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MicroRNA-142 promotes the development of nasopharyngeal carcinoma through targeting PTEN

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Abstract. – OBJECTIVE: To elucidate whether microRNA-142 could regulate the development of nasopharyngeal carcinoma (NPC) by mediating gene of phosphate and tension homology deleted on chromosome ten (PTEN) expression.

PATIENTS AND METHODS: The microRNA-142 expression in NPC tissues and paracancerous tissues was detected by the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Correlation between the microRNA-142 expression and the prognosis of NPC patients was analyzed. MicroRNA-142 expression in NPC cell lines was determined as well. By transfection of microRNA-142 inhibitor or negative control, biological performances of NPC cells were accessed through cell counting kit-8 (CCK-8), colony formation, wound healing, and transwell assay. Dual-luciferase reporter gene assay was conducted to verify the binding condition between microRNA-142 and its target gene PTEN. Rescue experiments were carried out by co-transfection of microRNA-142 inhibitor and si-PTEN, followed by detecting the invasive capacity of NPC cells. Protein expressions of relative genes in the PI3K/AKT pathway after the microRNA-142 knockdown in NPC cells were determined by Western blot.

RESULTS: MicroRNA-142 was highly expressed in NPC tissues than that of paracancerous tissues, which was correlated with poor prognosis of NPC patients. MicroRNA-142 was also highly expressed in NPC cells. Downregulated microRNA-142 inhibited proliferative, migratory, and invasive capacities of NPC cells. Dual-luciferase reporter gene assay verified that microRNA-142 could directly bind to PTEN. Knockdown of PTEN could reverse the inhibitory effect of microRNA-142 on invasive capacity of NPC cells. Finally, Western blot results demonstrated that the microRNA-142 knockdown inhibited the PI3K/AKT pathway in NPC cells.

CONCLUSIONS: MicroRNA-142 is highly expressed in NPC. MicroRNA-142 enhances the proliferative and invasive capacities of NPC cells by inhibiting PTEN expression, thus promoting NPC development.

Key Words
MicroRNA-142, NPC, PTEN, Proliferation.

Introduction

Nasopharyngeal carcinoma (NPC) is a highly malignant tumor derived from the nasopharyngeal epithelium and is common in China. The pathogenesis of NPC involves environmental factors, genetic susceptibility, and Epstein-Barr virus (EBV) infection. Early occurrence of distant metastasis and high rate of local metastasis are the main characteristics of NPC[1-5]. With the advanced technology in tumor treatments, radiotherapy and chemotherapy have greatly improved the therapeutic efficacy of NPC. However, about 30-40% of NPC patients have already progressed into the middle or advanced stage at the first time of diagnosis due to insidious symptoms of NPC. Some NPC patients experience distant metastasis and recurrence within a few years after comprehensive treatment because of drug tolerance, which greatly limits the therapeutic efficacy and clinical outcomes[6-8]. China is a high-risk area for NPC, accounting for about 15% of new cases worldwide each year[9,10]. Therefore, it is extremely urgent to comprehensively study the radiotherapy tolerance mechanism of NPC.

The occurrence and development of tumors are complex processes involving the coordinated expressions of multiple genes in time and space. In addition to protein-coding genes, some studies have found that microRNAs are closely related to tumor development[11]. MicroRNAs are highly conserved, temporal and tissue-specific non-coding RNAs that are widely present in organisms. They contain approximately 18-24 nucleotides

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in length. After being transcribed by RNA polymerase II, they inhibit translation or expressions at the post-transcriptional level through incompletely complementary to 3'UTR of target genes[12]. Abnormally expressed microRNAs are greatly involved in differentiation, migration, invasion, angiogenesis, and stem cell differentiation in various tumors[13-17]. Analyses of miRNA gene expression profiling also confirmed that a large number of microRNAs act as oncogenes or tumor-suppressor genes. Expression changes of these microRNAs further alter their target genes, thus affecting relative signaling pathways[18]. To our best knowledge, the specific role of microRNA-142 in NPC has not been reported yet.

Patients and Methods

Cell Culture

NPC cells (CNE-1, CNE-2, SUNE-1, and C666-1) and normal nasal mucosal epithelial cell line (NP69) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were maintained in a 5% CO_2 incubator at 37°C.

Cell Transfection

Until 40% of cell confluence, transfection plasmids (miR-NC, microRNA-142 inhibitor, si-PTEN or negative control) diluted in serum-free medium and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) diluted in Opti-MEM were gently mixed and maintained at room temperature for 15 min. After incubation for 4 h, medium containing 30% FBS was replaced and incubated for another 48 h.

Sample Collection

30 pairs of NPC tissues and paracancerous tissues were harvested from Otolaryngology Department, The First Affiliated Hospital of Anhui Medical University. All samples were harvested from the nasopharyngeal biopsy and pathologically diagnosed. NPC tissues studied in this work were non-keratinized undifferentiated squamous cell carcinoma. Enrolled NPC patients did not receive preoperative chemotherapy or radiotherapy. For the sample collection, the informed consent was obtained from the patients and/or their families. This study has been approved by the Ethics Committee.

Dual-Luciferase Reporter Gene Assay

After transfection of dual-luciferase reporter gene assay for 36 h, cells were lysed, followed by detecting luciferase activities of firefly luciferase and Renilla luciferase using the GloMaxTM 96 Microplate Illuminometer. Transfection efficacy was corrected by co-transfection of pRL-sv40 as the loading control.

Colony Formation Assay

200 cells were seeded in each well of 6-well plate. After cell culture for 2 weeks until visible colony formation, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Colonies were stained with crystal violet for 10-30 min and captured using the microscope.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 1000 cells per well. After cell culture for 0 h, 24 h, 48 h, and 72 h, respectively, the CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added in each well. 2 hours later, the optical density (OD) value at 450 nm of each well was measured using a microplate reader.

Transwell Assay

The cell suspension was added in the transwell chamber. 700 μL of medium containing 20% FBS was added to the bottom chamber. 48 hours later, cells were fixed with 4% paraformaldehyde for 15 min after removal of the chamber. Cells were stained with 0.2% crystal violet for 20 min. The inner layer cells were carefully removed. 10 randomly selected fields of each sample were captured for calculating the amount of penetrating cells.

Wound Healing Assay

Cells were seeded into 6-well plates. A sterile 10 μL micropipette tip was used to vertically scratch the cell plate until 90% of cell confluence. After removing the exfoliated cells with PBS, serum-free medium was placed for 48 h-incubation. Migratory cells were observed and captured under an inverted microscope, and the width of the scratch was measured and photographed.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was deter-

mined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibody, immunoreactive bands were exposed by enhanced chemiluminescence method.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from BC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan), and StepOne Plus Real-time PCR System (Applied Biosystems Foster City, CA, USA). Primers used in this study were: MicroRNA-142, F: AAAACATAAAGTAGAAAGCACTACT, R: TAACCAGGCCCGACCCTGCT; PTEN, F: TGGATTCGACTTAGACTTGACCT, R: GGTGGGTTATGGTCTTCAAAAGG.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between the two groups were analyzed using the *t*-test. The one-way ANOVA was conducted to analyze differences among multiple groups, followed by the Post-hoc test. $p<0.05$ was considered to be statistically significant.

Results

High Expression of MicroRNA-142 in NPC

We first detected the expression of microRNA-142 in 30 pairs of NPC and adjacent paracancerous tissues by qRT-PCR. The results showed that microRNA-142 is highly expressed in NPC tissues in comparison with that of paracancerous tissues (Figure 1A). NPC patients with higher expression of microRNA-142 presented a worse prognosis than those with lower expression (Figure 1B). MicroRNA-142 expression was also detected in NPC cell lines and normal nasal mucosal epithelial cell line. Compared with NP69 cells, microRNA-142 was highly expressed in NPC cell lines, especially in CNE-1 and CNE-2 cells (Figure 1C). Hence, we selected these two cell lines as research objects.

MicroRNA-142 Knockdown Inhibited Proliferative, Migratory and Invasive Capacities of NPC Cells

By liposomal transfection of microRNA-142 inhibitor, the expression level of microRNA-142 in NPC cells was markedly inhibited (Figure 2A). Subsequently, CCK-8 and colony formation assay were conducted to determine the proliferative capacity of NPC cells. MicroRNA-142 knockdown markedly inhibited the proliferative rate of NPC cells, which achieved the lowest level at 72 h (Figure 2B). Consistently, knockdown of microRNA-142 also inhibited the proliferative capacity of NPC cells through colony formation assay (Figure 2C). Wound healing assay was performed and the results indicated that the migratory capacity of NPC cells is reduced by microRNA-142 knockdown (Figure 2D). Furthermore, transwell assay

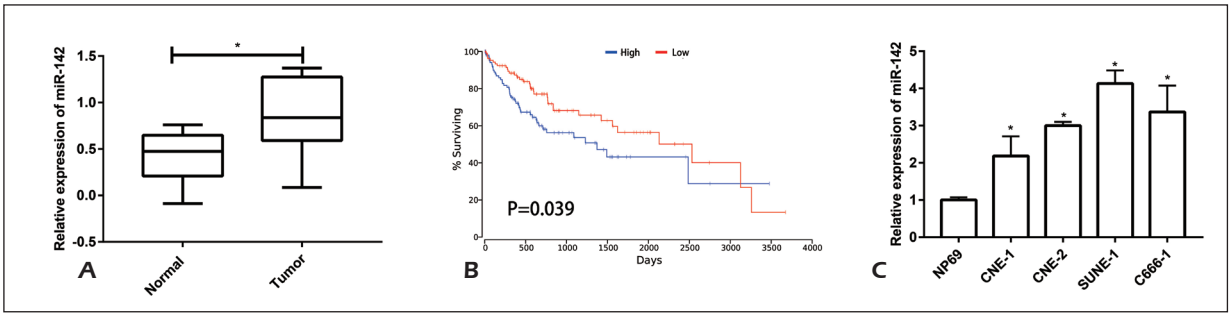

Figure 1. High expression of microRNA-142 in NPC. **A**, Expression of microRNA-142 in 30 pairs of NPC and adjacent paracancerous tissues detected by qRT-PCR. **B**, Correlation between microRNA-142 expression and prognosis of NPC patients. **C**, MicroRNA-142 expression in NPC cell lines and normal nasal mucosal epithelial cell line.

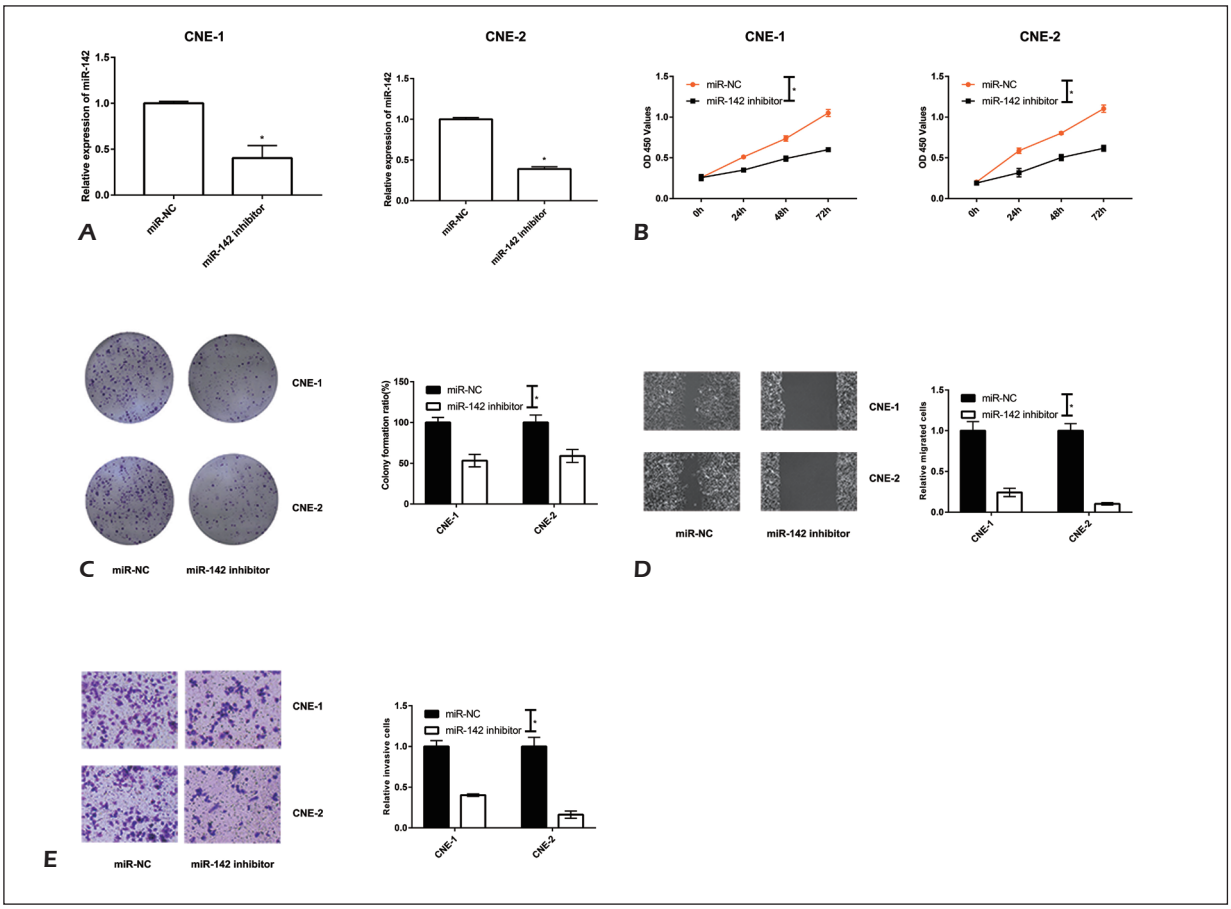

Figure 2. MicroRNA-142 knockdown inhibited proliferative, migratory and invasive capacities of NPC cells. **A**, Transfection efficacy of microRNA-142 inhibitor and negative control in NPC cells. **B**, CCK-8 assay showed proliferative rate of NPC cells after transfection of microRNA-142 inhibitor or negative control. **C**, Colony formation assay showed proliferative capacity of NPC cells after transfection of microRNA-142 inhibitor or negative control (Magnification: 40×). **D**, Wound healing assay showed migratory capacity of NPC cells after transfection of microRNA-142 inhibitor or negative control (Magnification: 10×). **E**, Transwell assay showed invasive capacity of NPC cells after transfection of microRNA-142 inhibitor or negative control (Magnification: 40×).

demonstrated the inhibited invasive capacity of NPC cells after transfection of microRNA-142 inhibitor (Figure 2E).

MicroRNA-142 Regulated PTEN Expression in NPC

As the potential target gene of microRNA-142 predicted by bioinformatics, PTEN was speculated to participate in NPC development. Dual-luciferase reporter gene assay verified that microRNA-142 overexpression markedly decreases the luciferase activity of wild-type PTEN, whereas the luciferase activity in mutant-type PTEN did not change (Figure 3A). It is suggested that microRNA-142 could directly bind to PTEN. QRT-PCR and Western blot results further confirmed the negative correlation between expressions of microRNA-142 and PTEN in NPC cells at mRNA and protein levels (Figure 3B and 3C). To elucidate whether microRNA-142 regulates NPC development through mediating PTEN expression, rescue experiments were carried out. Co-transfection of microRNA-142 inhibitor and si-PTEN reversed the inhibited invasive capacity of NPC cells (Figure 3D).

MicroRNA-142 Knockdown Inhibited PI3K/AKT Pathway in NPC

Previous researches have already confirmed the crucial role of the PI3K/AKT pathway in tumor development. Hence, we further explored the effect of microRNA-142 on the PI3K/AKT pathway. It is found that the PI3K/AKT pathway is inhibited both in CNE-1 and CNE-2 cells after microRNA-142 knockdown (Figure 4A and 4B). We believed that microRNA-142 regulates NPC development through the PI3K/AKT pathway.

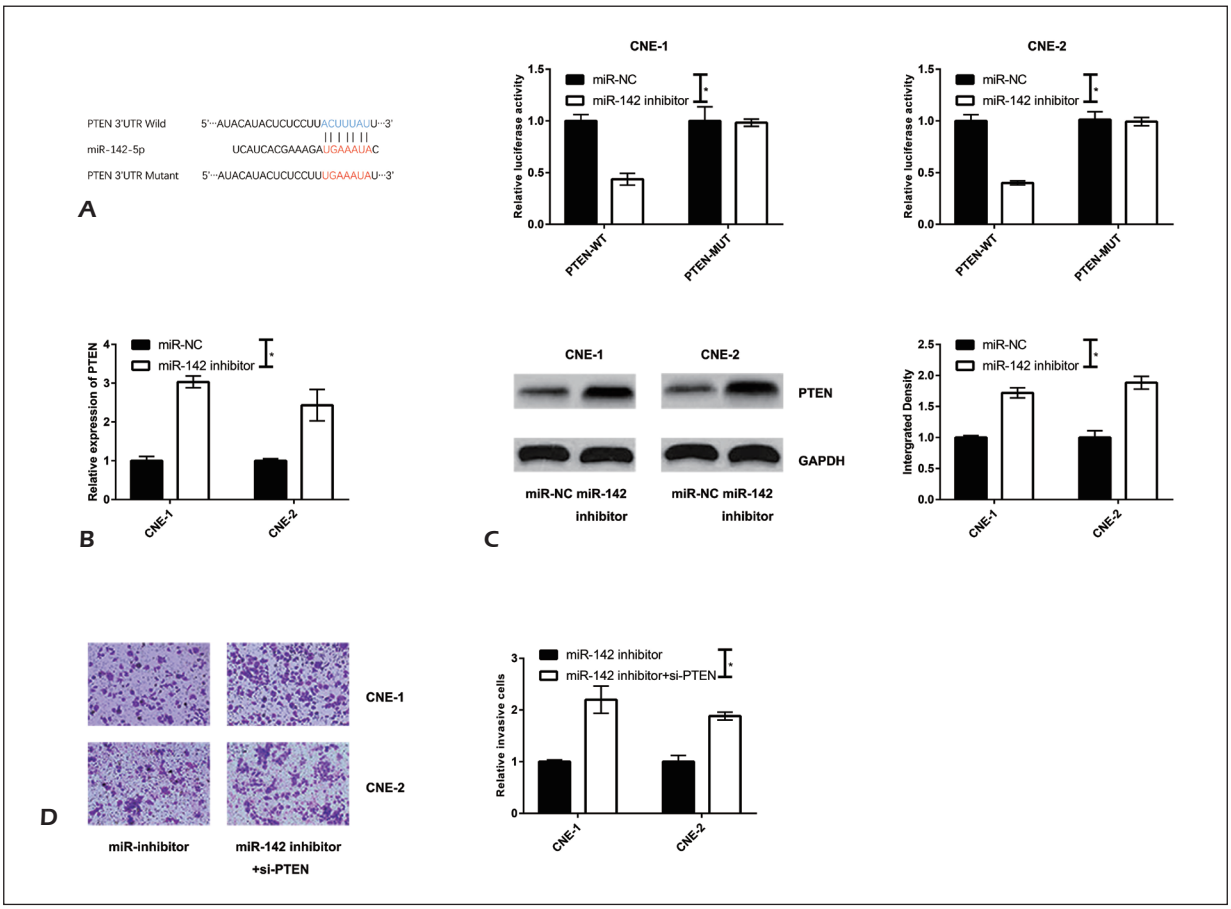

Figure 3. MicroRNA-142 regulated PTEN expression in NPC. **A**, Dual-luciferase reporter gene assay verified that microRNA-142 overexpression markedly decreases the luciferase activity of wild-type PTEN, whereas the luciferase activity in mutant-type PTEN did not change. **B**, QRT-PCR showed the negative correlation between mRNA expressions of microRNA-142 and PTEN in NPC cells. **C**, Western blot showed the negative correlation between protein expressions of microRNA-142 and PTEN in NPC cells. **D**, Invasive capacity of NPC cells after co-transfection of microRNA-142 inhibitor and si-PTEN (Magnification: 40×).

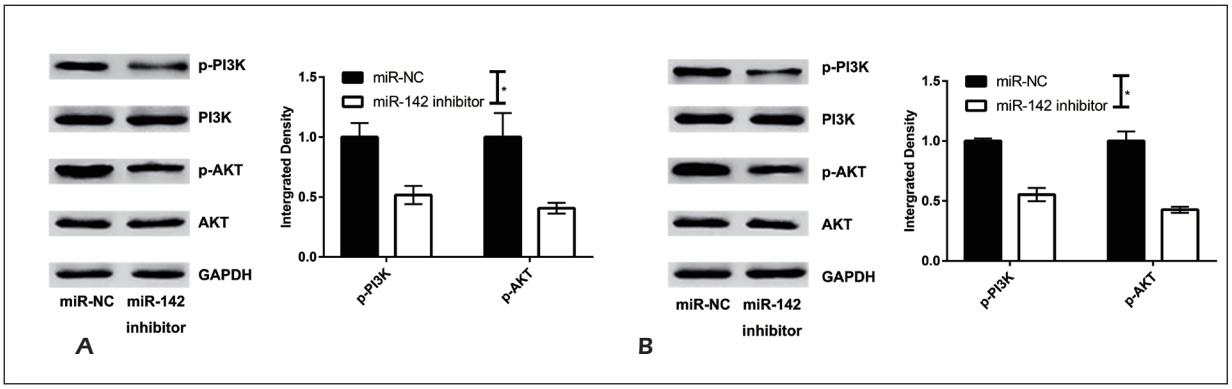

Figure 4. MicroRNA-142 knockdown inhibited PI3K/AKT pathway in NPC. **A**, PI3K/AKT pathway in CNE-1 cells after transfection of microRNA-142 inhibitor. **B**, PI3K/AKT pathway in CNE-2 cells after transfection of microRNA-142 inhibitor.

Discussion

With the rapid development of molecular biology, it is believed that the pathogenesis of NPC involves multiple oncogenes and tumor-suppressor genes. The imbalance between these genes is the molecular basis of NPC.

Previous studies on NPC-related microRNAs have pointed out that miR-320 overexpression inhibits NPC growth in xenograft mice. MiR-320 overexpression also inhibits migratory and invasive capacities of NPC cells by targeting BMI-1[19]. Additionally, overexpression of miR-451 inhibits cell viability, colony formation, migration, and invasion by targeting macrophage migration inhibitory factor (MIF)[20]. In addition to being a tumor-suppressor gene, microRNAs can also promote NPC development. Yu et al[21] reported that miR-378 significantly promotes proliferation, colony formation, migration, and invasion of NPC cells, as well as *in vivo* growth of NPC. As an oncogene, miR-141 affects biological performances of NPC cells through targeting BRD3, UBAP1, and PTEN by regulating RB/E2F pathway[22]. PTEN is found to be a crucial tumor-suppressor gene in various types of tumor[23,24]. It is reported that miR-205 is capable of downregulating PTEN but upregulating AKT[25]. Overexpression of miR-138 also inhibits proliferation and cell cycle progression in NPC cells[26].

In this study, we found that microRNA-142 is highly expressed in NPC tissues, and high expression of microRNA-142 can promote proliferative and invasive capacities of NPC cells. It is suggested that microRNA-142 exerts a tumor-promoting function in NPC. To explore the mechanism of microRNA-142 in the regulation of NPC development, we first predicted its target gene using bioinformatics and PTEN was screened out. MicroRNA-142 inhibited the expression of PTEN in NPC. Previous researches have already confirmed the crucial role of PTEN/PI3K/AKT pathway in tumor development. Hence, we further explored the effect of microRNA-142 on the PTEN/PI3K/AKT pathway.

As a new tumor-suppressor gene, PTEN (Phosphatase and tensin homolog) has been widely concerned in tumor researches since it was discovered in the late 20th century. PTEN is another important tumor-suppressor gene after the emergence of P53. It rapidly catalyzes the phosphatidylinositol substrate[27]. PIP3 is the most essential substrate for PTEN and promotes AKT (also known as PKB) activation. PIP3 dephosphorylation is mainly mediated by PTEN that allows PIP3 maintains at a low level. Afterwards, the PI3K/AKT pathway is inactivated under the normal circumstance[28]. PTEN deficiency and loss of function are observed in malignant tumor cells in the human body and *in vitro* cell lines[29]. Absent expression of PTEN would activate the PI3K/AKT pathway, and further phosphorylates AKT expression. The p-AKT exerts multiple biological effects, which promotes tumor cell growth and their malignant performances. As a result, PTEN deficiency leads to dysregulated PTEN/PI3K/AKT pathway in tumors and eventually results in the unrestricted proliferation and the loss of chemotherapy sensitivity of tumor cells. Our study showed that low expression of microRNA-142 can significantly inhibit the PTEN/PI3K/AKT pathway. Besides, low expression of PTEN reversed the inhibitory effects of microRNA-142 on invasive ability of NPC cells.

Conclusions

We found that microRNA-142 is highly expressed in NPC. MicroRNA-142 enhances proliferative and invasive capacities of NPC cells by inhibiting PTEN expression, thus promoting NPC development.

Conflict of Interests

The authors declare that they have no conflict of interest.

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