Inhibition of PTEN Gene Expression by Small Interfering RNA on PI3K/Akt/FoxO3a Signaling Pathway in Human Nasopharyngeal Carcinoma

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Abstract
The objective of this article is to study the effect of inhibiting phosphatase and tensin homolog deleted chromatosome 10 gene on phosphoinositide 3-kinase/protein kinase B (Akt)/Forkhead homeobox O3a signaling pathway in human nasopharyngeal carcinoma HK-1 cells. Nasopharyngeal carcinoma HK-1 cell lines were divided into PTEN gene interference group (siPTEN), nonspecific small interfering RNA group (siNC), empty vector group (Vector), and no transfection control group (Normal). The mRNA and protein expression levels of PTEN, PI3K, p-Akt, and FoxO3a were detected by real-time fluorescence quantitative polymerase chain reaction and Western blot. Immunofluorescence was used to detect the subcellular localization of PTEN, PI3K, p-Akt, and FoxO3a in HK-1 cells. The proliferation of HK-1 cells was detected by MTT assay, and the apoptosis of HK-1 cells was detected by flow cytometry. Compared with the siNC group, the expression levels of PTEN, FoxO3a messenger RNA, and protein in the siPTEN group were significantly decreased (P < .05), while the expression levels of PI3K, p-Akt messenger RNA, and protein were significantly increased (P < .05). The growth rate of HK-1 cells in the siPTEN group was significantly higher than the siNC group (P < .05), while the apoptosis rate was significantly lower than that of the siNC group (P < .05). Small interfering RNA can inhibit the expression of PTEN in HK-1 cells, and PTEN can participate in the development of NPC by affecting PI3K/Akt/FoxO3a signaling pathway.

Keywords
PTEN, phosphoinositide 3-kinase, protein kinase B, small interfering RNA, nasopharyngeal cancer

Abbreviations
cDNA, complementary DNA; DMSO, dimethyl sulfoxide; FoxO3a, Forkhead homeobox O3a; mRNA, messenger RNA; NPC, nasopharyngeal carcinoma; PBS, phosphate-buffered saline; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog deleted chromatosome 10; qRT-PCR, real-time fluorescence quantitative polymerase chain reaction; RNAi, RNA interference; V-FITC, Annexin V-fluorescein isothiocyanate

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Nasopharyngeal carcinoma (NPC) is a malignant tumor characterized by region and race. It occurs mostly in Southeast Asia and southern China. Nasopharyngeal carcinoma generally originates from nasopharyngeal epithelial squamous cells.1 There is no specific clinical manifestation in the early stage, so it is not easy to attract the attention of patients. Most patients have been in the middle or late stage when they visit the clinic. At this time, NPC has a high degree of invasiveness and metastasis, which makes treatment more difficult and seriously affects the quality of life of patients. Nowadays, radiotherapy and

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chemotherapy are still the standard therapy of NPC. But the related data showed that the 5-year survival rate for NPC patients is only 60%. Therefore, it has become most important to understand the growth mechanism of NPC cells and search for new molecular targeted therapies. Phosphoinositide 3-Kinase (PI3K) can activate protein kinase B (Akt), form PI3K/Akt signaling pathway by phosphorylated Akt, and regulate the accumulation of Forkhead homeobox O3a (FoxO3a) from nucleus to cytoplasm, leading to its inactivation, thus inhibiting the growth and migration of cancer cells. In NPC, the activation of PI3K/Akt/FoxO3a signaling pathway is usually closely related to the rapid growth of HK-1 cells. Moreover, previous studies found that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein expression in tumor cells was significantly lower than that in normal tissue cells in NPC, suggesting that PTEN may be involved in the development of NPC disease. However, it is unclear whether the abnormal expression of PTEN gene will participate in the disease progression of NPC by affecting the PI3K/Akt/FoxO3a signaling pathway. PTEN is a phosphorylase-mediated oncogene, which is prone to mutation or deletion. It has been proved that it can inhibit tumor cell proliferation and promote apoptosis in many tumor diseases. Therefore, this study intends to synthesize small interfering RNA in vitro by using RNA interference (RNAi), to study the effect of PTEN gene expression on NPC PI3K/Akt/FoxO3a signaling pathway.

Materials and Methods

Materials and Reagents

Nasopharyngeal carcinoma HK-1 cell line was purchased from ATCC (Washington D.C., USA) and preserved by our studying team; fetal bovine serum, RPMI-1640, and Opti-MEM medium were purchased from Gibco (Beijing, China); dimethyl sulfoxide (DMSO) was purchased from Sigma (Shanghai, China); phosphate-buffered saline (PBS) powder was purchased from Wuhan PhD Bioengineering Co., Ltd (Wuhan, China); Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Wuhan, China); Radio-Immuno-precipitation Assay (RIPA) lysate, phenylmethylsulfonyl fluoride, bicinechonic acid (BCA) protein concentration assay kit, SDS-polyacrylamide gelelectrophoresis (SDS-PAGE) gel rapid preparation kit (P0012 AC), PTEN, PI3K, p-Akt, FoxO3a first antibody and internal reference ACTB antibody, and goat antirabbit fluorescent second antibody were purchased from Beyotime Biotechnology.

Methods

Cell culture. HK-1 cells were incubated in RPMI 1640 medium containing 10% inactivated fetal bovine serum, incubated at a 37°C, 5% CO₂ constant-temperature incubator, and the medium was changed every 2 days. When the cell adherence grew logarithmically to cover 80% to 90% of the basement, 0.25% trypsin containing EDTA was used for digestion passage, and the cells with better growth condition were taken for subsequent experiments.

Cell transfection. Transfection was divided into 4 groups, including PTEN gene interference group (siPTEN), nonspecific small interfering RNA group (siNC), empty vector group (Vector), and nontransfected control group (Normal). The transfection reagent was Lipofectamine 2000, and the siRNA sequence of the siPTEN group was synthesized by Shanghai Jima Pharmaceutical Technology Co, Ltd. Its forward sequence was 5’-ACCAGGCCAGAGGAAACCT-3’, and the reverse sequence was 5’-TTTGTCAGGTGACACAGAG-3’. The HK-1 cells of the siNC group were treated with a nonspecific siRNA-Lipofectamine 2000, in which the nonspecific small interfering RNA (siRNA) base pair sequence was the forward sequence 5’-AUUGCUACUCGGAAGAG-3’, and the reverse sequence 5’-UCUCUGGUAGUGGCCCAAU-3’. One day before transfection, 0.4 to 1.0x10^5 cells/well were inoculated into a 24-well plate culture containing an appropriate amount of complete medium and were cultured at 37°C 5% CO₂ to 70% to 80% confluence; 1 hour before transfection, the 24-well plate culture was replaced with fresh, nonresistant complete medium. An amount of 0.8 μg siRNA plasmid was added into 50-μL Opti-MEM medium, and 4.8-μL Lip2000 reagent was diluted with 50-μL serum-free Opti-MEM, mixed and placed at room temperature for 5 minutes. The diluted siRNA and Lipofectamine 2000 reagents were mixed and placed at room temperature for 30 minutes to form siRNA/Lipofectamine 2000 complex. The 100-μL siRNA/Lipofectamine 2000 complex was added to the wells of the culture plate containing cells and medium, and the cell culture plate was shaken gently to and fro to make the siRNA/Lip2000 mixture cover the cells evenly. After the cells were incubated in CO₂ incubator at 37°C for 48 hours, the cells expressing green fluorescent protein were observed by fluorescence microscopy, and the transfection efficiency was observed.

Real-time fluorescence quantitative polymerase chain reaction. Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) was used for RNA quantitative analysis. First, total RNA was extracted by TRIzol reagent, and the OD value was determined by nucleic acid protein analyzer. When the ratio of OD260/OD280 was between 1.8 and 2.0, the prepared RNA was pure. Then complementary DNA (cDNA) was synthesized by single-stranded RNA reverse transcription according to the instructions of the cDNA kit. With ACTB as the internal reference, the quantitative PCR conditions followed: pre-denaturation at 95°C for 20 seconds, (95°C 10 seconds → 60°C 20 seconds → 72°C) x 40, and the relative expression level of the target gene was calculated by 2^-ΔΔCt. The primer sequences used in qRT-PCR were as follows: PI3K forward primer: 5’-AACTCTGGGATGACCTGGA-3’, reverse primer: 5’-AGCGGTCACAACACTCTCTA-3’; Akt forward primer: 5’-AGGGTTGCTGCAACCGCG-3’, reverse primer: 5’-GTTGGTTAGAGACACCGG-3’, FoxO3a forward primer: 5’-AAACCTTCTCAGGACTCTCTC-3’, reverse primer: 5’-
TAAAATCCCAACCCATCAgCATC-3’; ACTB forward primer: 5’-CAGCGACACCCACTCTC-3’, reverse primer: 5’-TGAGGTCACCCACCTGT-3’.

**Western blot detection.** Protein samples were lysed using RIPA proteolytic buffer to determine protein concentration using the BCA Protein Quantitation Kit according to the instructions. Then, the protein in the sample was separated by SDS-PAGE gel electrophoresis, 5 μL of protein loaded in each lane, and the pre-stained marker loaded on both sides, and the excess wells loaded with 1 × SDS-PAGE loading buffer. The wet system was used to transfer to PVDF film after the electrophoresis finished. After transfer completed, according to the prestained marker prediction target band interval, cut the PVDF membrane into the small grid, and the target protein was incubated corresponding to the primary antibody dilution PTEN (AP686, 1:1000) and PIIK (AF1549, 1:1000) at 60 to 70 rpm shaker at 4°C, p-Akt (AF1546, 1:1500), FoxO3a (AF609, 1:1000) overnight (12-16 hours). The goat antirabbit secondary antibody with HRP locus (A0208, 1:2000) was selected according to the source of the primary antibody and incubated for 1 hour at room temperature on a 60 to 70 rpm shaker and washed with tris buffered saline-Tween (TBS-T) buffer at 100 rpm for 10 minutes, 3 times; finally, the developer was added and developed by chemiluminescence imaging system.

**Immunofluorescence detection.** The well-crawled slides were immersed in PBS in the culture plate, fixed with 4% paraformaldehyde for 15 minutes; 0.5% cell penetrant (Triton X-100) was at room temperature for 20 minutes; normal goat serum were added dropwise on the slides, which was sealed at room temperature for 30 minutes. Each slide was added enough amount of diluted primary antibody PTEN (AP686, 1:1000), P13K (AF1549, 1:200), p-Akt (AF1546, 1:200), and FoxO3a (AF609, 1:200) and put into a wet box overnight at 4°C. Then, the diluted fluorescent secondary antibody (A0208, 1:200) was added and incubated at 20°C to 37°C for 1 hour in a wet box. 4’,6-diamidino-2-phenylnilidole (DAPI) was added to incubate for 5 minutes in the dark, and the samples were stained. Finally, seal the film with a sealing liquid containing an anti-fluorescence quencher to observe the collected image under the fluorescence microscope.

**MTT detection.** The HK-1 cells after transfection were treated with trypsin digestion and then seeded in 96-well plates. The marginal wells were filled with sterile water or PBS, and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, Thiazolyl Blue Tetrazolium Bromide (MTT) dilution was added to 37°C, incubated in a 5% CO2 incubator for 4 hours, and each group was set to 3 to 5 duplicate wells; then DMSO solution was added dropwise, and the OD value was measured at 490 nm for 3 days, and a growth curve was drawn.

**Flow cytometry detection.** Annexin V-fluorescein isothiocyanate (V-FITC) cell apoptosis detection kit (Keygen, No.KGA106) was used to detect the apoptosis of cells. EDTA-free trypsinase digestive cells were used to terminate digestion and centrifuged at 300g and 4°C for 5 minutes to collect cells; 0.5 mL cell suspension was added to resuspend, and the same amount of untreated live cells were mixed in addition to that. Cell suspension was added to 1.5 mL, divided into 3 tubes, one of which was a blank control tube and the 2 tubes were single-stained tubes; 5 μL Annexin V-FITC or 10 μL propidium iodide (PI) was added to the single-stained tubes, and the compensation of the fluorescent channels was adjusted with a single-stained tube. In the sample group, Annexin V-FITC (Ex = 488 nm; Em = 530 nm) was detected by FITC detection channel and PI was detected by PE detection channel.

**Statistical Processing Analysis**

The statistical software SPSS 19.0 was used for data analysis. The measurement data were expressed as mean ± standard deviation. The comparison among groups was analyzed by variance and the comparison between groups by q-snk. The difference was statistically significant at P < .05.

**Results**

**Detection of the Protein Level of PTEN, PI3K, p-Akt, and FoxO3a by Western Blot**

From Figure 1, PTEN, PI3K, p-Akt, and FoxO3a proteins were expressed in all 4 groups of cells. Compared with the siNC group, the PTEN and FoxO3a protein levels in the siPTEN group were significantly decreased (P < .05), the PI3K protein levels significantly increased (P < .05), and the Akt phosphorylation level significantly upregulated (P < .05).

**Detection of the Messenger RNA Expression Levels of PTEN, PO3K, p-Akt, and FoxO3a by qRT-PCR**

The expression levels of PTEN and FoxO3a messenger RNA (mRNA) in the siPTEN group were significantly lower than those in the siNC group (P < .05), while expression levels of PI3K and p-Akt mRNA were significantly higher than those in the siNC group (P < .05). There were no significant differences in the expression of PTEN, PI3K, p-Akt, and FoxO3a mRNA among the Normal group, the Vector group, and the siNC group (P > .05), as shown in Figure 2.

**MTT Assay for Proliferation of HK-1 Cells**

Within 24 hours, the growth rate of HK-1 cells in Normal group, Vector group, siNC group, and siPTEN group was not significantly different (P > .05), but after 24 hours, the growth rate of HK-1 cells in the siPTEN group was significantly higher than that in the siNC group (P < .05), and the difference became more obvious over time, as shown in Figure 3.
Immunofluorescence Detection of the Expression of PTEN, PI3K, p-Akt, and FoxO3a in HK-1 Cells

From Figure 4, PTEN, PI3K, and p-Akt proteins were mainly located in the cytoplasm and expressed in a small amount in the nucleus. However, after PTEN gene was interfered, the expression of PTEN, PI3K, and p-Akt proteins in the cytoplasm decreased significantly, while the expression of PTEN, PI3K, and p-Akt proteins in the nucleus increased significantly, that is, from the cytoplasm to the nucleus (Figure 4A-C). FoxO3a protein was mainly located in the nucleus and expressed in a small amount in the cytoplasm. However, after PTEN gene was interfered, FoxO3a protein moved from the nucleus to the cytoplasm (Figure 4D).

Flow Cytometry Detection of Apoptosis in HK-1 Cells

Normal HK-1 cells were not stained with FITC and PI and were in the left lower quadrant of flow cytometry. HK-1 cells in the early stage of apoptosis were stained with FITC and were in the right lower quadrant. HK-1 cells in the late stage of apoptosis were simultaneously stained with FITC and PI and were in the right upper quadrant. Necrotic or mechanically damaged cells were in the left upper quadrant. In the siPTEN group, the proportion of HK-1 cells at the early stage of apoptosis was 6.1% and the total apoptotic rate of HK-1 cells at the early stage of apoptosis was 7.2%, which was significantly lower than that in the siNC group ($P < .05$; Figure 5A-H).
Discussion

The occurrence of cancer is usually the result of a combination of several factors, such as genetics, environment, viral infection, diet, and so on. Nasopharyngeal carcinoma, as a malignant tumor with strong geographical distribution, is more affected by genetic factors than other cancers. Because NPC is highly sensitive to radiation therapy, the current standard treatment for this disease is still radiotherapy. Patients with stage III and IV need to undergo both radiotherapy and chemotherapy, but according to relevant data, using radiotherapy and chemotherapy for NPC, only short-term local control can be achieved and long-term prognosis is not effective. It is prone to adverse symptoms such as recurrence and metastasis, resulting in a decrease in patient survival rate. With the advancement of medical science and technology, molecular biology-based gene-level research has elevated the understanding of diseases to a new stage. The focus of NPC research has gradually shifted to related gene modification to control the development of the disease from a genetic perspective.

RNA interference technology refers to the effective degradation and silencing gene expression of homologous host mRNA by double-stranded RNA. After transcription, specific double-stranded RNA is processed into siRNA duplexes and loaded into RNA-induced silencing complexes. Small interfering RNA recognizes target mRNA by base pairing, for direct cleavage and degradation. RNA interference technology can precisely block specific gene expression and produce gene knockout effects, providing a new method for the majority of scholars to analyze gene function. In this study, the expression of PTEN gene in human NPC HK-1 cell line was inhibited by RNAi. The results of qRT-PCR and Western blot showed that the expression levels of PTEN mRNA and protein in the siPTEN group were significantly lower than those in normal and siNC group, indicating that RNAi technology successfully inhibited PTEN gene expression in human NPC HK-1 cell line. PTEN, also known as mutated in multiple advanced cancer, transforming growth factor (TGF)-regulated and epithelial cell-enriched phosphatase (TEP1), is a novel cancer suppressor
gene located on the human chromosome 10q23.3. It is encoded by 403 amino acid residues and was first discovered in 1997. PTEN belongs to casein phosphatase family and also has lipo-phosphatase activity. It can inhibit cell proliferation, induce apoptosis, and enhance immune stability to achieve anticancer effect. PTEN have been used in the study of endometrial cancer, renal cancer, lung cancer, ovarian cancer, and other cancer diseases. In this study, the results of qRT-PCR and Western blot showed that the expression levels of FoxO3a mRNA and protein in the siPTEN group were significantly lower than those in the siNC group. The expression level of PI3K, p-Akt mRNA, and protein in the siPTEN group was significantly higher than that of the siNC group, which suggested that inhibiting the expression of PTEN gene in HK-1 cells can upregulate the expression of PI3K, p-Akt mRNA, and protein and downregulate the expression of FoxO3a mRNA and protein. It also indicated that the PTEN gene could affect the expression of PI3K, p-Akt, and FoxO3a. PI3K is composed of a catalytic subunit and a regulatory subunit, which can be regulated by vascular endothelial growth factor, fibroblast growth factor, and other signal transducers, and autophosphorylation occurs in the medial side of the plasma membrane. It can catalyze phosphatidylinositol 4,5-trisphosphate (PIP2) to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3). Akt is a specific ser/thr protein kinase of the AGC family, which contains 3 domains of regulation, catalysis, and pH. On the one hand, Ser473 and Thr308, which are located on the regulatory and catalytic domain, respectively, can undergo phosphorylation under the regulation of PI3K to activate the PI3K/Akt signaling pathway. On the other hand, the PH domain of Akt has a natural affinity for PIP3 and thus binds to PIP3 to activate the PI3K/Akt signaling pathway. Studies have shown that PI3K/Akt signaling pathway is abnormally expressed in a variety of tumor diseases, including NPC, which can promote tumor cell proliferation, change the cell active, and participate in disease progression by phosphorylating a variety of related substrates. FoxO3a is located on human chromosome 6q21 and is encoded by 673 amino acids. It has a fork-head DNA-binding domain typical of the fork-shaped transcription factor family. It plays an important role in cell cycle regulation, DNA damage repair, and other cellular physiological processes, known as “tumor suppressor.” FoxO3a expression activity is closely related to subcellular localization. Under normal conditions, FoxO3a is localized in the nucleus, but in tumor cells, it can be phosphorylated by Akt stimulation in its upstream pathway, forming a complex with 14-3-3 protein and transferring to the cytoplasm, leading to inactivation.

The results of immunofluorescence assay showed that after the PTEN gene was interfered, the expression of PTEN in the cytoplasm was significantly decreased and the expression in the nucleus was significantly increased. PI3K and p-Akt proteins first migrated to the plasma membrane region and then redisperse in the cytoplasm and nucleus. FoxO3a protein was mainly located in the nucleus, but after the PTEN gene was interfered, FoxO3a protein moved from the nucleus to the cytoplasm, suggesting that the accumulation of FoxO3a in the nucleus may be related to the inhibition of PTEN. The phosphatase activity of PTEN could transform PIP3 into PIP2, which reduces the phosphorylation of PI3K to Akt by blocking the level of PIP3 to block the PI3K/Akt signaling pathway and

Figure 5. Flow cytometry shows that the proportion of HK-1 cells in the early stage of apoptosis in the siPTEN group was 6.1%, and the total apoptosis rate of HK-1 cells in the early stage of apoptosis was 7.2%, which was significantly lower than that in the siNC group. ***P < .001. A, Apoptotic rate. B, No fluorescein isothiocyanate (FITC) and propidium iodide staining. C, Only FITC staining. D, Only propidium iodide staining. E, Normal group. F, Vector group. G, The siNC group. H, The siPTEN group.
promote the expression of FoxO3a.\textsuperscript{18} When the PTEN gene was inhibited, the PI3K/Akt signaling pathway was enhanced, which promoted PI3K upregulation of Akt phosphorylation and inhibition of FoxO3a expression, resulting in decreased expression of FoxO3a mRNA and protein.

The results of MTT assay showed that the growth rate of HK-1 cells in the siPTEN group was significantly higher than that in the siNC group after 24 hours, and the difference was more obvious with time, suggesting that inhibition of PTEN gene expression could promote cell proliferation. The results of flow cytometry showed that in the siPTEN group, the proportion of HK-1 cells at the early stage of apoptosis was 6.1% and the total apoptotic rate of the siPTEN group was 7.2%, which was significantly lower than that in the siNC group, suggesting that inhibition of PTEN gene expression could inhibit apoptosis.

In conclusion, inhibition of PTEN gene expression in HK-1 cells upregulates PI3K and Akt protein and mRNA levels, activates PI3K/Akt signaling pathway, inhibits FoxO3a activity, and increases cell growth rate and slows down apoptotic rate, suggesting that PTEN can participate in the development of NPC disease by affecting the PI3K/Akt/FoxO3a signaling pathway.

**Authors’ Note**
Our study did not require an ethical board approval because it did not contain human or animal trials.

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