# Original Article Paeoniflorin exhibits antitumor effects in nasopharyngeal carcinoma cells through downregulation of NEDD4

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Abstract: Paeoniflorin (PF), which is isolated from the paeony root, possesses tumor suppressive function in a variety of malignancies. However, it is unknown whether PF possesses anticancer activity in nasopharyngeal carcinoma (NPC) and which molecular mechanism is involved in PF-triggered antitumor function. In the current study, we measured cell proliferation, apoptotic death, cell cycle, cell motility in NPC cells after PF exposure. We found that PF suppressed cell proliferation, stimulated apoptosis, triggered cell cycle arrest, and impeded cell migratory and invasive activity in NPC cells. Mechanistically, PF inhibited the expression of neural precursor cell expressed developmentally downregulated protein 4 (NEDD4). We further validated that PF induced antitumor activity via downregulation of NEDD4 in NPC. Our data suggest that PF might act as a potential inhibitor of NEDD4 for treating NPC.

Keywords: Paeoniflorin, nasopharyngeal cancer, NEDD4, invasion, viability

#### Introduction

Nasopharyngeal carcinoma (NPC) is a rare type of head and neck cancer [1]. NPC is mainly diagnosed in Southeast Asia and Southern China [2]. Genetic factors, smoking, Epstein Barr virus (EBV) infection, and certain foods including salted fish, hot spices, preserved food, and alcohol have been reported to contribute to NPC development [2]. Radiotherapy is a common method of treating NPC. Chemotherapy is often used for NPC patients with locoregional and distant metastases [3]. However, due to the highly invasive nature of NPC and the resistance to radiotherapy and chemotherapy, chemoradiotherapy outcomes are often unsatisfactory [4]. Thus, it is necessary to develop new compounds to improve the therapeutic benefit of NPC patients.

Neural precursor cell expressed developmentally downregulated protein 4 (NEDD4) is an E3 ligase that degrades multiple proteins to regulate various cell processes including fluid and electrolyte homeostasis [5], neuronal development [6] and cell survival [7]. In recent years, NEDD4 was reported to be involved in tumor development and progression. For example, higher expression of NEDD4 is existed in nonsmall-cell lung carcinoma [8], colon [9], prostate and bladder [10], gastric carcinoma [11], and mammary cancers [12]. Moreover, NEDD4 was revealed to play a crucial role in tumor metastasis [13, 14]. NEDD4 was identified to degrade the key tumor suppressor phosphatase and tensin homologue depleted on chromosome ten (PTEN) to exhibit its oncogenic function in breast cancer and prostate cancer [15]. Another tumor suppressor, large tumor suppressor kinase 1 (LATS1), is ubiquitinated and degraded by NEDD4, leading to inactivation of the Hippo pathway [16]. The haplotype of NEDD4 binding protein 2 (N4BP2) is associated with sporadic NPC [17]. Our previous study

demonstrated that NEDD4 participates in the acquisition of epithelial-mesenchymal transition (EMT) in cisplatin-resistant NPC cells [18]. These reports indicate that NEDD4 is an important oncoprotein in malignancies, indicating that targeting NEDD4 might be a putative approach for treating cancers.

Paeoniflorin (PF), which is isolated from the paeony root, possesses multiple activities including regulation of immunity, protection of convulsant, reduction of blood sugar, protection of low blood pressure [19, 20]. Accumulating evidence has shown that PF exerts its tumor suppressive function in various types of human cancers [21-24]. However, it is unclear whether PF possesses anticancer activity in NPC and which molecular mechanism is involved in PF-mediated tumor suppressive function. To address this question, we measured the cell proliferation, apoptotic death, cell cycle, cell motility in NPC cells after PF exposure. We further detected whether PF could inhibit the expression level of NEDD4 in NPC cells. We also determined whether PF has its antitumor activity via regulation of the NEDD4 pathway. Our study could provide evidence for the discovery of new agent PF for the treatment of NPC patients in the future.

#### Materials and methods

#### Cell culture and reagents

Human NPC cell lines CNE1 and CNE2 were obtained from Shanghai Chinese Academy of Sciences cell bank (Shanghai, China) and cultured in RPMI-1640 medium with 10% FBS at 37°C. Anti-NEDD4, anti-Akt, anti-pAKT, anti-PTEN, and anti-tubulin antibodies were bought from Cell Signaling Technology. The secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 was bought from Invitrogen. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-Htetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO). PF was bought from the Huanyu Company (Beijing, China).

#### Cell viability assay

NPC cells (5  $\times$  10<sup>3</sup>) were seeded in a 96-well plate overnight. Cells were exposed with different concentrations of PF for 48 hours and 72 hours. Cells were treated with 0.1% dimethyl sulfoxide (DMSO) as control group. Then, MTT assay was performed as described previously [25].

### Cell apoptosis analysis

The Annexin V-fluorescein isothiocyanate (FI-TC)/propidium iodide (PI) method was used to detect the apoptosis in NPC cells after treatment with different concentrations of PF. The detailed method for measuring cell apoptosis was described before [25].

### Cell cycle analysis

NPC cells were cultured in 6-well plate for overnight and treated with different doses of PF for 48 hours. Then, cells were collected and fixed with ice-cold 70% (v/v) ethanol and stored at 4°C overnight. Cell cycle was analyzed as described previously [25].

### Wound healing assay

NPC cells were grown in a 6-well plate until more than 90% confluence. Then, cells were scratched in a straight line using a sterile pipette tip to create rectangular lesion. The cells were exposed to PF for 20 hours. The images were captured at 0 hour and 20 hours.

# Transwell invasion assay

Matrigel coated Transwell filter was used to detect the cell invasion in NPC cells after exposure to different doses of PF for 20 hours. The detailed approach was described previously [18].

# Western blotting analysis

NPC cells were treated with different doses of PF for 72 hours and then were harvested, and lysed for Western blotting. The method of Western Blotting analysis was described previously [18].

#### Transfection

Cells were seeded in six-well plates and treated with PF in combination with transfection with NEDD4 small interfering RNA (siRNA) (GenePharma, Shanghai, China), or non-targeting control siRNA or NEDD4 cDNA constructor for 48 hours using Lipofectamine 2000. After the transfection and PF exposure, cells were used to measure cell viability, apoptosis, and motility.

# Statistical analysis

Statistical analyses were performed using the analysis of variance (ANOVA) to analyze the significant difference among multiple groups. Data are represented as means  $\pm$  SD. *P* values <0.05 has statistically significant.

#### Results

### PF inhibits cell viability

To determine whether PF treatment could inhibit cell viability in NPC cells, CNE1 and CNE2 cells were exposed to different PF concentrations for 48 hours and 72 hours. Cell proliferation was measured by MTT assay in NPC cells after PF exposure. PF inhibited cell viability in both NPC cell lines (**Figure 1A**). In fact, 20  $\mu$ M and 40  $\mu$ M PF exposures resulted in 40% and 70% reduction of cell viability in CNE1 cells at 72 hours, respectively (**Figure 1A**). Similarly, 20  $\mu$ M and 40  $\mu$ M PF exposures caused 50% and 75% reduction of cell viability in CNE2 cells, respectively (**Figure 1A**). Our data suggest that PF suppressed cell viability in NPC cells.

#### PF induces cell apoptosis

Next, to explore whether PF induces apoptosis in NPC cells, CNE1 and CNE2 cells were exposed to PF for 48 hours and reacted with Annexin V-FITC/PI. Our data showed that 20  $\mu$ M and 30  $\mu$ M PF exposures resulted in apoptosis rates from 4.05% to 14.85% and 26.53% in CNE1 cells, respectively (**Figure 1B**). The apoptosis rates elevated from 5.64% to 13.04% and 21.35% in CNE2 cells with 20  $\mu$ M and 30  $\mu$ M PF exposures, respectively (**Figure 1B**). Our results indicated that PF stimulated apoptosis that could contribute to the reduction of cell viability.

#### PF induces cell cycle arrest

Cell cycle analysis was performed in NPC cells after PF treatment. CNE1 and CNE2 cells were exposed to PF for 48 hours and stained with PI to measure DNA content. We observed that PF exposure led to cell cycle arrest at G2/M phase in NPC cells. The G2/M phase fraction increased from 13.4% to 27.50% in CNE1 cells with 30  $\mu$ M PF treatment, from 17.59% in the control group to 29% in CNE2 cells with 30  $\mu$ M PF exposures (**Figure 1C**). These data suggest that PF induced cell cycle arrest at the G2/M phase in NPC cells.

### PF inhibits cell migration and invasion

PF inhibits cell motility in human cancer cells. Here, we determined whether PF could regulate cell motility in NPC cells. A wound healing assay was used to measure cell migration in NPC cells after PF exposure. We found that cell migration was significantly inhibited in NPC cells after PF treatment for 20 hours (**Figure 2A** and **2B**). We further defined whether PF could retard cell invasion in NPC cells. Our Transwell chamber assay results demonstrated that PF impeded cell invasive activity of NPC cells (**Figure 2C**). Our results clearly showed that PF retarded cell motility in NPC cells.

### PF downregulates NEDD4 expression

NEDD4 is a pivotal oncoprotein in tumorigenesis. In order to investigate the molecular insight into PF-triggered antitumor activity, western blot analysis was used to measure the expression of NEDD4 in NPC cells after PF exposure. Our Western blotting data revealed that PF inhibited the expression of NEDD4 in NPC cells (Figure 2D). PTEN is an important target of NEDD4 in human cancer. Thus, we measured the expression of PTEN in NPC cells after PF treatment. We found that PF treatment led to the upregulation of PTEN in NPC cells (Figure 2D). Moreover, our western blotting results showed that PF treatment inhibited the expression of pAkt in NPC cells (Figure 2D). Therefore, PF might exhibit antitumor via suppression of NEDD4 and subsequent upregulation of PTEN and downregulation of pAkt in NPC cells.

Overexpression of NEDD4 rescues PFmediated inhibition of cell viability and motility

To further validate whether PF exposure led to anticancer feature via inhibition of NEDD4 in NPC cells, NEDD4 was upregulated in NPC cells via transfection with NEDD4 cDNA. Cells were transfected with pcDNA 3.1 as a control group. The MTT results showed that NPC cells transfected with the NEDD4 cDNA construct had enhanced cell growth (Figure 3A). Importantly, NEDD4 cDNA transfection abrogated PF-involved cell viability reduction in NPC cells (Figure 3A). Our wound healing assay results dissected that upregulation of NEDD4 increased cell migratory activity in both NPC cell lines (Figure 3B). Moreover, upregulation of NEDD4 rescued PF-mediated inhibition of cell migration (Figure 3B). Furthermore, our Transwell invasion assay



**Figure 1.** Effect of PF on NPC cell viability, apoptosis and cell cycle. A. MTT assay was used to detect cell viability in NPC cells after PF exposure. \*\*P<0.05 vs control. B. Apoptosis was detected by flow cytometry using Annexin V-FITC/PI in NPC cells after PF exposure. C. Cell cycle was analyzed by flow cytometer in NPC cells following PF exposure.

data showed that upregulation of NEDD4 increased cell invasion in NPC cells (**Figure 3C**). Consistently, upregulation of NEDD4 abolished PF-induced retardation of cell invasion in NPC cells (**Figure 3C**). Altogether, PF inhibited cell growth and motility via the downregulation of NEDD4. Overexpression of NEDD4 abolished PFinduced cell apoptosis

Apoptosis was analyzed in cells after PF exposure in combination with NEDD4 cDNA transfection. We found that overexpression of NE-DD4 inhibited cell apoptosis in both NPC cell



**Figure 2.** Effect of PF on motility of NPC cells. A. A wound healing assay was used to detect migration of NPC cells after PF exposure. B. Quantitative results were illustrated for the wound healing assay. \*P<0.01 vs control. C. A Transwell assay was used to detect invasion of NPC cells following PF exposure. D. Left panel: Western blotting was used to detect the protein levels of NEDD4, Akt, and PTEN NPC cells after PF exposure. Right panel: Quantitative results are illustrated for the left panel. \*P<0.05 vs control.



**Figure 3.** Overexpression of NEDD4 abolished PF-induced cell viability inhibition and motility suppression. A. Cell viability was measured by MTT assay in NPC cells with NEDD4 overexpression following PF exposure. Both: PF plus NEDD4 cDNA transfection. B. Left panel: A wound healing assay was used in NPC cells with NEDD4 overexpression following PF treatment. Right panel: Quantitative results are illustrated for left panel. \*P<0.05 vs Control; #P<0.05 vs PF treatment alone or NEDD4 cDNA transfection alone. C. Left panel: A Transwell chamber assay was used in NPC cells with NEDD4 overexpression following PF treatment. Right panel: Quantitative results are illustrated for left panel: A Transwell chamber assay was used in NPC cells with NEDD4 overexpression following PF treatment. Right panel: Quantitative results are illustrated for left panel. \*P<0.05 vs Control; #P<0.05 vs PF treatment. Right panel: Quantitative results are illustrated for left panel. \*P<0.05 vs Control; #P<0.05 vs PF treatment. Right panel: Quantitative results are illustrated for left panel. \*P<0.05 vs Control; #P<0.05 vs PF treatment. Right panel: Quantitative results are illustrated for left panel. \*P<0.05 vs Control; #P<0.05 vs PF treatment alone or NEDD4 cDNA transfection alone.

lines (Figure 4A). Notably, up-regulation of NEDD4 abolished PF-induced cell apoptosis (Figure 4A). Our western blotting data showed that NEDD4 cDNA transfection increased the expression of NEDD4 in NPC cells (Figure 4B and 4C). Importantly, upregulation of NEDD4 reduced PTEN levels and upregulated pAkt levels in NPC cells (Figure 4B and 4C). Notably, overexpression of NEDD4 rescued the inhibition of pAkt and upregulation of PTEN induced by PF exposure (Figure 4B and 4C). These results implied that PF induced apoptosis in part via down-regulation of NEDD4 in NPC cells.

# Downregulation of NEDD4 enhances PFmediated antitumor activities

To further confirm whether NEDD4 is involved in PF-exerted anticancer effects in NPC cells, cells were transfected with NEDD4 siRNA plus PF treatment. Our MTT data showed that downregulation of NEDD4 by siRNA inhibited cell growth in CNE1 and CNE2 cells (Figure 5A). Moreover, NEDD4 siRNA transfection in combination with PF treatment led to a greater degree of cell growth inhibition compared with PF treatment or siRNA transfection alone (Figure 5A). Our wound healing assay data demonstrated that downregulation of NEDD4 impeded cell migration and enhanced PF-mediated reduction of cell migration (Figure 5B). Transwell invasion assay results indicated that PF-mediated cell invasion inhibition was enhanced by NEDD4 siRNA transfection (Figure 5C). In addition, downregulation of NEDD4 enhanced PFinduced apoptosis in NPC cells (Figure 6A). The western blotting analysis data showed that downregulation of NEDD4 enhanced PF-mediated inhibition of pAKT and upregulation of PTEN in NPC cells (Figure 6B and 6C). Taken together, the downregulation of NEDD4 promoted PF-induced tumor suppressive functions in NPC cells.

# Discussion

PF plays a tumor suppressive role in human malignancies. However, the biological functions of PF and the underlying mechanism in NPC have not been studied. Thus, we aimed to determine the role of PF in NPC and explore the molecular basis of PF-mediated antitumor activity. We found that PF suppressed cell viability, stimulated apoptosis, triggered cell cycle arrest, and impeded cell motility in NPC cells. Moreover, PF inhibited the expression of NEDD4 and validated that PF-induced anticancer activity in part via reduction of NEDD4 in NPC. Our findings suggest that PF could be a potential natural compound to treat NPC.

Increasing evidence has shown that PF exerts antitumor activity in cancers. For example, PF inhibited cell proliferation via induction of cell cycle arrest and activation of the Fas/Fas ligand-mediated apoptotic pathway in nonsmall-cell lung cancer cells [26]. PF induced cell apoptosis via modulating Bcl-2 family proteins and caspase-3 in cervical cancer cells [27]. One study showed that PF inhibited the growth of human colorectal carcinoma in vitro and in vivo via activation of p53 [28]. Another study reported that PF induced apoptosis through suppression of prostaglandin E receptor EP2 in human hepatoma cells [29]. PF inhibited tumor invasion and metastasis via decreased expression of metalloproteinase-9 (MMP-9) and extracellular signal regulated kinases (ERK) and increased expression of E-cadherin in hepatocellular carcinoma cells [30]. PF inhibited cell proliferation and induced apoptosis via suppression of MMP-9 in glioma cells [31]. Similarly, PF inhibits cell proliferation through inactivation of phosphatidylinositol 3kinase (PI3K)/Akt and signal transducers and activators of transcription 3 (STAT3) signaling in gastric cancer cells [32]. PF suppressed proliferation and invasion via inactivation of the Notch-1 pathway in breast cancer cells [33]. Our study identified that PF suppressed the expression of NEDD4 in NPC cells, leading to inhibition of cell growth and invasion.

NEDD4 plays an oncogenic role in a variety of human cancers including NPC [17, 18]. In this study, we found that downregulation of NEDD4 in NPC cells inhibited cell viability, migration and invasion, whereas upregulation of NEDD4 enhanced cell viability and motility. Therefore, suppression of NEDD4 might be a helpful strategy for treating NPC. Several studies have identified multiple inhibitors of NEDD4 for the potential treatment of human malignancies. For example, the chemopreventative agent indole-3-carbinol interacts with NEDD4 protein and disrupts PTEN degradation, resulting in antiproliferative effects on human melanoma cells [34]. Recently, I3C analogues were developed as potent inhibitors of NEDD4, and these compounds inhibit the proliferation of melanoma



**Figure 4.** Overexpression of NEDD4 abrogates PF-mediated apoptotic death in NPC cells. (A) Apoptotic death was measured by flow cytometry in NPC cells with NEDD4 overexpression following PF exposure. (B) Western blotting was used to measure the expression of NEDD4 and its downstream targets in NPC cells with NEDD4 overexpression following PF exposure. (C) Quantitative results are illustrated for (B). \*P<0.05 vs Control; #P<0.05 vs PF treatment alone or NEDD4 cDNA transfection alone. Both: PF exposure plus NEDD4 cDNA transfection.



**Figure 5.** Downregulation of NEDD4 enhanced PF-mediated antitumor activities. A. MTT assay was used to detect cell viability in NPC cells with NEDD4 downregulation following PF exposure. Both: PF exposure plus NEDD4 siRNA transfection. B. Left panel: A wound healing assay was used in NPC cells with NEDD4 downregulation following PF exposure. Right panel: Quantitative results are illustrated for left panel. \*P<0.05 vs Control; #P<0.05 vs PF treatment alone or NEDD4 siRNA transfection alone. Both: PF exposure and NEDD4 siRNA transfection. C. Left panel: A Transwell chamber assay was used in NPC cells with NEDD4 downregulation following PF exposure. Right panel: Quantitative results are illustrated for left panel. \*P<0.05 vs PF treatment: A Transwell chamber assay was used in NPC cells with NEDD4 downregulation following PF exposure. Right panel: Quantitative results are illustrated for left panel. \*P<0.05 vs PF treatment alone or NEDD4 siRNA transfection alone. \*P<0.05 vs Control; #P<0.05 vs PF treatment alone or NEDD4 siRNA transfection. C. Left panel: Quantitative results are illustrated for left panel. \*P<0.05 vs PF treatment alone or NEDD4 siRNA transfection. C. Left panel: Quantitative results are illustrated for left panel. \*P<0.05 vs Control; #P<0.05 vs PF treatment alone or NEDD4 siRNA transfection alone.



**Figure 6.** Downregulation of NEDD4 enhanced PF-mediated cell apoptosis. (A) Apoptotic death was detected flow cytometry in NPC cells with NEDD4 downregulation following PF exposure. (B) Western blotting was used to detect the protein levels of NEDD4 and its downstream targets in NPC cells with NEDD4 downregulation following PF exposure. (C) Quantitative results are illustrated for (B). \*P<0.05 vs Control; #P<0.05 vs PF treatment alone or NEDD4 siRNA transfection alone. Both: PF exposure and NEDD4 siRNA transfection.

cells [35]. Curcumin was found to downregulate the expression of NEDD4 in glioma cancer cells and pancreatic cancer cells, leading to antitumor activity [36, 37]. Therefore, the discovery of new NEDD4 inhibitors is pivotal for human cancer treatment. Our study indicates that PF could be a potent inhibitor of NEDD4 for NPC, although further exploration is required to define the mechanism and side-effects of PF.

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### Disclosure of conflict of interest

None.

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