ray film.

Statistical analysis. All data are expressed as the mean ± standard deviation of at least three independent experiments or samples. The statistical difference was determined using a two-tailed Student t-test or one-way analysis of variance followed by a Bonferroni post-hoc test. All data analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-222 expression is upregulated in NPC tissues and cells. The expression levels of miR-222 in NPC and adjacent normal tissues was measured by RT-qPCR. As presented Fig. 1A, the level of miR-222 expression in NPC tissues was significantly higher than that of adjacent normal tissues (P<0.01). The expression of miR-222 in three NPC cell lines (C666-1, HONE-1 and TWO3) and the NP69 nasopharyngeal epithelial cell line was subsequently examined. miR-222 expression was significantly upregulated in the three NPC cell lines compared with NP69 cells (P<0.05; Fig. 1B). This data suggests that miR-222 may be involved in the initiation and progression of NPC.

miR-222 overexpression increased cell viability and colony formation, and inhibits the apoptosis of NPC cells. To examine the biological effects of miR-222 in NPC cells, C666-1 cells were transfected with miR-222 mimic or anti-miR-222 and the biological function of miR-222 in the C666-1 cells was subsequently evaluated. RT-qPCR analysis confirmed that C666-1 cells transfected miR-222 mimic had an upregulation of miR-222 expression, whereas transfection with anti-miR-222 resulted in a downregulation in miR-222 expression compared with NCs (Fig. 2A). MTT assay indicated that miR-222 mimic transfected C666-1 cells had increased cell proliferation compared with miR-NC. Conversely, a significant decrease in proliferation was observed in cells transfected with anti-miR-222 compared with anti-miR-NC (Fig. 2B). Similar results were obtained in the colony formation assay. As presented in Fig. 2C, upregulated miR-222 expression promoted colony formation, whereas downregulated miR-222 expression inhibited colony formation in C666-1 cells compared with the negative controls. Flow cytometry was subsequently used to examine the role of miR-222 in apoptosis (data not presented). It was demonstrated that downregulated miR-222 expression induced apoptosis, whereas upregulated miR-222 expression decreased cell apoptosis compared with the negative control groups (Fig. 2D). These results suggest that miR-222 functions as an oncomir in NPC cells.

miR-222 confers radioresistance in NPC cells. It is well established that certain miRNAs can regulate the radioresistance of cancer cells (11-13,17,18). Thus, the effect of miR-222 on NPC cell radioresistance was investigated. C666-1 cells were transfected with miR-222 mimic prior to treatment with different doses of radiation. Colony formation assays were subsequently performed and survival curve parameters were counted. The survival rate of miR-222 mimic transfected cells significantly increased, whereas the survival rate of miR-NC transfected

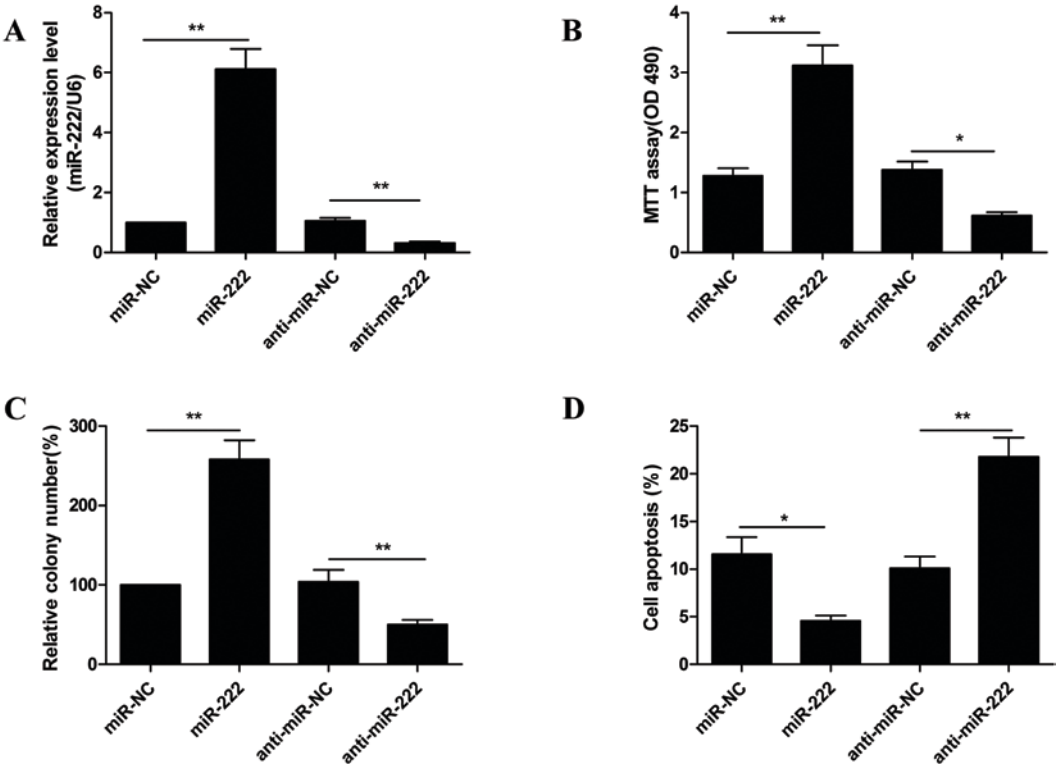

Figure 2. Effect of miR-222 on cell viability, colony formation and apoptosis of NPC cells. (A) Reverse transcription-quantitative polymerase chain reaction was performed to quantify miR-222 expression in C666-1 cells transfected with miR-222 mimic or anti-miR-222. (B) Cell viability, (C) colony formation and (D) apoptosis were determined in C666-1 cells transfected with miR-222 mimic or anti-miR-222. *P<0.05, **P<0.01. miR-222, microRNA-222; anti-miR-222, miR-222 inhibitor. NC, negative control; OD 490, optical density 490 nm.

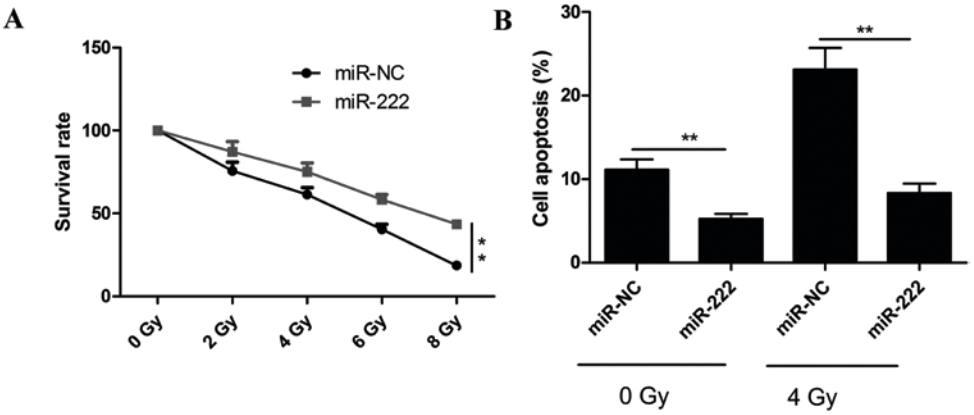

Figure 3. miR-222 confers radioresistance in NPC cells. (A) Clonogenic survival assays of C666-1 cells treated with miR-222 mimic or miR-NC followed by increasing doses of irradiation. Surviving fractions were calculated using a Kaplan–Meier plot. (B) Cell apoptosis was determined in C666-1 cells treated with miR-222 mimic or miR-NC, followed by 4 Gy of X-ray radiation. **P<0.01. miR-222, microRNA-222; miR-NC, miR-222 negative control.

cells significantly decreased (Fig. 3A). The apoptotic rate in cells overexpressing miR-222 following IR was also examined by flow cytometry (data not presented). miR-222 overexpression significantly decreased radiation-induced apoptosis in C666-1 cells at 0 Gy and 4 Gy (Fig. 3B), suggesting that miR-222 may increase radioresistance in NPC cells.

PTEN is a direct target of miR-222 in NPC cells. PTEN has been previously identified as a direct target of miR-222 in several types of cancer (17,18). However, the link between miR-222 and PTEN in NPC remains unclear. To verify if PTEN is a direct target of miR-222 in NPC, a human PTEN 3'UTR fragment containing the binding sites or mutated binding sites of miR-222 (Fig. 4A) was fused to a luciferase reporter vector and co-transfected with miR-222 mimic or miR-NC into C666-1 cells. The luciferase reporter assay was subsequently performed. As presented in Fig. 4B, the relative luciferase activity was reduced following co-transfection with miR-222 mimic and Wt-PTEN-3'UTR, compared with co-transfection with miR-222 mimic and Mut-PTEN-3'UTR. To determine whether miR-222 expression affected endogenous PTEN mRNA and protein expression, miR-222 mimic, miR-NC, anti-miR-222 and anti-miR-NC were transfected into C666-1 cells for 48 h and were subsequently

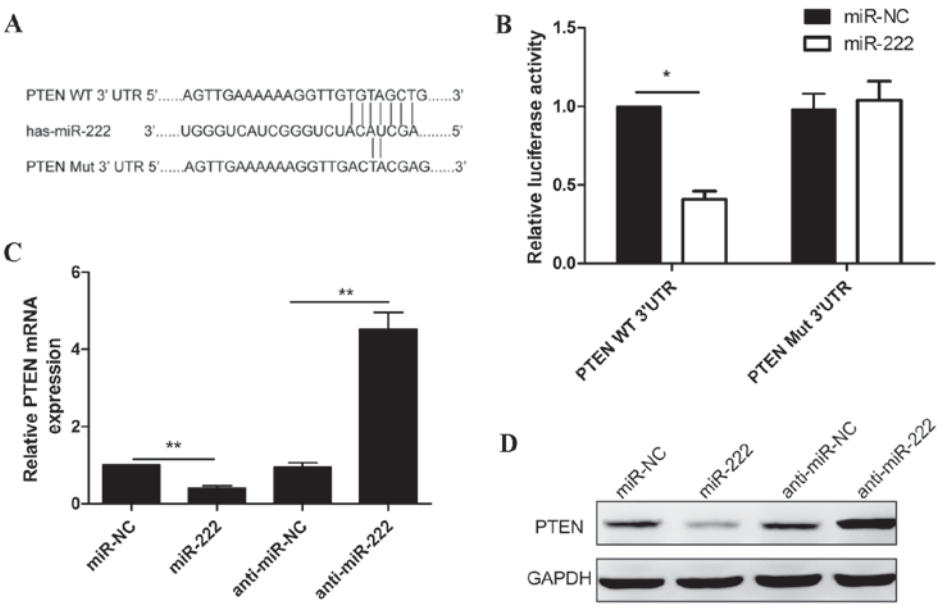

Figure 4. miR-222 targets the 3'UTR of PTEN and downregulates its expression in NPC cells. (A) The predicted binding sites for miR-222 in the 3'UTR of PTEN and the mutations in the binding sites are presented. (B) C666-1 cells were co-transfected with PTEN WT or Mut reporter plasmids along with miR-222 mimic or miR-NC. Relative luciferase activity was measured. (C) PTEN mRNA expression and (D) protein expression was determined in C666-1 cells following transfection with miR-222 or anti-miR-222. GAPDH was used as an internal control. *P<0.05, **P<0.01. PTEN, phosphatase and tensin homolog; WT, wild type; 3'UTR, 3'untranslated region; miR-222, microRNA-222; Mut, mutant; miR-NC, miR-222 negative control; anti-miR-222, miR-222 inhibitor.

analyzed with RT-qPCR and western blotting. The results revealed that miR-222 overexpression inhibited PTEN mRNA and protein expression in C666-1 cells, whereas reduced miR-222 expression increased PTEN mRNA and protein expression in C666-1 cells (Fig. 4C and D), suggesting that PTEN is a target of miR-222 in NPC cells.

miR-222 regulates the PI3K/AKT signaling pathway in NPC cells. PTEN has been reported to act as a negative regulator of the PI3K/AKT pathway, via the dephosphorylation of phosphatidylinositol (3,4,5)-triphosphate (20). To investigate if miR-222 could regulate the PI3K/AKT pathway, PI3K, AKT and p-AKT protein levels were detected by western blot in miR-222 mimic or anti-miR-222 transfected C666-1 cells. PI3K and p-AKT protein expression levels were increased in miR-222 mimic transfected cells, and decreased in anti-miR-222-transfected cells compared with the negative controls (Fig. 5). AKT protein expression was unchanged among the groups (Fig. 5). These findings suggest that the promotion of cell proliferation, migration and invasion by miR-222 may be mediated via the activation of the PI3K/AKT signaling pathway.

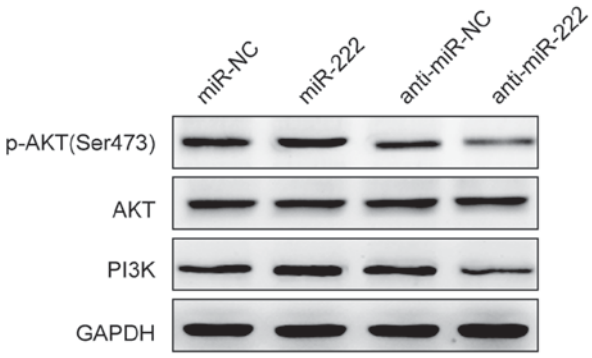

Figure 5. miR-222 regulates the PI3K/AKT signaling pathway. PI3K, AKT and p-AKT protein expression was determined in C666-1 cells after transfection with miR-222 mimic or anti-miR-222. GAPDH was used as an internal control. miR, microRNA; NC, negative control; anti-miR-222, miR-222 inhibitor; AKT, protein kinase B; p-AKT, phosphorylated-AKT; PI3K, phosphoinositide 3-kinase.

Discussion

Recent studies have demonstrated the involvement of several miRNAs in NPC initiation and development, through the regulation of target gene expression (11-13,21). The present study revealed that miR-222 expression was increased in clinical NPC tissues and cell lines, compared to adjacent normal tissues and cell lines. miR-222 was demonstrated to be involved in NPC progression through the regulation of proliferation, colony formation and cell apoptosis. Furthermore, miR-222 was observed to increase radioresistance in NPC cells. PTEN was verified as a direct, functional target of miR-222 in NPC cells. These findings contribute to the understanding of NPC development and radioresistance, and suggest miR-222 as a potential target in NPC therapy.

As an oncomir, miR-222 has been widely reported to be upregulated in numerous types of human cancer, including non-small cell lung cancer (22), bladder cancer (23), breast cancer (24), glioblastoma (25) and hepatocellular carcinoma (26). Accumulating evidence suggests that miR-222 contributes to tumor development, progression, metastasis, and may be an effective biomarker or therapy target (15-18,23-26). Previous studies have demonstrated that miR-222 is involved in the radioresistance of glioblastoma and gastric cancer cells (17,18). However, the role of miR-222 in NPC remains elusive. In the present study, miR-222 expression was upregulated in NPC

tissues and cell lines, and miR-222 overexpression promoted tumor growth and the radioresistance of NPC cells, suggesting that miR-222 functions as an oncomir in NPC cells.

Located on chromosome 10q23.3, PTEN is downregulated in numerous types of human cancer and functions as a tumor suppressor (27). Recent studies have revealed that PTEN expression is downregulated in NPC tissues, and that normal PTEN expression may suppress NPC development (28,29). It has been reported that PTEN can regulate radioresistance in cancer cells (17,18) and the PTEN/PI3K/AKT pathway (30). PTEN has previously been identified as a direct target gene of miR-222 in glioblastoma cells and gastric cancer cells (17,18). Consistent with these results, the present study identified PTEN as a potential target of miR-222 in NPC cells. miR-222 was also demonstrated to regulate the PI3K/AKT pathway. These results suggest that miR-222 can promote tumor growth and confer radioresistance in NPC cells by directly targeting PTEN and thus indirectly regulating the PI3K/AKT signaling pathway.

In conclusion, the present study provides evidence that miR-222 expression is upregulated in NPC tissues and cell lines, and that miR-222 may promote cell proliferation and colony formation, decrease cell apoptosis and confer radioresistance in NPC cells. Furthermore, PTEN was identified as a crucial target gene of miR-222. It was demonstrated that mir-222 inhibited PTEN expression and positively regulated the PI3K/AKT signaling pathway, suggesting that miR-222 functions as an oncomir in NPC and may be a potential therapeutic target in NPC.

Acknowledgements

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