The Role of PTEN Protein Phosphatase in Nasopharyngeal Carcinoma: The Ability of TGF-β1 Inducing Migration and Invasion was Inhibited by PTEN Protein Phosphatase through Down-regulating p-P38

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Abstract

Background: The phosphatase and tensin homolog gene (PTEN) is a crucial cancer suppressor gene in nasopharyngeal carcinoma (NPC), which encodes the protein Including lipid phosphatase and protein phosphatase. p-P38 plays a vital role in the development process of cancers. However, whether and how PTEN protein phosphatase inhibit p-P38 expression and regulate migration and invasion in nasopharyngeal carcinoma is still fully elucidated.

Methods: The abilities of migration and invasion were analyzed using Scratch, Transwell, Boyden experiments in NPC cells. Western Blot was utilized to explore the expression of E-cadherin, N-cadherin and VIMENTIN in Epithelial-mesenchymal transition (EMT), P38, p-P38 (Thr180/Tyr182), AKT, p-AKT (Ser473), PTEN and its mutants. qPCR was done to detect the mRNA expression of PTEN and PTEN mutants. Immunofluorescence localization assay and Co-immunoprecipitation assay was used to explore the mutual combination of PTEN and P38.

Results: We verified that transforming growth factor-β1 (TGF-β1) could induce migration, invasion, and EMT in NPC again, and appropriate concentration was 5ng/ml. Interestingly, we demonstrated that 5ng/ml TGF-β1 enhanced the level of...
Further more, after overexpression of PTEN WT and various mutants including PTEN-C124S (lacking of both lipid p-hosphatase and protein phosphatase), PTEN-G129E (only lacking of lipid phosphatase but remaining protein phosphatase), PTEN-Y138L (lacking of protein phosphatase but remaining lipid phosphatase), and PTEN 1-353 (Lack of c-tail structure), we observed that PTEN protein phosphatase reduced the ability of migration, invasion by inhibiting TGF-β1-induced p-P38, but the PTEN c-tail did not involve in this process. At last, we confirmed PTEN could bind to P38 each other.

**Conclusions:** The assay in the study indicated that PTEN protein phosphatase might be anticancer, where it was able to inhibit the ability of TGF-β1 inducing migration and invasion by reducing p-P38 in NPC cells.

**Keywords:** TGF-β1, PTEN protein phosphatase, p-P38, Migration, Invasion, NPC

**Background**

Nasopharyngeal carcinoma (NPC) is prevalent in South-East Asia, and mainly affects the middle-aged population [1-2]. Radiation therapy can cure early stage of NPC, but NPC distant metastasis and local recurrence is still the pivotal obstacles which affecting the prognosis of NPC [3-6]. And distant metastasis accounted for 59.8% of total mortality [7]. Therefore, we need to study the protein mechanism of NPC metastasis for reducing death-rate.

However, a number of studies argue PTEN may affect migration and invasion of tumor cells [8-12]. PTEN, a tumor suppressor gene, normally plays a important role in malignant tumor progression, which could frequently lost or mutated in human malignant tumor. And PTEN encodes protein including lipid phosphatase and protein phosphatase activity [13-15]. The structural pattern of PTEN protein is referred as Figure 1, PTEN is mainly divided into four parts, PBM, Phosphatase domain (PD), C2 domain and c-tail. Among them, the amino acid sequence of p-AKT (Ser473) and p-P38 (Thr180/Tyr182).
PD region ranges from 7-185, C2 region contains 186-351 amino acid sequence, and c-tail has 353-403 amino acid sequences. In addition, its mutant forms include PTEN C124S, PTEN G129E, PTEN Y138L and PTEN 1-353[16-17]. Among them, PTEN C124S, which lacks of PTEN protein phosphatase and lipid phosphatase activity. PTEN G129E remains protein phosphatase activity but abolishes lipid phosphatase. Whereas, PTEN Y138L remains lipid phosphatase but eliminates protein phosphatase activity. PTEN 1-353, which has no c-tail[18].

TGF-β is a secreted cytokine and exists in three isomeric forms, from β1 to β3, in human[19]. TGF-β1 plays a pleiotropic regulator in tumorigenesis and development such as metastasis, proliferation, survival, etc[20].

Especially in the terms of TGF-β1-induced EMT in malignant tumor cells[21-23]. The positive rates of TGF-β1 protein in NPC tissues obviously exceed that in nasopharyngeal inflammation tissues, and may correlate with NPC distant metastases[24-25]. In fact, recent researches show TGF-β1 could participate in EMT of nasopharyngeal carcinoma cells[26-29]. TGF-β1-induced migration requires RAC1-dependent activation of P38 in pancreatic carcinoma[30]. Interestingly, PTEN can inhibit Rac1 activity[10, 31].

Thus, P38 can also regulate the progress of nasopharyngeal carcinoma[32], which is serine and tyrosine kinase, and can be phosphorylated[33]. In fact, p-P38 for malignant tumor growth, metastasis and apoptosis also plays a very important role[34-35].

Therefore, we speculate that PTEN might directly regulate activation of P38 and affect migration and invasion in NPC cells.

**Materials and methods**

**Cell culture.** NPC cell lines including HONE1, SUNE-1, CNE-1, CNE-2, 5-8F and nasopharyngeal epithelial immortalized cell line NP69 were obtained from Central Laboratory of TCM-Integrated Cancer of Southern Medical University. Above mentioned cells were co-cultured into RPMI-1640(BI) with 10% fetal bovine serum(BI) with different concentrations(0ng/ml, 1ng/ml, 5ng/ml and 10ng/ml)
of TGF-β1 (Proteintech). NP69 was cultured in keratinocyte free serum medium (Gibco). All NPC cells were cultured in a incubator of 5% CO2 with 37°C.

**Plasmids transfection.** NC, PTEN WT, PTEN C124S, PTEN G129E and PTEN Y138L plasmids (Addgene) were transfected into 5-8F and HONE1 cells with Lipofectamine 2000 (Invitrogen) in serum-free conditions. 6–8h later, the medium was changed to the 10% serum medium with TGF-β1. Transfected cells were cultured 24–48h before qPCR, and 48h before Western Blot.

**qPCR.** Total RNA was extracted using Trizol reagent (Invitrogen). And cDNA was synthesized with the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). PCR analyses was carried out by SYBR Premix Ex Taq (TaKaRa). The forward primers of PTEN: 5′-TGACCAGGGATGAGACCAAC-3′ and the reverse one: 5′-CAGCACATCCCTGTATGGC-3′. GAPDH were used as the control, its two primers (including forward and reverse primer) were as follows: 5′-AT GGGGAAGGTGAAGGTCGGA-3′ and 5′-TTACTCCTGGAGGCCATGTGGG-3′.

**Western blot.** NPC cells were lysed in RIPA buffer (Beyotime, Shanghai, China) with protease inhibitors (Cwbio, Beijing, China) and phosphatase inhibitors (Cwbio). 10% SDS-PAGE was used to separate the protein of each sample, and the SDS-PAGE with separated protein was transferred to PVDF membranes (Millipore, Billerica, MA, USA). Next, the PVDF membranes were incubated with primary antibodies for PTEN (1:1000 dilution), p-P38 (Thr180/Tyr182) and P38 (1:1000 dilution), p-AKT (Ser473) and AKT (1:1000 dilution) (Above-mentioned primary antibodies from Cell Signaling Technology), E-cadherin (1:1000 dilution), N-cadherin (1:1000 dilution), VIMENTIN (1:1000 dilution), GAPDH (1:2000 dilution) (each antibody from Proteintech) at 4°C overnight. Immunocomplexes of the PVDF membranes were exposed using a PharosFX Plus molecular imager (MiniChem i 620, SAGECREATION, Beijing, China).

**Migration and invasion assay.** Scratch as assay was performed using a 200μl pipette tip and ruler to obtain the straight lane in cell-culture dish. Next, the cells were washed twice with D-hanks (8.0g NaCl, 0.4g KCl, 0.08g
Na$_2$HPO$_4$$\cdot$12H$_2$O,0.06g KH$_2$PO$_4$,0.35g NaHCO$_3$,ddH$_2$O 1000ml,PH 7.2),and the cells was cultured with serum-free medium.12h,24h,36h and 48h later, the change of the straight lane was observed and photographed under a inverted microscope(Nikon,To-kyo,Japan) at a specific location of cell-culture dish. Migration and invasion assay was carried out using Standard 24-well chemotaxis chamber (8µm pore size;Corning). For cell invasion assay, Matrigel(BD Biosciences) was added to the transwell chamber, which was as upper well. Briefly, $2\times10^5$ cells in 100µl serum-free media were moved to the upper chamber and 500µl media containing 10% FBS was put into the lower well. The cells were cultured at 37°C for 16-20h for migration assay and 36-40h for invasion assay. Those cells passed through the polycarbonate membrane were signed by Giemsa stain, and photographed by the inverted fluorescence microscope(Nikon). In five random highpower felds, thenumber of cells was calculated. All assays were repeated independently at least for three times.

**Immunoflfluoenscence (IF) Confocal Microscopy Analysis.** Cells were plated in the special small dish(sorfa,Zhejiang,China) of IF at a density of $5\times10^5$ cells/mL per well. 6h later, the cells were washed twice with 200µl D-hanks and replaced fresh medium. 24h later, 200µl D-hanks was used to wash the cells three times. And then the cells were permeabilized with 0.1% Triton for 10 minutes and blocked in 3% Bovine Serum Albumin(BSA) for 1h. The cells were signed with primary antibodies against PTEN(1:50, Cell Signaling Technology) and P38(1:50, Cell Signaling Technology) at 4°C overnight. Afterward, the cells with primary antibodies were stained with DyLight 488 Affinipure Goat Anti-Rabbit IgG, DyLight 594 Affinipure Goat Anti-Rabbit IgG(ybiotech, Shanghai, China) secondary antibodies (1:1000) for 1h, and the cell nucleus were signed with DAPI (Beyotime, Shanghai, China). Then, photographed using a confocal microscopy(Olympus, Japan). Colocalization efficiency was quantified through Image J software(NIH, Beth-esda, MD, USA).

**Co-Immunoprecipitation(co-IP) Assay.** In brief, PTEN WT plasmids were transfected into 5-8F cells. 48h later, D-hanks was used to wash the cells, twice. Then,
those cells were lysed in 800 µL of cell Lysis/Washing buffer (ybiotech) at 4°C for 30 minutes. After cell lysates was centrifugated at 12,000×g at 4°C for 10 minutes, the supernatant was taken as IP, and the rest was used as Input. The PTEN interacting proteins were enriched by co-immunoprecipitation using 10µl PTEN antibody (Cell Signaling Technology). The protein complex was analyzed by Western Blot using corresponding antibodies.

**Statistical analysis.** All statistical data were gained from at least three independent experiments and analyzed by SPSS (version 20.0, IBM, USA). Student T test, One-way ANOVA test and Dunnett’s multiple comparison test was performed for two groups and multiple groups, respectively. Two-way ANOVA test was performed for two factors data. Data were presented as the mean±sem. P<0.05 was considered statistically significant.

Result

**TGF-β1 induces metastasis of NPC cells.**

TGF-β1 plays a key role in the control of metastatic capacity of many malignancies [30,36]. To demonstrate the function of TGF-β1 on NPC cells, 5-8F and HONE1 cells were cultured in 10% FBS medium with 0ng/ml, 1ng/ml, 5ng/ml, 10ng/ml of TGF-β1, respectively. After 48h, Scratch, Transwell and Boyden asasay was used to detect the change in ability of migration and invasion (Figure. 2A,B, C,D,E,F). As for 5-8F and HONE1 cells, metastatic capacity was enhanced in a concentration-dependent behavior. But, the difference between the experimental groups and the control group (0ng/ml TGF-β1 group) was statistical significance when the concentration of TGF-β1 was ≥5ng/µl. And Western Blot was used to prove the change of EMT transformation, we found that the expression of E-cadherin was reduced, whereas, N-cadherin and VIMENTIN was increased (Figure. 3 A,B). The results showed, compared to 0ng/ml TGF-β1, 5ng/ml or 10ng/ml TGF-β1 promoted migration, invasion and EMT in NPC cells, and we gave that appropriate stimulus concentration of TGF-β1 was 5ng/µl.

**TGF-β1 promotes the increasing of p-AKT and p-P38.**
The metastasis ability of malignant tumor cells is correlated with the phosphorylation levels of AKT(Ser473) and P38(Thr180/Tyr182) kinase [37]. To confirm whether TGF-β1 could induce the increasing level of p-AKT(Ser473) and p-p38 (Thr180/Tyr182), we need the 5ng/μl TGF-β1 to stimulate 5-8F and HONE1 for 48h. Western Bolt was used to detect p-AKT(Ser473) and p-p38(Thr180/Tyr182) (Figure 4). And the result demonstrated that TGF-β1 enhanced the expression of p-AKT(Ser473) and p-P38(Thr180/Tyr182).

**PTEN protein phosphatase inhibits EMT in NPC cells by down-regulating TGF-β1-induced p-p38.**

**Overexpression of PTEN WT and its mutations.**

Previous studies reported that PTEN expression was down-regulated in NPC cells and biopsies [38-41].

We overexpressed PTEN WT, mutant PTEN C124S without lipid phosphatase and protein phosphatase activity, PTEN G129E with only protein phosphatase activity, PTEN Y138L with only lipid phosphatase activity, and PTEN 1-353 lacking c-tail structure in NPC cells. As shown in Figure 5 A, B, PTEN WT and each mutant was the efficiency of instant transfer.

**PTEN protein phosphatase inhibits TGF-β1-induced migration, invasion and EMT in NPC.**

In order to verify the functions of different enzyme activities and structures of PTEN, on the basis of various PTEN overexpression, 5-8F and HONE1 cells were treated in 10% FBS medium with 5ng/μl TGF-β1 for 48h. Transwell and Boyden assays were used to test the migration and invasion ability.

Compared to NC group, when the plasmids of PTEN WT, PTEN G129E with protein phosphatase were overexpression, the ability of migration and invasion was declined. Concurrently, those plasmid with lipid phosphatase activity, PTEN WT, PTEN Y138L, the transfer ability of NPC cells was also down-regulated. And PTEN C124S, neither protein phosphatase nor lipid phosphatase, did not down regulate migration and invasion (Figure 6 A, B, C, D). PTEN WT with c-tail and PTEN 1-353 without c-tail both inhibited migration and invasion, but those
two group had no difference (Figure 6 A, B, C, D). Those results indicated that both protein phosphatase and lipid phosphatase of PTEN could inhibit the migration and invasion of NPC cells, but PTEN c-tail did not.

To confirm whether two phosphatase activities of PTEN are able to inhibit EMT of NPC. After NPC cells were treated for 48h with 5ng/μl TGF-β1, Western Blot was used to detect the expressions of E-cadherin, N-cadherin and VIMENTIN. Compared with NC, E-cadherin was enhanced by PTEN WT, PTEN G129E, PTEN Y138L and PTEN 1-353, N-cadherin and VIMENTIN was lowered, but PTEN C124S not (Figure 7 C, D). Therefore, PTEN both lipid phosphatase and protein phosphatase could inhibit EMT in NPC cells. But PTEN c-tail was not involved in the EMT process of NPC cells.

Those results proved that both the protein phosphatase and lipid phosphatase of PTEN could inhibit migration, invasion and EMT in NPC. And c-tail of PTEN did not participate in those processes.

**PTEN protein phosphatase reduced TGF-β1-induced p-p38.**

To further clarify the mechanism of PTEN both lipid phosphatase and protein phosphatase inhibiting metastasis of NPC cells, Western Blot was used to detect the expressions of AKT, p-AKT (Ser473), P38 and p-P38 (Thr180/ Tyr182). The increase of TGF-β1-induced p-P38 was prevented by PTEN WT, PTEN G129E and PTEN 1-353 compared with the NC, but PTEN C124S not. The expression of TGF-β1-induced p-AKT was declined by PTEN WT, PTEN Y138L and PTEN 1-353. Therefore, those results explained that PTEN protein phosphatase could lower the expression of TGF-β1-induced p-p38, but its lipid phosphatase did not. On the contrary, PTEN lipid phosphatase only could inhibit the expression of p-AKT, but its protein phosphatase didn’t (Figure 7 A, B). So we argued that TGF-β1 inducing migration and invasion was inhibited by PTEN protein phosphatase through reducing p-P38 in NPC.

**PTEN could bind to P38 each other**

Yet, How do PTEN and P38 directly interact? We conducted immunofluorescence localization experiments in 5-8F, which confirmed the localization of
PTEN was in cytoplasm and nucleus. And P38 was in the nucleus, the two proteins co-located with the nucleus (Fig. 8A). Further more, PTEN and P38 were detected simultaneously by co-immunoprecipitation (Fig. 8B). Those results shown that PTEN could bind to P38 each other.

**Discussion**

Witte D. etl found that TGF-β1-induced cell migration requires RAC1 and NOX4-dependent activation of P38 in pancreatic carcinoma cells [30]. Interestingly, Liliental J. etl think that genetic deletion of the PTEN promotes cell motility by activation of Rac1 and Cdc42 GTPases [31]. Perhaps, PTEN could down-regulate TGF-β1-induced p-P38 by inhibiting the activity of RAC1, thereby inhibit the metastasis ability of tumor cells. We illuminate PTEN protein can directly act on P38 and regulate the migration and invasion ability of cancer cells under the stimulation of TGF-β1.

We confirmed under the stimulus of TGF-β1, PTEN protein phosphatase can inhibit NPC cell metastasis, and PTEN c-tail does not seem to be involved in the metastasis process of nasopharyngeal carcinoma cells, but this paper was lacking some experiments, for instance, we are unable to determine the definite working location of PTEN protein and P38, and our animal experiments also failed to undertake.

**Conclusion**

In this study, we determined PTEN protein phosphatase could inhibit the migration and invasion of NPC cells in TGF-β1 microenvironment, which correlated with PTEN combined with P38 and down-regulated p-P38 (Thr180/Tyr182). To our knowledge, this is the first report to describe p-P38 is the downstream substrate of PTEN.

To better understand the influence of different enzyme activities of PTEN on the migration, invasion and EMT of NPC and possible mechanisms, we did those assays. There is a growing realization about the metastasis process of NPC. May-
be we can use this as a breakthrough point to guide clinicians in the diagnosis and treatment of tumors.

**List of abbreviations**

NPC: Nasopharyngeal carcinoma; PTEN: Phosphatase and tensin homolog deleted on chromosome ten; P38: P38 mitogen-activated protein kinases; AKT: Protein kinase B; TGF-β1: Transforming growth factor-β; co-IP: Co-Immunoprecipitation; IF: Immunofluorescence

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Written informed consent for publication was obtained from all participants.

**Availability of data and materials**

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

JZ and NZ conducted the cell experiments, collected the data and wrote the manuscript. SW, XZ, CH, and NZ analyzed the data from the experimental results. PG and HP were involved in the conception and design of the study, and revised the manuscript. All authors have read and approved the final version of the manuscript.
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**Figure legends**

Figure 1. The structure of PTEN. PTEN protein is divided into four parts, PBM, Phosphatase domain (PD), C2 domain and c-tail. Among them, PBM region contains 1-7 amino acid sequences, the amino acid sequence of PD region ranges from 7-185, C2 region contains 186-351 amino acid sequences, and c-tail has 354-403 amino acid sequences. PTEN C124S, PTEN G129E, PTEN Y138L, PTEN 1-353, these are the mutant forms.

Figure 2. The changes in migration and invasion ability of nasopharyngeal carcinoma cells were stimulated by TGF-β1 at 0ng/ml, 1ng/ml, 5ng/ml and 10ng/ml. (A), (B) Scratch test confirmed the transfer capacity in 5-8F and HONE1
respectively with TGF-β1 at 5ng/ml or 10ng/ml were more than 0ng/ml. (C)(D) Transwell experiments tested that TGF-β1 promoted the migration ability of 5-8F and HONE1 when the concentration of TGF-β1 was at 5ng/ml and 10ng/ml. (E) (F) Boyden experiments confirmed that TGF-β1 promoted the invasion of 5-8F and HONE1. Original magnification, ×200; One-way ANOVA, mean±sem, N=3. *P< 0.05, **P<0.01, ***P<0.001.

Figure 3. Western Blot showed different concentration of TGF-β1 could induce EMT. (A)(B) Respectively indicated that the EMT of 5-8F and HONE1 cells. When TGF-β1 was at 5ng/ml or 10ng/ml, the expression of E-cadherin was significantly enhanced than 0ng/ml. However, the levels of N-cadherin and VIMENTIN were lower. The data were shown as the mean±sem, One-way ANOVA, *P<0.05, **P<0.01.

Figure 4. Western Blot showed TGF-β1 induced phosphorylation of related kinase proteins. When the concentration of TGF-β1 was 5ng/ml, the phosphorylation levels of P38 (Thr180/Tyr182) and AKT (Ser473) was more increase compared to 0ng/ml. The data were shown as the mean±sem Student’s t-test, *P<0.05, **P<0.01.

Figure 5. (A) Western Blot was used to verify the plasmid transfection efficiency of NC, PTEN WT, PTEN C124S, PTEN G129E, PTEN Y138L and PTEN 1-353
in 5-8f cells. (B) Western Blot showed those plasmid overexpressions in HONE1 cells. The data were shown as the mean±sem, One-way ANOVA, *P<0.05, **P<0.01, ***P<0.001.

Figure 6. After NPC cells transfected with NC, PTEN WT or mutants, the changes of migration and invasion were detected by Transwell and Boyden assays respectively with 5ng/ml TGF-β1. (A) (C) Transwell assays showed PTEN WT, PTEN G129E, PTEN Y138L and PTEN 1-353 restrained 5ng/ml TGF-β1-induced migration. (B) (D) Boyden assays showed PTEN WT, G129E, Y138L and PTEN 1-353 inhibited 5ng/ml TGF-β1-induced invasion. Two-way ANOVA test. Mean±sem, N=3, *P<0.01, **P<0.01, ***P<0.001.

Figure 7. After NPC cells transfected with NC, PTEN WT or mutants, in 5ng/ml TGF-β1 condition, the changes of relative proteins of kinases and EMT were detected by Western Blot. (A) Western Blot indicated that PTEN WT, PTEN G129E and PTEN 1-353 inhibited the expression of TGF-β1-induced p-p38 (Thr180/Tyr182) compared with NC and PTEN C124S in 5-8F cells, the expression of P38 was invariable. PTEN WT, PTEN Y138L and PTEN 1-353 Inhibited the expression of p-AKT(Ser473), but PTEN G129E did not inhibit the expression of p-AKT, and the protein level of AKT did not change. Simultaneously, (C) PTEN WT, PTEN G129E, PTEN Y138L and PTEN 1-353
inhibited TGF-β1-induced EMT process compared with NC and PTEN C124S. (B)(D) Western Blot showed changes accordingly in HONE1 cells. Two-way ANOVA test. Mean±sem, N=3,**P<0.01,***P<0.001.

Figure 8. PTEN associates with P38. (A) Immunofluorescence assay (IF) showed PTEN and P38 bound to the nucleus. Red light represented PTEN protein, which expressed not only in the cytoplasm but also nucleus. Green light represented P38, which expressed in the nucleus. DAPI was in the nucleus, which is blue. The combination of red fluorescence and green fluorescence was yellowish green. (B) Co-Immunoprecipitation (IP) verified the mutual binding of PTEN and P38.