



Tumor cell-derived exosome RNF126 affects the immune microenvironment and promotes nasopharyngeal carcinoma progression by regulating PTEN ubiquitination

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Abstract

This study aimed to investigate the role and regulatory mechanism of RNF126 in nasopharyngeal carcinoma. Firstly, the expression and prognosis of RNF126 were analyzed by TCGA database. The expression of RNF126 was further verified by NPC tissue samples and cells. An ectopic xenograft model was constructed to verify the regulatory role of RNF126 in NPC tumor progression. The regulatory effect of RNF126 on macrophage polarization and migration was verified by co-culture of tumor cells and THP-1 cells. The role of RNF126 in tumor exosomes involved in intercellular communication was further verified by nanoparticle tracking technology, western blotting and immunofluorescence assays. QRT-PCR, half-life assay and WB assay were used to verify the regulatory effect of RNF126 on PTEN ubiquitination and PI3K/AKT pathway. Finally, an in vivo assay was used to verify the regulation of exosomes on tumor growth and metastasis. In summary, we found for the first time that tumor-derived exosomal PTEN degrades PTEN through ubiquitination to regulate the tumor immune microenvironment and promote NPC growth and metastasis. These results provide the basis for the screening of early markers of NPC and targeted therapy.

Keywords Nasopharyngeal carcinoma · RNF126 · Ubiquitination · Autophagy · Macrophage polarization

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck malignancies in southern China and Southeast Asia [1]. There are significant regional differences in the incidence of NPC, with high incidence in the Arctic, North Africa, and Southeast Asia. It is the third most common malignant tumor in southern China [2]. Globally, there are 80,000 new cases and approximately 50,000 deaths each year [3]. Existing studies have found that tumor-associated macrophages (TAM) play an important role in the growth, invasion and metastasis of tumors; this is also the case in NPC [4]. Cancer cell co-culture up-regulates the

expression of early cellular inflammatory factors, and causes the expression of metastasis-related genes and interferon-activated genes in the later stage of co-culture, indicating that nasopharyngeal cancer cells "tame" tumor-associated macrophages to play a tumor-promoting role, especially these factors induced by tumor-associated macrophages can promote the migratory ability of nasopharyngeal carcinoma cells [5, 6].

Current research shows that tumor cell-derived exosomes (Exo) can promote tumorigenesis and metastasis by affecting other types of cells in the tissue microenvironment [7]. At the same time, tumor-derived exosomes are the key carriers for the communication between tumor cells and stromal cells, and they play an important role in tumor growth and metastasis [8, 9]. Exosomes exert corresponding biological effects by carrying mRNA, protein, ncRNA and other substances [10, 11]. This finding provides a basis for us to explore the mechanism of exosomes and the delivery mode of related molecules.

Phosphatase and tension homologs (PTEN), known as tumor suppressors, are frequently aberrantly or highly mutated in a variety of cancers. In addition, PTEN has been

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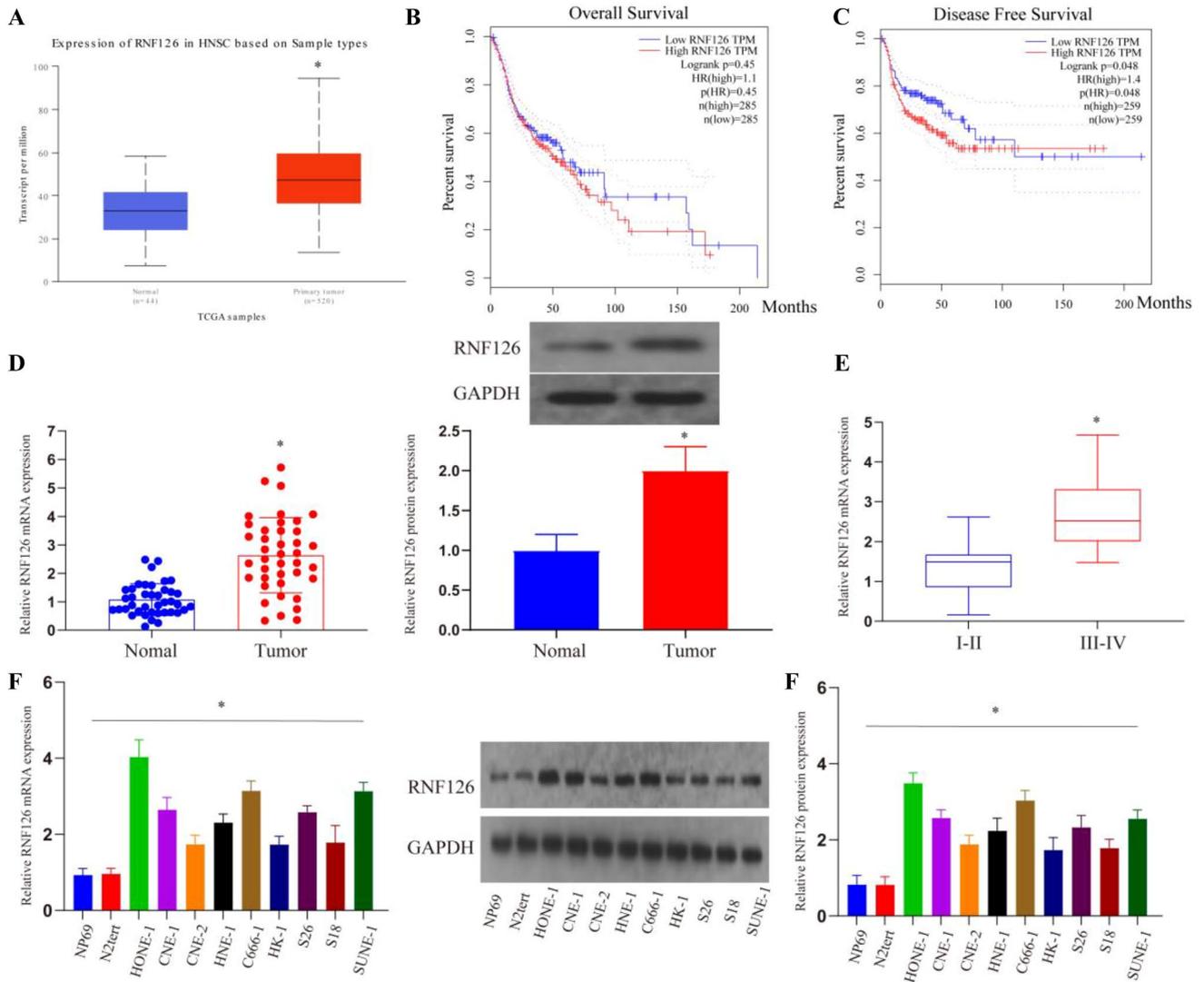
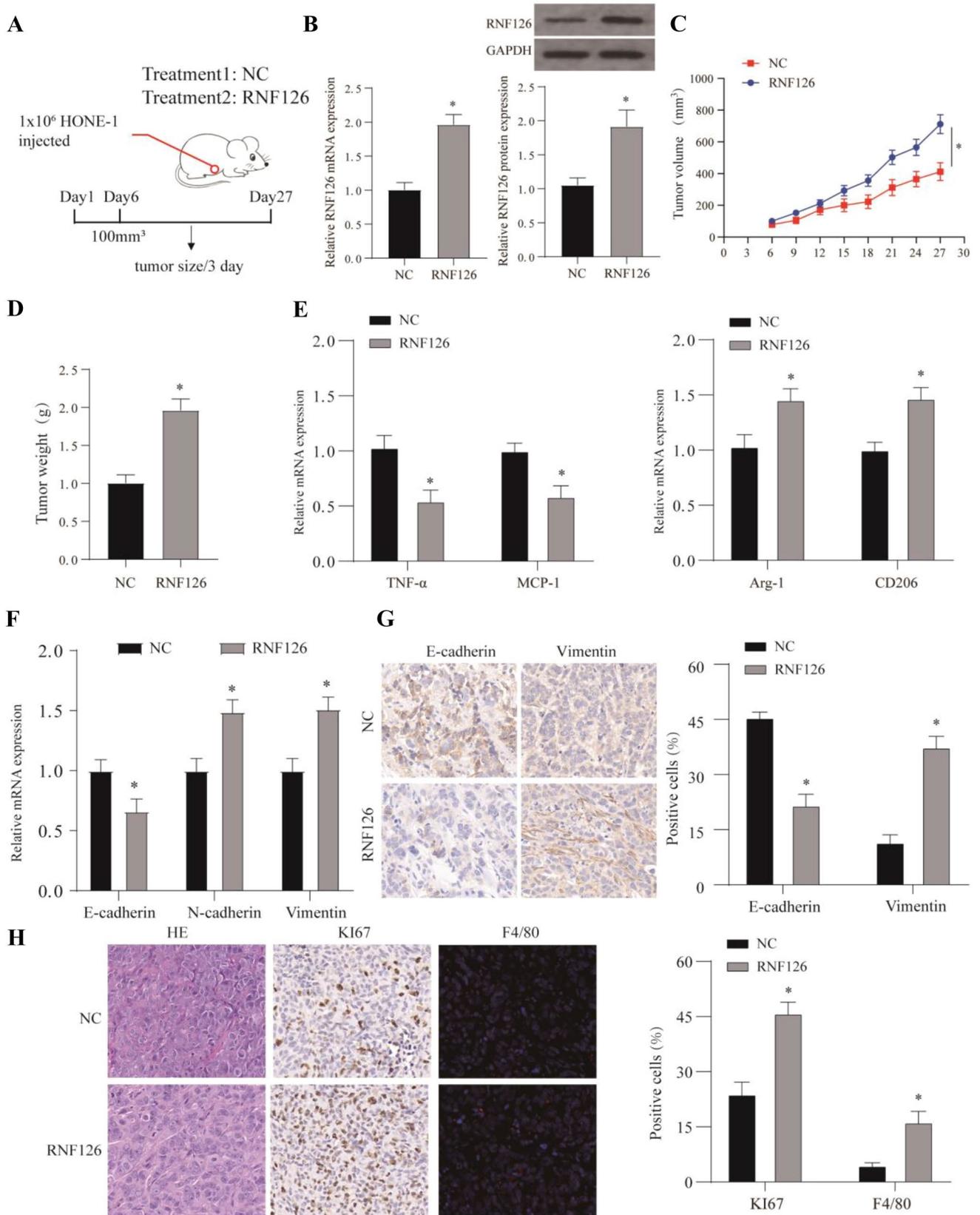


Fig. 1 Expression of RNF126 in nasopharyngeal carcinoma tissues and cells. **A** Expression of RNF126 in NPC tumors in TCGA database; **B** Kaplan–Meier survival analysis of RNF126 expression for overall survival. **C** Kaplan–Meier survival analysis of RNF126 expression for disease free survival (DFS). **D** The mRNA and pro-

tein expression of RNF126 in 40 pairs of clinical samples. **E** The expression of RNF126 in different of tumor stages. **F** The mRNA and protein expression of RNF126 in various NPC cells was detected by QRT-PCR. * $p < 0.05$, compared with relative control group

confirmed to be involved in the regulation of malignant tumor cell proliferation, migration and EMT by regulating the phosphatidylinositol 3-kinase-protein kinase B (PI3K/AKT) signaling pathway, thereby regulating tumor radiotherapy and chemotherapy resistance and pathogenesis [12]. Current studies have shown that various post-translational modifications (PTMs) such as acetylation, phosphorylation, oxidation, and ubiquitination/deubiquitination processes regulate the expression of PTEN proteins. This may be one of the mechanisms of abnormal expression of PTEN in tumors

Fig. 2 RNF126 promotes NPC growth process in vivo. **A** Differently treated HONE-1 cells were injected when tumors grew to 100 mm³. **B** The expression of RNF126 in tumor tissue was detected by QRT-PCR. **C** The growth trend of tumor volume in different treatment groups. **D** Tumor weights in different treatment groups. **E** The expression of M1/M2 macrophage markers in different treatment groups was detected by QRT-PCR. **F** The expression of EMT markers in different treatment groups was detected by QRT-PCR. **G** Expression of E-cadherin and Vimentin proteins in tumor tissues of each treatment group. **H** Histological staining (HE, KI67 and immunofluorescence) was used to detect tumor tissue proliferation and macrophages, $\times 200$. * $p < 0.05$, compared with relative control group



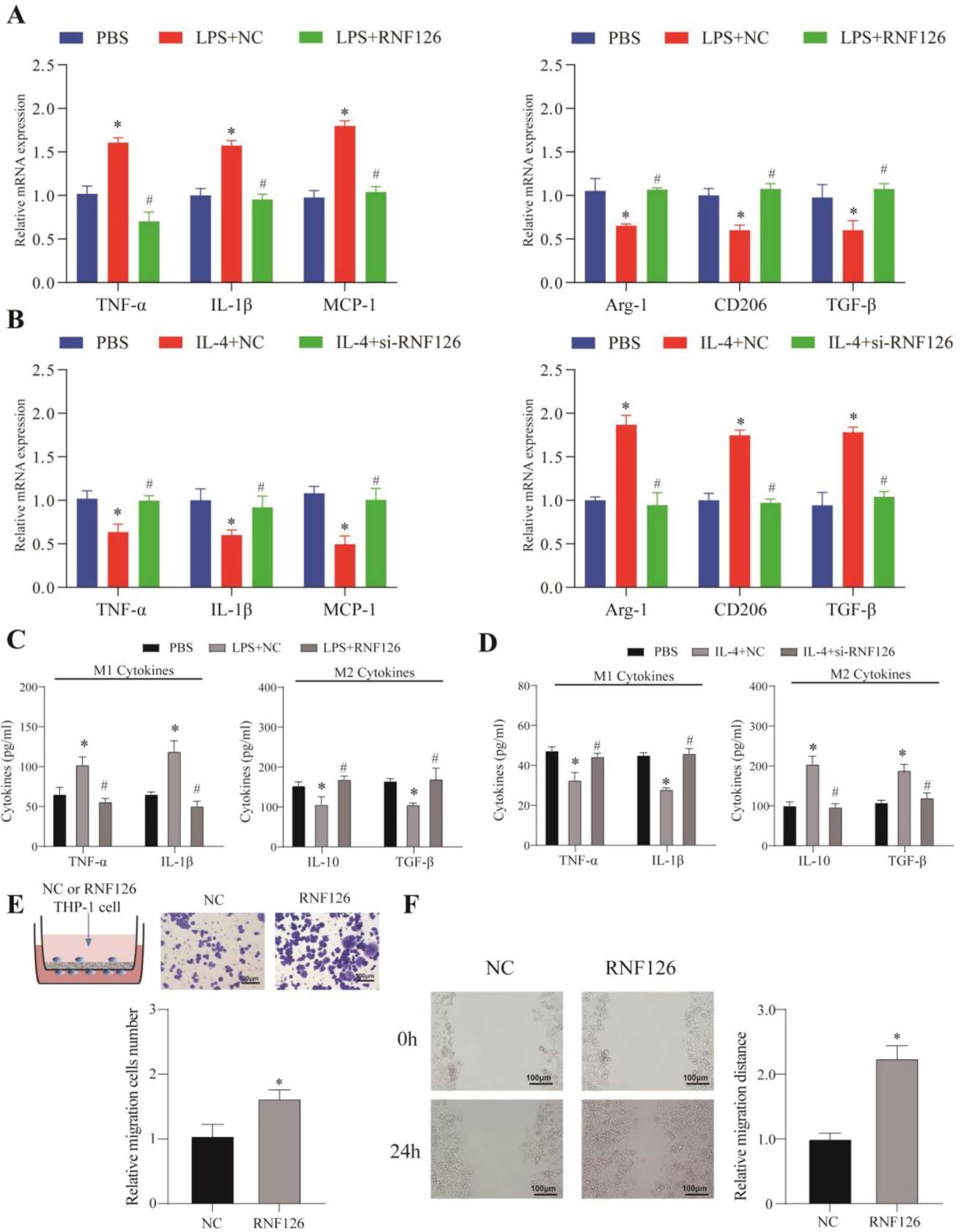


Fig. 3 Co-culture of HONE-1 and THP-1 cells to verify the regulatory effect of RNF126 on macrophage polarization. **A** The expression of M1/M2 macrophage markers in different treatment groups was detected by QRT-PCR. **B** The expression of M1/M2 macrophage markers in different treatment groups was detected by QRT-PCR. **C** The expression of M1/M2 related cytokines in each treatment group was detected by enzyme-linked immunosorbent assay. **D** The expression of M1/M2 related cytokines in each treatment group was detected by enzyme-linked immunosorbent assay. **E** Transwell was used to detect the cell invasion ability of THP-1 cells treated with exosomes overexpressing RNF126. **F** wound healing assay was used to detect the cell migration ability of THP-1 cells treated with exosomes overexpressing RNF126. * $p < 0.05$, compared with relative control group

[13, 14]. These PTMs modulate the activity and stability of PTEN to inhibit the PI3K/AKT signaling pathway. Ubiquitination is an important post-translational modification of proteins. It participates in the regulation of cell signaling, autophagy, cell cycle and DNA damage repair by affecting protein stability, cellular sublocation or enzymatic activity, biological process [15]. The regulation of PTEN by ubiquitination modification is mainly manifested in regulating PTEN protein stability, inhibiting phosphatase activity, and changing its subcellular localization. NEDD4-1 is the first reported E3 ubiquitin ligase to regulate the ubiquitination of PTEN, which can regulate the stability of PTEN protein at the cellular level [16]. However, studies have shown that NEDD4-1 does not significantly regulate PTEN protein stability at the level of tumor samples and mice [17]. Subsequently, E3 ubiquitin ligases such as XIAP, WWP2 and CHIP were successively reported to be able to ubiquitinate PTEN, thereby affecting the stability of PTEN [18, 19]. In addition to the regulation of PTEN protein stability, ubiquitination modifications can also modulate PTEN phosphatase activity. For example, TRIM27 can affect the activity of the PI3K/AKT pathway by mediating the ubiquitination of PTEN [20]. It has been reported that NEDD4-1-mediated monoubiquitination of Lys13 and Lys289 of PTEN can promote its nuclear localization [21]. Although many E3 proteins can participate in the regulation of PTEN protein, most experiments are based on cell experiments, so it is more realistic and convincing to analyze the correlation between PTEN and E3 from the level of mouse models and clinical tumor samples. However, the regulation of PTEN is complex, and the mechanisms that regulate stable levels in NPC remain poorly understood.

RNF126, an E3 ubiquitination ligase, has acted as an oncogene in various cancers including breast, prostate,

gastric, leukemia and tongue cancer [12]. However, the role of RNF126 in nasopharyngeal carcinoma remains unclear. Here, we reveal a novel mechanism by which RNF126 promotes nasopharyngeal carcinoma and identify PTEN as a novel RNF126 substrate. We have described that RNF126 interacts with PTEN and mediates its ubiquitination and proteasomal degradation. In addition, TAM is involved in the process of NPC through the regulation of autophagy mediated by the PI3K/AKT pathway.

Material and methods

Clinical sample

Forty patients with nasopharyngeal carcinoma who were treated in our hospital from January 2017 to December 2020 were selected. This study was approved by the ethics committee of our hospital. The collection of all samples obtained the informed consent of the patients and signed the informed consent. This study was approved by the ethics committee of the hospital. All included individuals signed the written informed consent. This study followed the Declaration of Helsinki. No radiotherapy and chemotherapy were performed before tissue biopsy, and each tissue specimen was placed in a -80°C freezer for cryopreservation. The clinical sample information is detailed in Supplementary Table 1.

Cell culture and transfection

NPC cell lines including HONE-1, CNE-1, CNE-2, HNE-1, C666-1, HK-1, S26, S18 and SUNE-1 were collected from the Cellbank of the Chinese Academy of science (Shanghai, China). The human immortalized nasopharyngeal epithelial cell lines (NP69 and N2Tert) served as a control. NP69 cells were cultured in K-SFM complete medium and NPC cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). The culture conditions were 37°C , 5% CO_2 . In this study, cells were divided into NC group, RNF126 group and si-RNF126 group. RNF126, si-RNF126 and their control sequences were transfected into NPC and THP-1 cells using Lipofectamine 2000 (Biosharp, China) according to the instructions of transfection reagents for subsequent detection.

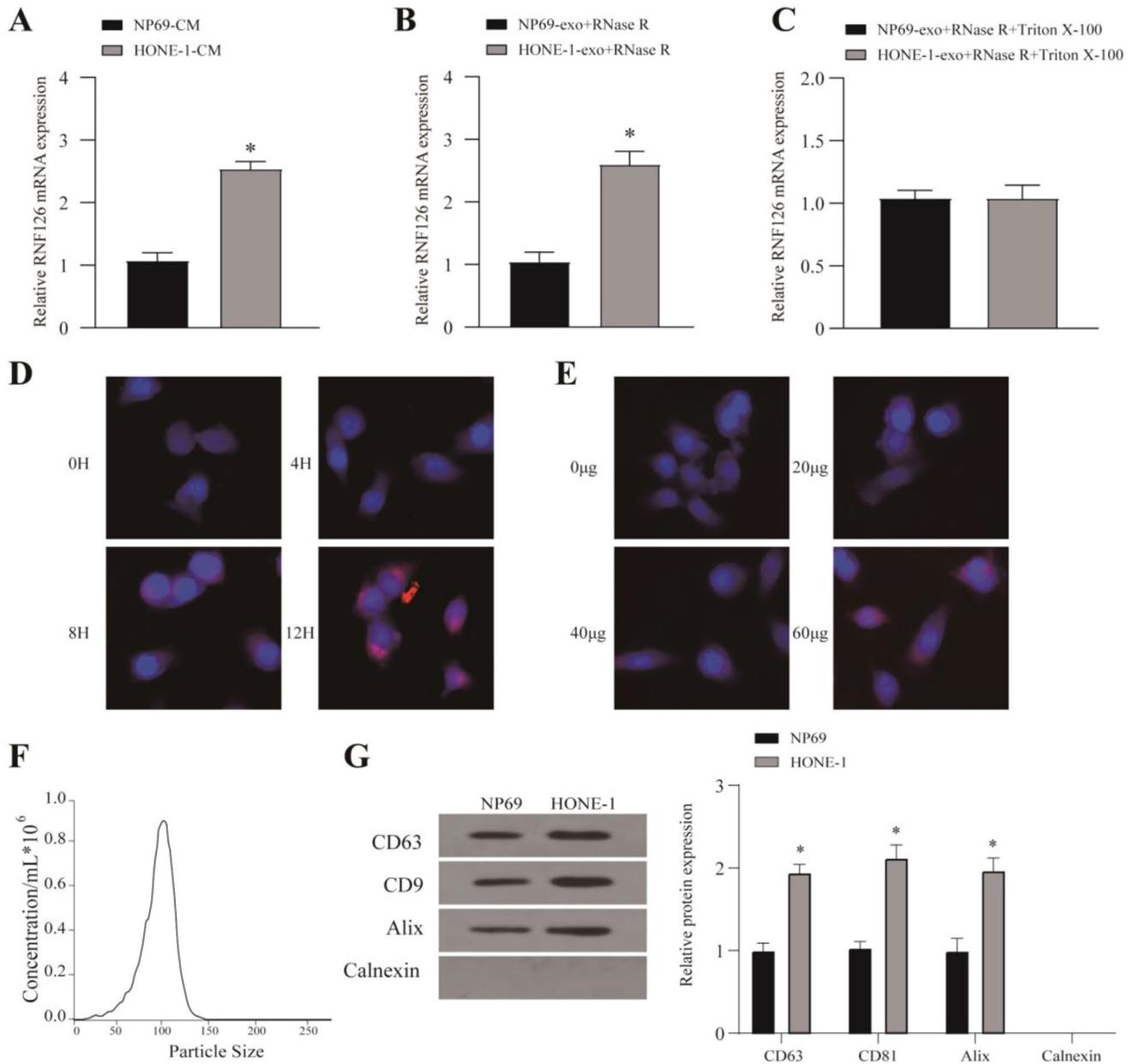


Fig. 4 The role of exosomes in the delivery of RNF126. **A** Expression of RNF126 in cell culture medium detected by QRT-PCR. **B** The level of RNF126 expression in the exosomes extracted with RNase A (0.1 mg/ml) after in different group. **C** The level of RNF126 in the exosomes destroyed by Triton X-100 (0.3%) and RNase A treated with different group was detected by RT-PCR. **D–E** The endocytosis

of PKH67-labeled exosomes in THP-1 cells was detected by immunofluorescence at different treatment times and doses. **F** NTA was used for particle size and concentration analysis of tumor cell-derived exosomes. **G** The expression of exosome markers in different cells was detected by WB. * $p < 0.05$, compared with relative control group

Exosome isolation and observation

The cell culture medium of each treatment group was collected. The exosome extraction was carried out according to the kit instructions. In other words: centrifuge the samples at $2000\times g$ for 20 min, transfer the supernatant to a new tube, add exosome separation agent and mix well, then let stand at $4\text{ }^{\circ}\text{C}$ for 30 min to 2 h; then centrifuge at $15,000\times g$ for

30 min at $4\text{ }^{\circ}\text{C}$; discard the supernatant. The clear liquid is exosomes. Take $50\text{ }\mu\text{L}$ of exosomes resuspended in PBS, adsorb them on a copper mesh with a polymethylvinyl acetate support membrane, stain with 2% phosphotungstic acid, and observe the size using a transmission electron microscope. The particle size and concentration of exosomes were analyzed using nanoparticle tracking technology.

Nanoparticle tracking analysis (NTA)

NanoSight uses a 45-mW 405-nm laser and an electron multiplying charge-coupled device (EMCCD) to determine particle size distribution by tracking the Brownian motion of individual particles. 50-fold dilution to detect the smallest beads, using high sensitivity settings (dilution 1:50; shutter 26.67 ms; gain 650; threshold 22; tracking 1.8×10^3 particles) and low sensitivity settings (undiluted; shutter 1.67 ms; gain 100; threshold 10; track 1.1×10^4 particles). We multiplied the concentrations provided by the NTA software by the ratio between the expected and measured concentrations of the calibration beads. This concentration calibration was performed using 102-nm and 203-nm polystyrene beads, using high sensitivity settings (dilution 1:500; shutter 26.67 ms; gain 650; threshold 19; tracking 1.0×10^3 vesicles) and low sensitivity Settings (dilution 1:100; shutter 26.67 ms; gain 400; threshold 10; tracked 1.1×10^3 vesicles). Use 105-nm and 206-nm silica beads for concentration calibration, setting the concentration to 1×10^8 mL for high and low sensitivity, because the refractive indices of silica and vesicles are close.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from NPC cells or tumor tissues in each group with TRIzol reagent, and qRT-PCR results were obtained in the form of $2^{-\Delta\Delta CT}$ values according to the manufacturer's recommended protocol, and each sample was repeated 3 times. The mRNA expression levels of RNF126, LC3, ATG10, Beclin-1, M1 macrophage markers (TNF- α , IL-1 β and MCP-1), M2 macrophage markers (Arg-1, TGF- β and CD206) and markers of EMT progression (E-cadherin, N-cadherin and Vimentin) were performed with GAPDH as an internal reference, and PCR reactions were performed according to the qRT-PCR kit manufacturer's instructions: 50 °C for 2 min, 95 °C for 10 min; 95 °C for 30 s, 60 °C for 30 s, 40 cycles. All primer sequences are detailed in Supplementary Table 2.

Western blot (WB)

The expression of related proteins was detected by Western blotting. The cell pellets of each group were collected, the cells were lysed by RIPA to extract total protein, and the BCA kit was used for protein quantification. Prepare polyacrylamide gel, load 30 μ g total protein/well, electrophoresis for 2 h, transfer membrane for 1.5 h, and block for 1 h. Add anti-GAPDH monoclonal antibody (1:4 000, ab8245, Abcam), anti-PTEN monoclonal antibody (1:500,

ab267787, Abcam), anti-RNF126 monoclonal antibody (1:1000, ab234812, Abcam), anti-p-PI3K monoclonal antibody (1:1000, ab182651, Abcam), total PI3K Antibody (1:1000, ab32089, Abcam), anti-p-AKT monoclonal antibody (1:1000, ab38449, Abcam), total AKT antibody (1:1000, ab179463, Abcam), anti-CD63 antibody (1:2000, ab134045, Abcam), anti-Alix (1:1000, ab275377, Abcam) and anti-calnexin (1:1000, ab133615, Abcam) incubated overnight at 4 °C; washed with TBST, HRP-labeled goat anti-rabbit IgG (1:5 000, ab6721, Abcam), incubated at room temperature for 1 h; ECL luminescent solution, developed. The gray values of the bands were analyzed and quantified using image J software.

Induction of M1/M2 macrophages

THP-1 cells (5×10^5 cells/mL) were stimulated with 100 ng/mL PMA for 24 h, the medium was changed after adhering, 40 ng/mL IL-4 or 100 ng/mL LPS was added, and the cells were cultured for 48 h. The cells were photographed under a phase contrast microscope. The cell pellets were collected, and the mRNA expression levels of relevant macrophage markers were detected by qRT-PCR.

Transwell chemotaxis assay

The Transwell chamber was placed in a 24-well plate, 3×10^4 THP-1 cells were added to the upper chamber, and then 100 ng/mL PMA was added for 24 h. After THP-1 was transformed into adherent cells, the medium in the upper chamber was replaced, and the medium in the lower chamber was replaced. 2×10^4 HONE-1 and RNF126-overexpressing HONE-1 cells were added to co-culture for 24 h. The upper chamber was taken out, washed 3 times with PBS, fixed with methanol for 30 min, washed again with PBS, stained with 1% crystal violet solution for 15 min, washed 3 times with PBS, gently wiped off the cells inside the upper chamber with a cotton swab, and randomly selected 5 fields of view under the microscope Observe and take pictures, and count the number of pierced cells in each group. Three replicate wells were set in each group, and the experiment was repeated three times independently.

Wound healing assay

Differently treated THP-1 cells were seeded on a 6-well plate. When the cell confluence reached 80–90%, use a 200 μ l pipette tip to draw a straight line in the center to form a single layer of cells. The cell healing distance after 24 h was observed by microscope.

Fig. 5 RNF126 regulates PTEN ubiquitination in macrophages. **A** We used the Ubiquitin database and the BioGRID protein interaction database to predict the regulatory substrates of RNF126 and found that PTEN may be a downstream regulatory protein of RNF126. **B** The expression level of PTEN protein after overexpression of RNF126 was detected by WB. **C** The correlation between the expression of PTEN and RNF126 in head and neck tumor tissues was analyzed in Starbase database. **D** Immunofluorescence for RNF126 and PTEN localization in THP-1 cells. **E** Co-IP was used to detect the interaction between RNF126 and PTEN. **F** THP-1 cells were transiently transfected with HA-tagged RNF126 and Myc-tagged PTEN. **G** CHX was used for PTEN protein half-life assay. **H** HEK-293 cells were co-transfected with HA-RNF126 or mutated HA-RNF126 vectors and vectors expressing PTEN and His-ubiquitin (His-Ub), respectively. Cells expressing RNF126 showed increased PTEN ubiquitination compared to cells transfected with control vector. * $p < 0.05$, compared with relative control group

Enzyme-linked immunosorbent assay (ELISA)

The cell-free supernatants from THP-1 cell line was collected. The levels of TNF- α , IL-1 β , IL-10 and TGF- β in the supernatant of cell culture medium were detected using enzyme-linked immunosorbent assay (ELISA, Beyotime Biotechnology, China) according to the manufacturer's instructions.

Nude mouse xenograft model

BALB/c nude mice were purchased from Charles River Laboratories (Beijing, China). In this study, different treated HONE-1 cells (2×10^6) were injected subcutaneously into the lower abdomen of nude mice. Tumor volume was measured every 3 days after completion of the injection. Tumor volume calculation formula: volume = (width² × length) / 2. When the tumor volume reached the 100mm³, we injected 10 μ g/50 μ L EVs or 50 μ L PBS into the tumor tissue. This study was injected once a day for 5 days. After the experiment, the mice were euthanized, and a part of the extracted tissue was fixed with 4% paraformaldehyde for subsequent histological staining. Another portion was quickly placed in liquid nitrogen and stored for qRT-PCR and WB detection.

Ki-67 and TUNEL staining

In this study, the tumor tissues of mice in each treatment group were paraffin-embedded and sliced, and then the tissues were stained with the Ki-67 immunohistochemistry kit (E607235, Sangon Biotech, China) and TUNEL analysis kit (06432344001, Roche, USA) referring to the manufacturer's method.

Hematoxylin and eosin (HE) and F4/80 staining

In this study, hematoxylin and Shuo were used to stain paraffin sections of mouse tumor tissues after deparaffinization and dehydration. In addition, this study performed immunohistochemistry on tumor tissues using antibodies against F4/80 (macrophage marker) (10 μ g/ml, ab6640, abcam). Finally, observe the cell morphology under a microscope.

Statistical analysis

All data were statistically analyzed by SPSS Statistics 22.0 statistical software, data were expressed as $\bar{x} \pm s$, independent samples *t* test was used, and three or more groups were compared by one-way analysis of variance, and $p < 0.05$ was considered statistically significant.

Result

Expression of RNF126 in nasopharyngeal carcinoma tissues and cells

First, we used the UALAN database to find that the expression of RNF126 in head and neck tumors was significantly higher than that in the control group (Fig. 1A). After extended analysis of TCGA data, it was found that RNF126 expression was up-regulated in a variety of tumors (Supplementary Fig. 1). Further survival analysis showed that high expression of RNF126 was associated with poor prognosis in disease free survival (Fig. 1B, C). In addition, we found that the expression of RNF126 was significantly up-regulated in nasopharyngeal carcinoma tissues and advanced tumor tissues (Fig. 1D, E). Similarly, the high expression of RNF126 was also verified in 9 strains of NPC cells (Fig. 1F). These results suggest that RNF126 may play an important role in NPC progression.

RNF126 promotes NPC growth process in vivo

To verify the effect of RNF126 on tumor growth, we constructed RNF126 overexpressing cells and control cells and injected them into nude mice (Fig. 2A, B). We measured the volume every 3 days of tumor growth and weighed the mice at the end. The results showed that the cell growth rate and tumor weight were significantly increased after RNF126 overexpression treatment compared with the NC

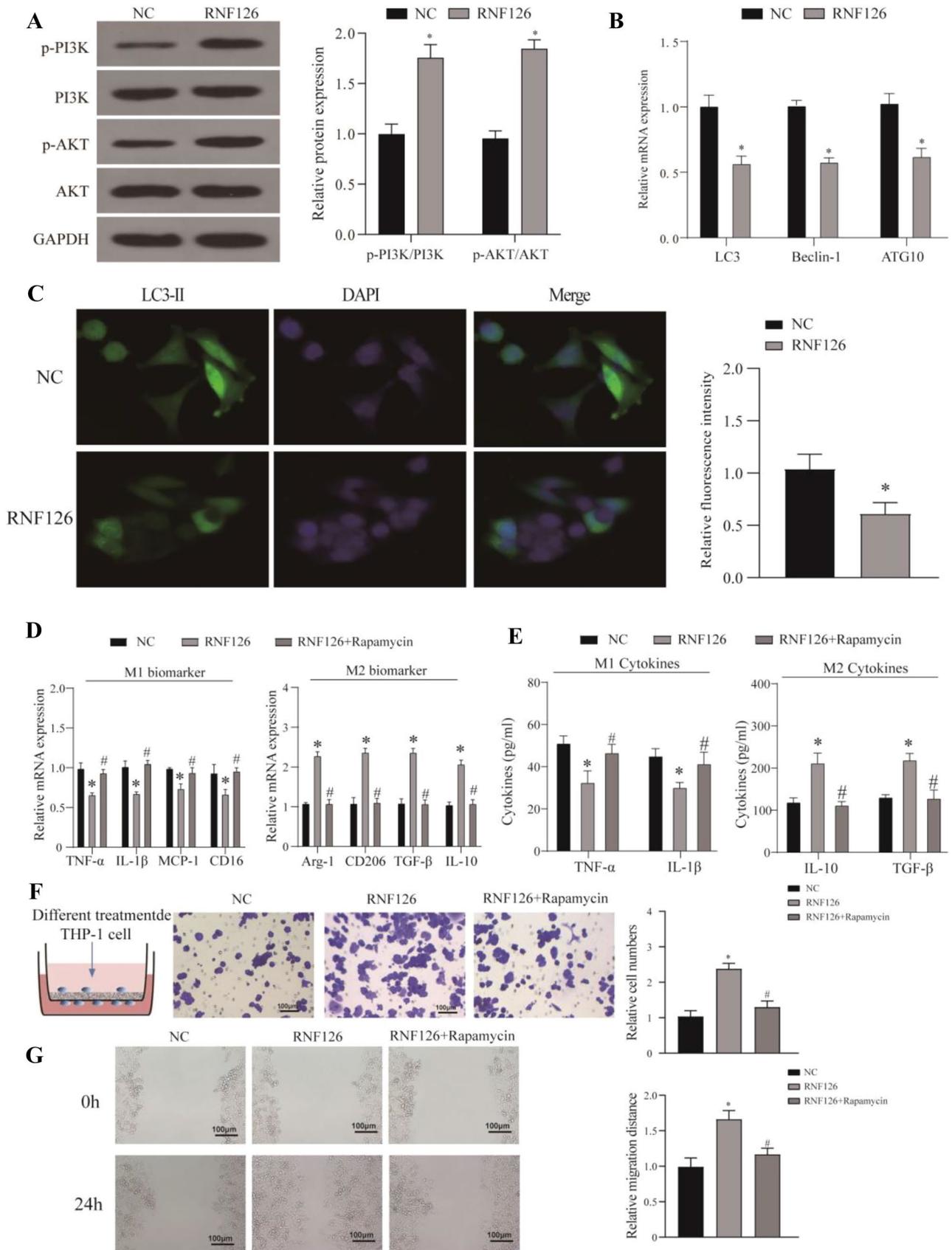


Fig. 6 The role of PI3K/AKT pathway and autophagy in the regulation of macrophage polarization by RNF126. **A** WB was used to detect the regulatory effect of RNF126 overexpression on the activation of PI3K/AKT pathway. **B** QRT-PCR was used to detect the regulatory effect of RNF126 on autophagy levels. **C** The expression of LC3-labeled autophagosomes was detected by immunofluorescence. **D–E** QRT-PCR and ELISA were used to detect the expression of M1/M2 macrophage markers after different treatments. **F** Transwell was used to detect the cell invasion ability of THP-1 cells treated with exosomes overexpressing RNF126 and rapamycin. **G** Wound healing assay was used to detect the cell migration ability of THP-1 cells treated with exosomes overexpressing RNF126 and rapamycin. * $p < 0.05$, compared with relative control group

group (Fig. 2C, D). Current studies have shown that M2 macrophage polarization can promote tumor progression. Therefore, we further tested M1/M2 macrophage markers and found that overexpression of RNF126 could promote M2 polarization (Fig. 2E). Epithelial-mesenchymal transition (EMT) process is an important mechanism of tumor metastasis. Therefore, we further detected EMT process-related genes and found that overexpression of RNF126 can significantly promote EMT process (Fig. 2F, G). Finally, we used HE, KI67 and immunofluorescence to verify the role of RNF126 in promoting tumor proliferation. Furthermore, we found that the number of RNF126 deposited in macrophages was compared with that in the NC group with F4/80-labeled macrophages in immunofluorescence (Fig. 2G). This suggests that it may promote tumor growth by promoting macrophage migration and M2 polarization.

Co-culture of HONE-1 and THP-1 cells to verify the regulatory effect of RNF126 on macrophage polarization

We have seen the effect of RNF126 on macrophages in *in vivo* experiments, so we further constructed a cell co-culture model after RNF126 interference to further verify this phenomenon. We used LPS to induce M1 polarization and IL-4 to induce M2 polarization, respectively, and found that overexpression of RNF126 could reverse the LPS-induced M1 polarization process. However, IL-4-induced M2-type polarization was also inhibited when RNF126 expression was inhibited (Fig. 3A, B). This was also confirmed in the verification of cytokines in culture medium (Fig. 3C, D). In addition, we verified that the co-culture of HONE-1 cells overexpressing RNF126

with THP-1 could significantly promote the invasive and migratory abilities of THP-1 cells (Fig. 3E, F). These data further validate the regulatory role of RNF126 on TAM.

The role of exosomes in the delivery of RNF126

As an important carrier of cell communication, exosomes can participate in the regulation of tumor progression [7, 10]. Therefore, in order to determine whether exosomes can deliver RNF126, we detected two sources of cell culture medium (CM) and found that RNF126 was highly expressed in HONE-1-CM. However, RNase R treatment could not reverse this high expression phenomenon. This high expression was reversed when RNase R was treated with Triton X-100, which disrupts membrane structure (Fig. 4A, C). This suggests that RNF126 may play a role in the entry of exosomes into THP-1 cells.

To verify the transfer of RNF126 to recipient cells via exosomes, we incubated THP-1 macrophages with PKH67-labeled exosomes derived from HONE-1 cells. These exosomes were found to enter macrophages in a time- and concentration-dependent manner (Fig. 4D, E). Finally, we verified the dose and concentration of HONE-1-exo and found that the number of HONE-1 exosomes secreted was higher than that of NP69 cells (Fig. 4F, G). This can also further explain the role of RNF126 in the regulation of macrophages.

RNF126 regulates PTEN ubiquitination in macrophages

As mentioned above, ubiquitination plays an important role in tumor progression. In addition, RNF126 can participate in tumor ubiquitination regulation as an E3 ubiquitination ligase [22, 23]. Therefore, in this study, we used the Ubiquitin database and the Biocrid protein interaction database to predict the regulatory substrates of RNF126 and found that PTEN may be a downstream regulatory protein of RNF126 (Fig. 5A). First, we verified that PTEN protein was significantly down-regulated in THP-1 cell line overexpressing RNF126 (Fig. 5B). The expression of PTEN and RNF126 were analyzed by Strabase database and found that there was a significant negative correlation between the two in tumor tissue (Fig. 5C). Furthermore, we performed subcellular localization of the two proteins by immunofluorescence and found that the two proteins

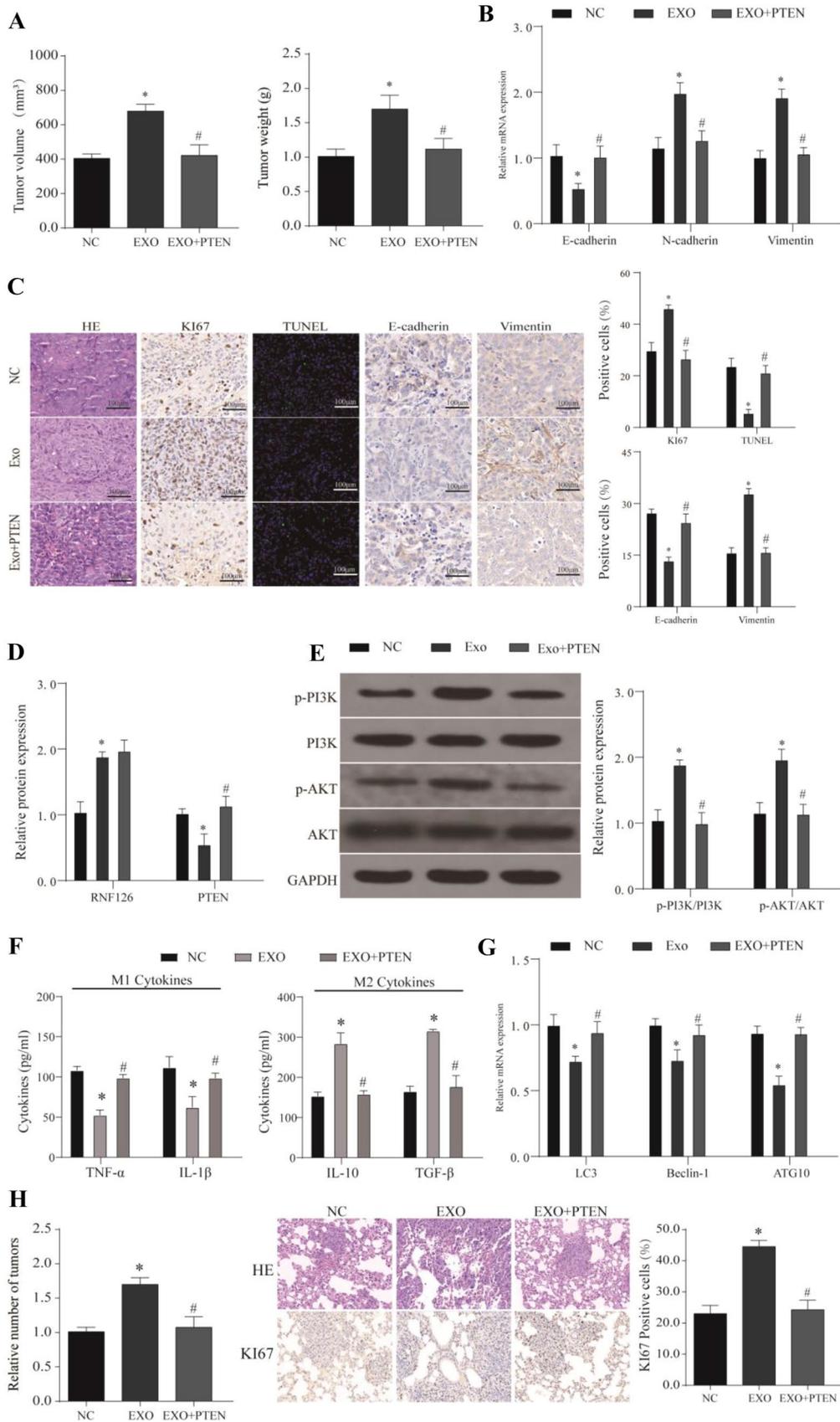


Fig. 7 In vivo assays verified the role of exosomes in tumor growth and metastasis. **A** The tumor volume and weights in different treatment groups. **B** The expression of EMT markers in different treatment groups was detected by QRT-PCR. **C** Histological staining (HE, KI67 and immunofluorescence) was used to detect tumor tissue proliferation and migration, $\times 200$. **D** The expression of RNF126 and PTEN in different treatment groups was detected by WB. **E** WB was used to detect the regulatory effect of exo on the activation of PI3K/AKT pathway. **F** The expression of M1/M2 related cytokines in each treatment group was detected by enzyme-linked immunosorbent assay. **G** QRT-PCR was used to detect the regulatory effect of exo on autophagy levels. **H** The lung metastasis model was used to verify the regulatory effect of exo on lung metastasis. $*p < 0.05$, compared with relative control group

were expressed in the cytoplasm and in the same location, which provided a spatiotemporal basis for their interaction (Fig. 5D). Further, we used Co-IP to test the combination of the two to verify our conjecture (Fig. 5E). To investigate the direct association of RNF126 and PTEN, we performed a proximity ligation assay. PLA analysis indicated that RNF126 is directly related to PTEN in THP-1 macrophages (Fig. 5F).

Importantly, knockdown of endogenous RNF126 increased the half-life of PTEN protein in THP-1 macrophages. To further verify the regulatory role of ubiquitination, an in vivo ubiquitination assay was employed. HEK-293 cells were co-transfected with HA-RNF126 or mutated HA-RNF126 vectors and vectors expressing PTEN and His-ubiquitin (His-Ub), respectively. Cells expressing RNF126 showed increased PTEN ubiquitination compared to cells transfected with control vector (Fig. 5G, H). These results demonstrate that PTEN plays an important role in the regulation of macrophages by RNF126.

The role of PI3K/AKT pathway and autophagy in the regulation of macrophage polarization by RNF126

PTEN is known to inhibit the activation of the PI3K/AKT pathway and this phenomenon was validated in our study. This indicated that RNF126 could activate the PI3K/AKT pathway through the degradation of PTEN (Fig. 6A). Previous studies have found that the PI3K/AKT pathway can inhibit autophagy, and autophagy inhibits M2-type polarization. The present study has found that RNF126 is able to promote M2-type macrophage polarization, so this finding is beneficial to explain our results (Fig. 6B, C). Furthermore, we co-treated THP-1 cells with rapamycin (an autophagy activator) and RNF126 and found that rapamycin reversed the promoting effect of RNF126 on M2-type polarization. Furthermore, we found the same effect on the ability of macrophages to migrate and invade (Fig. 6D–G). This indicates that the regulation of RNF126 on macrophages is due to the regulation of PI3K/AKT pathway mediated by PTEN.

In vivo assays verified the role of exosomes in tumor growth and metastasis

Finally, we verified the tumor-promoting effect of exosomes in vivo and found that tumor exosomes overexpressed RNF126 could significantly promote tumor growth and EMT progression, and this promotion was effectively reversed by overexpression of PTEN (Fig. 7A, B). Further morphological staining confirmed our phenomenon (Fig. 7C). This is yet another corroboration of our results in the previous section.

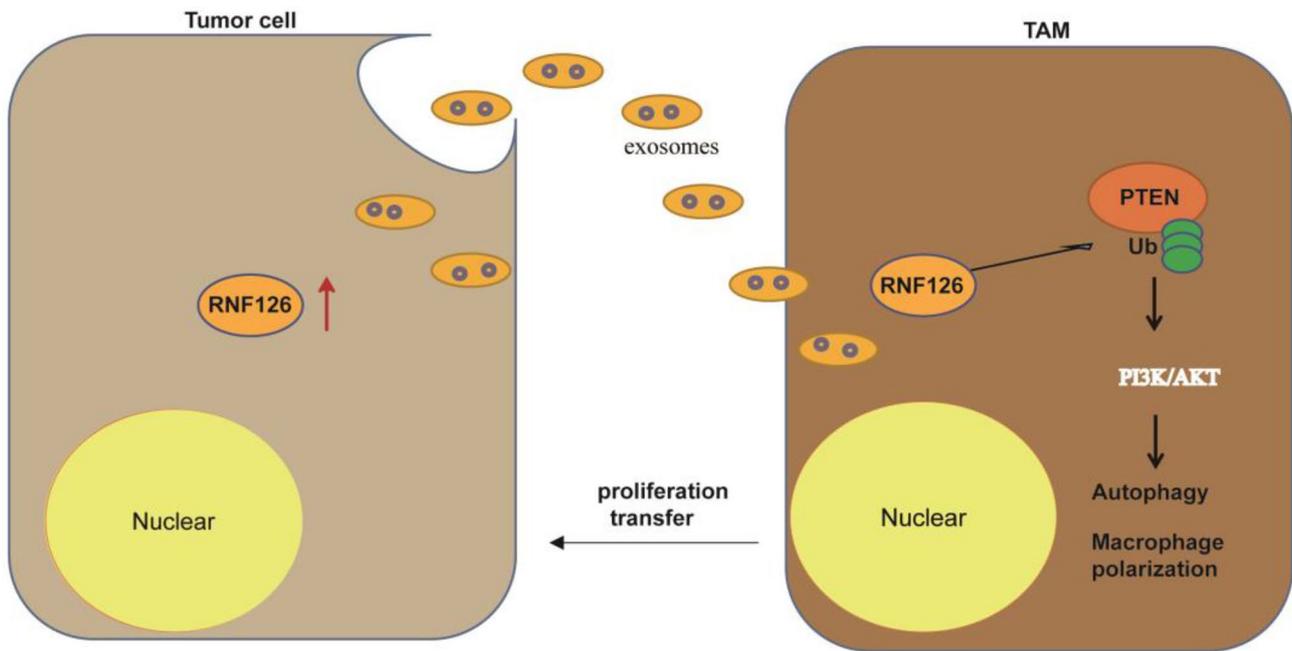


Fig. 8 Mechanism of tumor cell-derived exosomal RNF126 affecting the immune microenvironment and promoting nasopharyngeal carcinoma progression by regulating PTEN ubiquitination

Furthermore, we verified the expression of the PI3K/AKT pathway and found that exosomes could activate the pathway while this activation was reversed by concomitant overexpression of PTEN (Fig. 7D, E). Finally, we detected M1/M2 polarization-related inflammatory factors in this model and found that exosomes promoted the expression of anti-inflammatory factors that were reversed by PTEN. This behavior was reversed at the level of autophagy (Fig. 7F, G).

In order to verify the regulatory effect of our exosomes on the EMT process, we constructed a lung metastasis model and found that the number of tumor nodules in exosome-induced lung metastasis was higher than that in the control group and PTEN + EXO group (Fig. 7H). These phenomena were also confirmed by morphology. This indicates that tumor-derived exosomes can activate the PI3K/AKT pathway to promote tumor proliferation and metastasis through the degradation of PTEN by RNF126 (see Fig. 8).

Discussion

Changes in the tumor microenvironment can promote tumor cells to overcome inappropriate extracellular conditions and develop towards proliferation and metastasis. Recent reports have identified a critical role for exosomes in promoting the tumor microenvironment [24, 25]. Exosomes are involved in the regulation of various diseases including tumors by transmitting information to nearby or distant cells by delivering proteins and RNAs [26, 27]. Nasopharyngeal carcinoma is

an important head and neck tumor. In-depth study of tumor proliferation and metastasis mechanism has important guiding significance for improving the prognosis of patients. In this study, it was found that NPC cell-derived exosome RNF126 was involved in the communication between tumor cells and macrophages, resulting in changes in macrophage autophagy and polarization. It promotes tumor progression through this mechanism. First, RNF126 directly interacts with PTEN to degrade its protein expression after entering macrophages through tumor cell exosomes. Finally, PTEN degradation activates the PI3K/AKT pathway and inhibits autophagy, thereby promoting macrophage migration and M2-type polarization.

Previous studies have found that the E3 ubiquitin ligase RNF126 (ring finger protein 126) is highly expressed in various cancers and is closely associated with tumorigenesis [12]. Our results in the TCGA database also verify this phenomenon. However, its role in the progression of nasopharyngeal carcinoma remains unclear. Our results showed that the expression of RNF126 was significantly elevated in NPC tissues and cells. In addition, we validated the tumor-promoting effect of RNF126 using a nude mouse tumorigenic model. Surprisingly, we found that macrophages in the RNF126-overexpressing group developed a marked M2-type polarization. At present, studies have confirmed that M2-type polarization of tumor-associated macrophages can significantly promote tumor growth, but its regulatory mechanism in NPC is still unclear. Ubiquitination as an important mechanism of tumor regulation

has been reported in a variety of tumors. First of all, our database prediction results found that PTEN may function as a substrate protein of RNF126 and this result was confirmed by our data. Similarly, previous studies in bladder cancer found the same phenomenon, RNF126 can lead to the degradation of PTEN through ubiquitination. But this phenomenon was first reported in NPC.

Autophagy is a process that engulfs its own cytoplasmic proteins or organelles and coats them into vesicles, and fuses with lysosomes to form autophagolysosomes, degrading their encapsulated contents, thereby fulfilling the cell's own metabolic needs and the renewal of certain organelles [28]. Autophagy can be seen in the physiological and pathological processes of the body, and whether its role is positive or negative has not been fully elucidated, especially in the study of tumors, which deserves attention [29]. The current study found that PTEN can inhibit the activation of PI3K/AKT pathway. In addition, the role of PI3K/AKT pathway in autophagy has also been confirmed [12]. Therefore, we hypothesized that autophagy plays an important role in tumor-derived exosome-mediated macrophage polarization. We used rapamycin to activate autophagy and found that the role of exosomes in promoting M2 polarization was reversed. These data suggest that RNF126 can affect macrophage polarization and invasion ability by regulating the level of macrophage autophagy. However, although our findings were corroborated by *in vivo* and *in vitro* data, we lacked in-depth clinical diagnosis and associated prognostic data. Therefore, we still need to further explore the application of this mechanism in clinical diagnosis and the development of targeted drugs.

In summary, we found for the first time that tumor-derived exosomal PTEN degrades PTEN through ubiquitination to regulate the tumor immune microenvironment and promote NPC growth and metastasis. These results provide the basis for the screening of early markers of NPC and targeted therapy.

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Declarations

Conflict of interest The authors report no conflict of interests.

Ethical approval All animal experiments were approved by the Institutional Animal Care and Use Committee at the First Affiliated Hospital of Zhengzhou University (Approved number: 2020-KY-0399-002). The study followed the Health's Guide for the Care and Use of Laboratory Animals (National Research Council).

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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