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Original Article

Autophagy regulates anti-angiogenic property of lenvatinib in thyroid cancer

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Abstract: We aimed to explore the role of lenvatinib-mediated autophagy in papillary thyroid cancer (PTC). K1 and BCPAP, were tested for cell viability, proliferation, and apoptosis after treatment with lenvatinib or chloroquine (CQ) or both. The levels of angiogenesis vascular endothelial growth factor A (VEGFA) were measured by ELISA. Transwell and wound-healing assays were performed using endothelial HUVECs cells. The dynamics of microvessels were detected by tubular formation assay. Western blotting was used to determine the expression of LC3-I/II and Atg-7 and alterations in the PI3K/Akt/mTOR and MEK/ERK pathways. *In vivo* tumor growth assay and immunohistochemical staining (IHC) was also performed. The results showed that lenvatinib inhibited the viability of K1 and BCPAP cells and caused apoptosis. We further showed that lenvatinib also upregulated autophagy levels in thyroid cancer cells in a dose-dependent manner through the PI3K/Akt/mTOR and MEK/ERK pathways. Co-administration of lenvatinib with CQ resulted in a greater decrease of VEGFA in the tumor supernatant than with either lenvatinib or CQ alone. Autophagy inhibition enhanced the cytotoxicity and anti-angiogenic ability of lenvatinib, which was supported by the HUVECs migration, wound healing, and tube formation assays. Inhibiting autophagy chemically or genetically enhanced lenvatinib's cytotoxic effects and anti-angiogenic efficacy in thyroid cancer cells *in vitro* and *in vivo*. In conclusion, lenvatinib inhibited cell viability and induced apoptosis and autophagy in human PTC cells. Significantly, the combination of lenvatinib and autophagy inhibition may represent a novel and effective treatment option for PTC, which may be able to overcome drug resistance.

Keywords: Lenvatinib, autophagy, anti-angiogenesis, PTC, VEGFA

Introduction

The incidence of thyroid cancer, the commonest malignant tumor of the head and neck, has increased in recent years [1]. According to the 2020 International Agency for Research on Cancer survey, thyroid cancer accounts for around 3% of all human malignancies, with approximately 586 000 new cases each year globally [2]. The most common form of thyroid cancer is papillary thyroid carcinoma (PTC), which accounts for around 90% of all thyroid cancers [3]. PTC is a differentiated thyroid cancer (DTC). Although most PTC patients have a good prognosis, their mortality rate is still about 10% due to advanced local disease and distant metastases [4]. Surgery, radioactive iodine (RAI), and external beam radiotherapy are conventional treatments for thyroid cancer [5].

The 2018 NCCN Guidelines for Thyroid Cancer pointed out that molecular targeted therapy showed significant clinical effects in locally recurring, inoperable, metastatic medulla thyroid cancer and RAI refractory differentiated thyroid cancer (RR-DTC). lenvatinib is a VEGFR1-3, FGFR1-4, PDGFR, RET, and c-KIT multi-target tyrosine kinase inhibitor (TKI) [6]. The United State Food and Drug Administration approved its use for RR-DTC in 2015 following the significant improvement in progression-free survival (PFS) and tumor remission [7]. Autophagy is the phenomenon in which cells induce intracellular

activities to maintain cell homeostasis under hypoxic, energy deficient, and toxic drug conditions. In malignant tumors, autophagy is involved in tumor development, metastases, and treatment [8]. In the rapid growth phase of the initial or advanced tumor, angiogenesis in the tumor microenvironment does not meet the tumor's demand for energy, amino acids, oxygen, and growth factors. A series of metabolic stresses such as starvation, hypoxia, and accumulation of reactive oxygen species ensures cell survival by inducing autophagy [9]. In addition, autophagy is associated with the resistance to anticancer agents [10]. Inhibiting autophagy can reduce the adaptability of tumor cells and enhance the effect of chemotherapy and radiotherapy to kill tumor cells [11].

Angiogenesis is a physiological process in which new blood vessels are formed from sprouting mature blood vessels. Studies have shown that autophagy promotes the proliferation of vascular smooth muscle cells, which plays an important role in maintaining vascular wall structure and function [12]. The effect of autophagy on fibroblasts and extracellular and vasculogenic mimicry is also crucial for angiogenesis and tumor blood supply [13].

Anti-angiogenic therapy inhibits the formation of blood vessels in tumor tissues and thus arrests tumor growth [14]. Specific anti-VEGF targeted therapy can significantly inhibit the angiogenesis process in tumor tissues and reduce the uptake of nutrients such as oxygen and glucose by tumor cells. Under the influence of unfavorable factors such as lack of nutrition and oxygen, and the side effects of radiotherapy and chemotherapy, tumor cells can clear damaged organelles and biological macromolecules by initiating autophagy. On the other hand, tumor cells can obtain energy from autophagy to maintain their growth and promote metastases [15].

lenvatinib, a novel TKI, is widely used to treat thyroid cancer [6, 7, 16, 17]. However, the mechanism by which TKI induces autophagy in thyroid cancer remains unknown. This study sought to address the question of whether lenvatinib affects the level of autophagy in thyroid cancer, which in turn affects the development of tumors and angiogenesis.

Materials and methods

Chemicals

Lenvatinib was purchased from LC Laboratories (LC Laboratories, Woburn, MA). Chloroquine (CQ), rapamycin (RAPA), hygromycin, MEK/ERK inhibitor U0126, and the PI3K/Akt inhibitor LY294002 were all obtained from Sigma (Sigma, St. Louis, MO). Lipofectamine 3000 was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA).

Cell lines and cell cultures

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (Type Culture Collection, Manassas, VA). Human papillary thyroid cancer cell lines K1 and BCPAP were kindly gifted by Dr. Frédérique Savagner (University of Angers, France) and Dr. Mingzhao Xing (Johns Hopkins University, School of Medicine, USA). K1, BCPAP, and HUVECs cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Genetimes Technology Inc., China). The GFP-LC3 lentivirus was a gift from Professor Chris KP Mok (The Chinese University of Hong Kong, Hong Kong).

Construction of stable cell lines

To express GFP-LC3, K1 and BCPAP cells were plated at a density of 1×10^5 cells per 60-mm dish for 24 h and then infected with the GFP-LC3 lentivirus in DMEM without FBS. Forty-eight hours after infection, the cells were replated and selected with 150 μg/ml hygromycin for 5 days. The resulting hygromycin-resistant cells were expanded for further analysis.

GFP-LC3 puncta formation assay

Cells expressing GFP-LC3 were treated as indicated and fixed in 4% paraformaldehyde. Quantification of cells with GFP-LC3 puncta formation was performed using a fluorescence confocal microscope. At least 200 cells were counted on each slide, and the percentage of cells containing GFP-LC3 puncta was calculated.

Western blot analysis

Western blotting was performed on cultured cells as previously described [18]. The antibodies against Atg7, HRP-linked anti-IgG, and GAPDH were purchased from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX). The antibody against microtubule-associated protein 1 light chain 3b (LC3b), phospho-ERK (Thr-202/Tyr-204), total-ERK, phospho-mTOR, and total-mTOR were obtained from Cell Signaling (Cell Signaling Technology, Danvers, MA). Antibody-antigen complexes were detected using the ChemiDoc Imaging Systems (Bio-Rad, Hercules, CA). The gray analysis of bands was quantified by ImageJ software, version 1.5 (National Institutes of Health, Bethesda, MD).

RNA interference

Atg7 siRNA (sc-41447) and control siRNA (sc-37077) were purchased from Santa Cruz (Biotechnology, Dallas, TX). Lipofectamine 3000 (Invitrogen) was used to transfect cells with siRNAs according to the manufacturer's instruction.

MTT cell viability assay

Cells were seeded into 96-well plates at a density of 5000/well and cultured for 24 h, 48 h, and 72 h. After adding MTT solution (Merck, Darmstadt, Germany) to each well at a final concentration of 0.2 mg/ml, the cells were incubated at 37°C for an additional 4 h. After the incubation, 200 μl dimethyl sulfoxide (Sigma, San Antonio, TX) was added to each well to dissolve the formazan, and the absorbance at 570 nm was determined using a spectrophotometer. Prism 10.0 software was used to calculate the median inhibitory concentration (San Diego, CA).

Colony formation assay

Monolayer culture was used to conduct the colony formation assay. 1500 cells per well of a 6-well dish were plated. Cells were treated with the indicated drugs or interventions for 7-14 days following plating. Colonies were defined as those with a minimum of 50 cells. After staining with crystal violet solution, colonies were counted (Santa Cruz Biotechnology, Dallas, TX). All experiments were conducted in triplicate.

Transwell assay

Transwell assay was used to determine HUVECs motility (migration). After 24 h of treatment with lenvatinib alone or in combination with CQ, the supernatants of K1 and BAPAP cells were collected for further investigations. An Invasion Chamber (Corning, Corning, NY) was used for the transwell assay that was performed according to the manufacturer's instruction. To summarize, 1×10^5 HUVECs were seeded with the tumor cell supernatant in the top chamber, while the bottom chamber was seeded with DMEM 10% FBS and cultured for 24 h. Under the microscope, invasive and migratory cells on the lower surface were stained with 0.5% crystal violet and counted in 5 random fields.

Scratch wound healing assay

HUVECs were seeded in 6-well plates with the tumor supernatant (FBS-free) as the culture medium. When cells reached about 80-90% confluence, a sterile pipette tip was used to create a scratch across the midline of the cell layer. After 48 h, pictures were obtained through a microscope and the distance of cell movement was estimated using Image J software (National Institutes of Health, version 1.5).

ELISA assay

The concentration of VEGFA in K1 and BCPAP cell culture supernatants was determined using double antibody sandwich human VEGFA ELISA kits (Genetimes Technology Inc., China). Generally, the diluted supernatant was added to the 96-well plates with antibodies for 1 h. Finally, after washing 3-5 times, TMB hydrogen peroxide urea solution was used as the chromogenic substrate. The kit was measured under 450 nm wavelength by a spectrometer.

Tubular formation assay

A 96-well plate was coated with 50 μl Matrigel (Corning Incorporated, Glendale, AZ) for each well. HUVECs were seeded onto the plate and cultured with K1 cell supernatant (FBS-free). The cells were then incubated at 37°C in 5% CO_2. Images of tube length were captured by microscopy 6 h post-seeding and analyzed using ImageJ software, version 1.5 (National Institutes of Health, Bethesda, MD).

Apoptosis analysis

Apoptosis of cells was determined by an Apoptosis Detection kit (BioLegend, San Jose, CA) through flow cytometry. Cells treated as indicated were trypsinized and the culture supernatants were obtained by centrifugation at 1,500 rpm for 5 min. Each cell pellet was washed once with PBS and resuspended with 100 μL binding buffer. Each cell pellet was stained with 5 μL Annexin V/FITC and 10 μL propidium iodide (PI) at 37°C for 15 min in the dark and diluted with 400 μL binding buffer. A total of 10,000 events were counted for each sample and were analyzed within 1 h by the BD LSRFortessa cell analyzer (BD Biosciences, San Jose, CA). Cell apoptosis profiles were analyzed with FlowJo software, version 10.0 (FlowJo, Ashland, OR). Data were obtained from 3 independent experiments.

Xenograft experiments

Four to six weeks old nude mice were obtained from the Laboratory Animal Services Centre, the Chinese University of Hong Kong. Mice were raised in a pathogen-free environment. 5×10^6 K1 cells (in 100 μL of FBS-free DMEM) were subcutaneously injected into the right flank of each mouse. When the tumors were palpable and had reached about 3×3 mm^2, the mice were randomly assigned to four groups (four mice per group). They were administered PBS, lenvatinib (30 mg/kg/day orally for 14 days), CQ (50 mg/kg/day intraperitoneally for 14 days), or a combination of lenvatinib and CQ. lenvatinib was dissolved in a standard saline solution. Four weeks after the K1 cell injection, mice were sacrificed and the xenograft tumors were measured. The tumor size and the mouse body weight were measured every three days. All animal experiments complied with the National Institutes of Health guide for the care and use of laboratory animals, and all experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Immunohistochemical staining

The antibody against VEGFA was purchased from ABclonal Technology (ABclonal Technology, Woburn, MA). The antibodies of CD31 and c-Myc were obtained from Santa Cruz Santa (Cruz Biotechnology, Dallas, TX). The staining assay was carried out according to the standard protocol on formalin-fixed paraffin sections with a thickness of 5 μm. After staining, an immunoreactive score system was used to score the staining intensities by a pathologist and an investigator who were blind to the study design [19].

Statistical analysis

Numerical data are presented as mean ± SD. The differences between the two groups were analyzed using a student's t-test (two-tailed). *P* values less than 0.05 were considered statistically significant. SPSS Software version 25.0 (IBM, Armonk, NY) was used for all statistical analyses.

Results

Lenvatinib suppressed proliferation and induced apoptosis of PTC cells

As an antitumor drug, lenvatinib was first tested for its cytotoxicity on tumor cells. To examine the *in vitro* anticancer activity of lenvatinib, human PTC K1 and BCPAP cells were treated with various doses of lenvatinib (0, 1, 5, 10, 20, 30, 40, and 50 μM) for 24, 48, and 72 h and then subjected to MTT assay. Lenvatinib significantly inhibited the proliferation of K1 and BCPAP cells in a dose- and time-dependent manner ($P < 0.05$), producing IC50 values of 26.35 ± 2.60, 18.28 ± 1.82, and 11.89 ± 1.15 μM for K1 cells and 18.60 ± 2.56, 9.56 ± 0.92, and 7.95 ± 0.41 μM for BCPAP cells under lenvatinib treatments at 24, 48, and 72 h, respectively (**Figure 1A**). To further examine the cytotoxic effects of lenvatinib, colony formation assays were performed on both cell lines. Cells were treated with 0, 10, and 25 μM lenvatinib for 48 h. The analysis of the stained colonies indicated that lenvatinib treatment significantly abrogated the clonogenic ability of K1 and BCPAP cells in a dose-dependent manner (**Figure 1B**). To test the apoptotic effect induced by lenvatinib, flow cytometry assays were performed. Both cell lines were treated with 0, 5, 25, and 50 μM lenvatinib for 24 h. Apoptosis was measured by Annexin V-FITC and propidium iodide (PI) staining and analyzed by the flow cytometry. The rate of lenvatinib-induced apoptosis was significantly higher in both cell lines than that in the control group in a dose-dependent manner (**Figure 1C**).

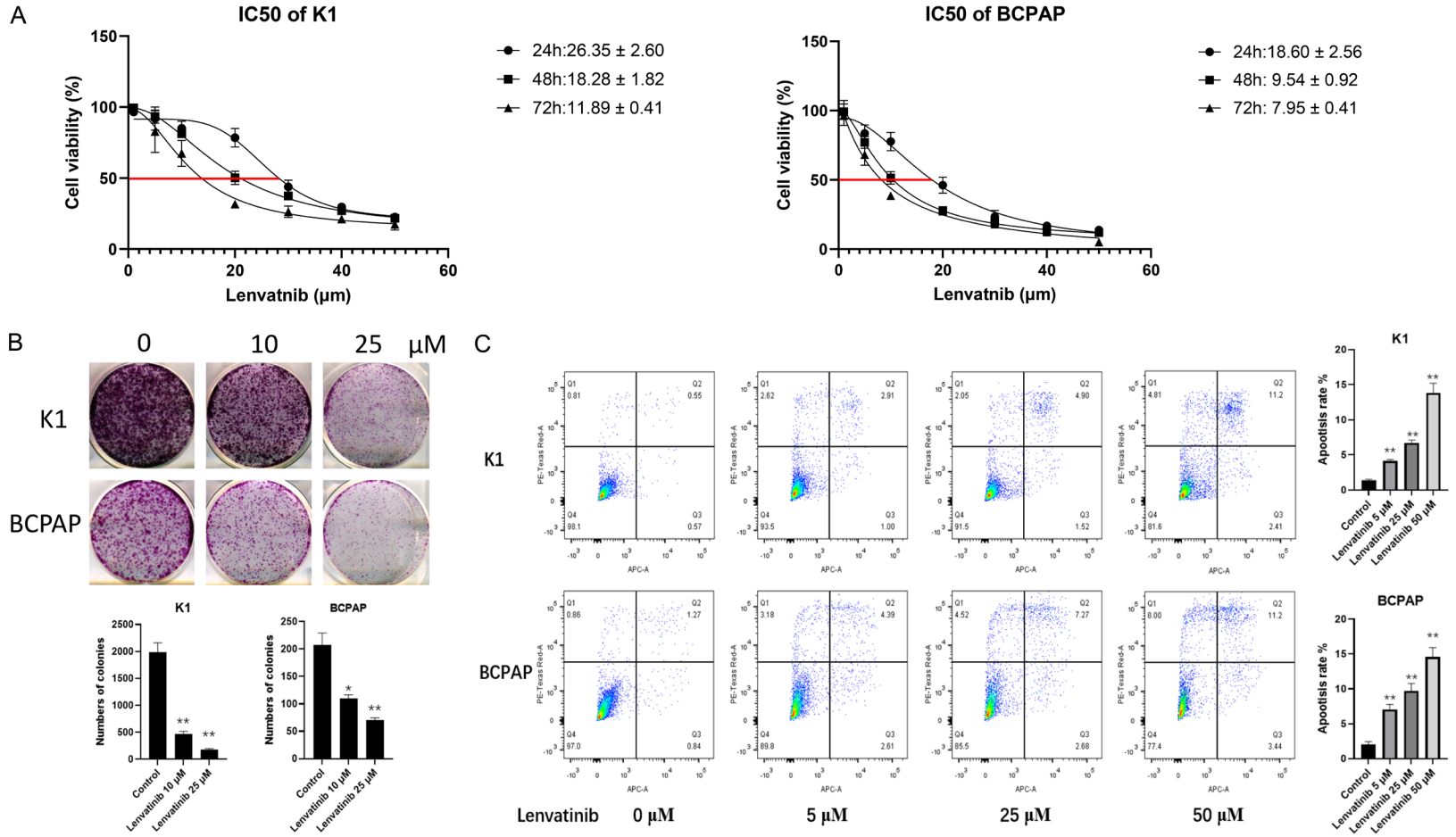

Figure 1. Lenvatinib inhibited the proliferation of PTC cells. A. K1 and BCPAP cell dose-response curves in response to lenvatinib treatment. Cells were treated with various concentrations of lenvatinib for 24, 48, and 72 h, and cell viability was determined using the MTT assay. B. Representative images of clone formation assay. C. Lenvatinib induced cancer cell apoptosis. K1 and BCPAP cells were treated with 0 μM, 5 μM, 25 μM, and 50 μM lenvatinib for 24 h. Apoptotic rate was determined by Annexin V-FITC and propidium iodide (PI) staining through flow cytometry. The columns represent means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Each experiment was performed in triplicate.

Lenvatinib promoted autophagy in PTC cells

The levels of autophagy were determined in human PTC cell lines K1 and BCPAP by measuring GFP-LC3. LC3, a human homolog of yeast Atg-8, is a unique protein that emerges during the early stages of autophagy. The cytoplasmic form of LC3-I is transformed into the membrane-bound lipoprotein LC3-II form during autophagy [20]. These cells were established by the stable reintroduction of functional GFP-LC3 into K1 and BCPAP cells. K1-GFP-LC3 and BCPAP-GFP-LC3 cells were treated with 25 μM lenvatinib or DMEM with FBS10% for 24 h, or with the autophagy activator RAPA as a positive control. GFP-LC3 fluorescence puncta were counted, and results showed that the autophagy level of thyroid cancer cells treated with lenvatinib or RAPA was significantly higher than untreated controls (**Figure 2A**). Western blotting was also used to determine the accumulation of LC3-II to confirm the occurrence of autophagy. As seen in **Figure 2B**, the expression of LC3-II was dose-dependently increased by lenvatinib treatment.

Two well-established signaling pathways are known to regulate autophagy in mammalian cells: the class I PI3K/Akt/mTOR signaling pathway and the MEK/ERK pathway. The Akt/mTOR signaling pathway plays an inhibitory role to downregulate autophagy, whereas the MEK/ERK signaling pathway can upregulate autophagy [21]. lenvatinib effectively inhibited Akt/mTOR signaling in K1 cells and promoted MEK/ERK pathway in K1 and BCPAP cells. In both cell lines, the phosphorylation level of mTOR was significantly lower, and the phosphorylation level of ERK1/2 was increased compared to the control groups ($P < 0.05$) (**Figure 2C**). However, the expression of total mTOR and ERK1/2 remained stable.

To further determine whether activation of the PI3K/Akt/mTOR and MEK/ERK pathways contribute to lenvatinib-induced autophagy in thyroid cancer cells, cells were pretreated with U0126 (a selective MEK/ERK inhibitor), LY294002 (a selective PI3K/Akt/mTOR inhibitor), or DMSO as a control. Before treatment with lenvatinib. U0126 significantly decreased the protein levels of LC3-II in K1 and BCPAP cells, but LY294002 significantly increased their levels ($P < 0.05$). Similar results were observed in BCPAP cells ($P < 0.05$) (**Figure 2D**). In conclusion, these findings have suggested that lenvatinib-induced autophagy is highly related to the PI3K/Akt/mTOR and MEK/ERK pathways in PTC cells.

Inhibition of autophagy by CQ enhanced the therapeutic effectiveness of lenvatinib for PTC

To elucidate the role of autophagy in lenvatinib-treated thyroid cancer cells, CQ was used to inhibit the transition of the autophagosome to the autolysosome. K1 and BCPAP cells were treated with lenvatinib 25 μM and CQ 50 μM with or without CQ in DMEM with 10% FBS for 24 h, and the expression of LC3-II was detected by Western blotting. It was found that CQ increased LC3-II expression ($P < 0.05$) (**Figure 3A**), indicating that CQ could inhibit the autophagy induced by lenvatinib in thyroid cancer cells. As illustrated in **Figure 3B**, lenvatinib inhibited the proliferation of K1 cells; moreover, when in combination with CQ, lenvatinib-induced inhibition of cell proliferation was enhanced. To further confirm the role of autophagy in lenvatinib-induced inhibition, Atg-7 expression was knocked down using its siRNA (**Figure 3C**). Atg-7 is a ubiquitin enzyme essential for autophagosome formation [22]. The effect of lenvatinib on K1 cells transfected with Atg-7 siRNA was much more obvious than controls (**Figure 3D**). These findings indicated that lenvatinib could inhibit the proliferation of human PTC cells, and the inhibition of autophagy via a chemical (CQ) or genetic (Atg-7 siRNA) methods could enhance this effect.

Inhibition of autophagy enhanced the anti-angiogenic properties of lenvatinib in PTC

In the carcinogenic process, angiogenesis contributes to the formation of new blood vessels that are thought to aid cancer development. Angiogenesis has become a major step of many carcinogenic processes, and autophagy can provide sufficient oxygen and energy for pathological tissues to promote angiogenesis [23]. VEGF and its receptors have been considered to be the most critical factors in tumor angiogenesis, with VEGFA playing a pivotal role [23]. We, therefore, hypothesized that the inhibition of excessive or avoidable autophagy would inhibit tumor angiogenesis.

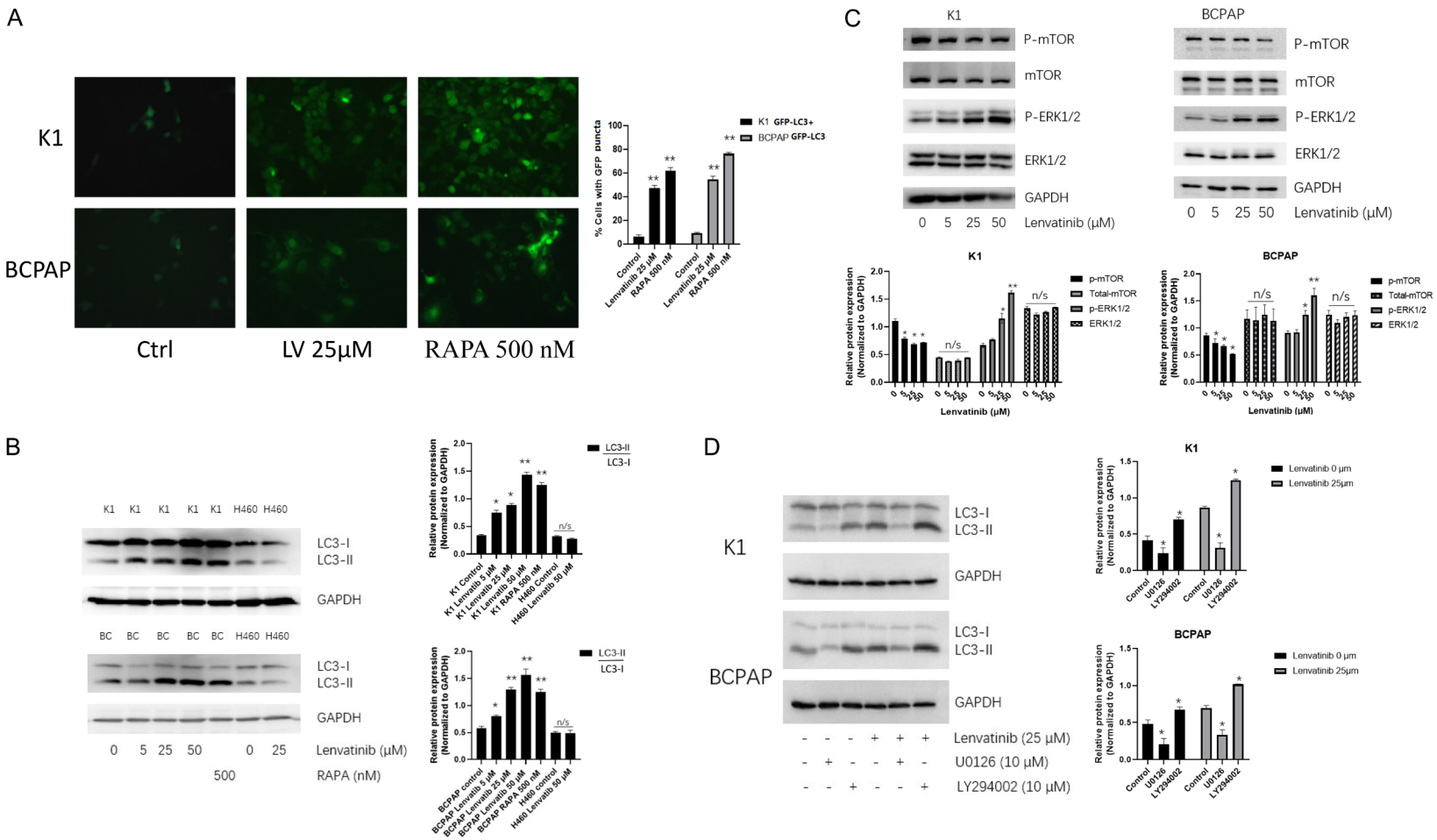

Figure 2. Lenvatinib induced autophagy in PTC cells. A. K1 and BCPAP cells were infected with the GFP-LC3 lentivirus, then selected by hygromycin, grown in DMEM supplemented with 10% FBS for 24 h. Cells were subsequently treated with lenvatinib (25 μM), RAPA (500 nM), or DMSO for 24 h, and then observed using a fluorescent microscope. Cellular autophagic levels were assessed by quantifying the percentage of cells with GFP-LC3 puncta, and the results are shown in the histogram. Magnification 100×. B. K1, BCPAP, and H460 cells were treated with lenvatinib 0-50 μM or with RAPA 500 nM for 24 h, and LC3-I/II expression levels were determined by Western blotting. C. Immunoblotting was used to detect the expression of mTOR, p-mTOR, ERK1/2, p-ERK1/2, and Atg-7 in thyroid cancer cells following 1 h treatment with the concentration gradient of lenvatinib. D. Inhibition of ERK activity attenuated lenvatinib-induced autophagy in K1 and BCPAP cells. K1 and BCPAP cells were pretreated with 10 μM U0126 (a selective MEK/ERK inhibitor), 10 μM LY294002 (a selective PI3K/Akt inhibitor), or DMSO for 1 h before exposure to lenvatinib 25 μM for 24 h. At the end of the treatment, autophagic flux was monitored by detecting the protein level of LC3-II using Western blotting analysis. $^{*}P < 0.05$, $^{**}P < 0.01$. Each experiment was performed in triplicate.

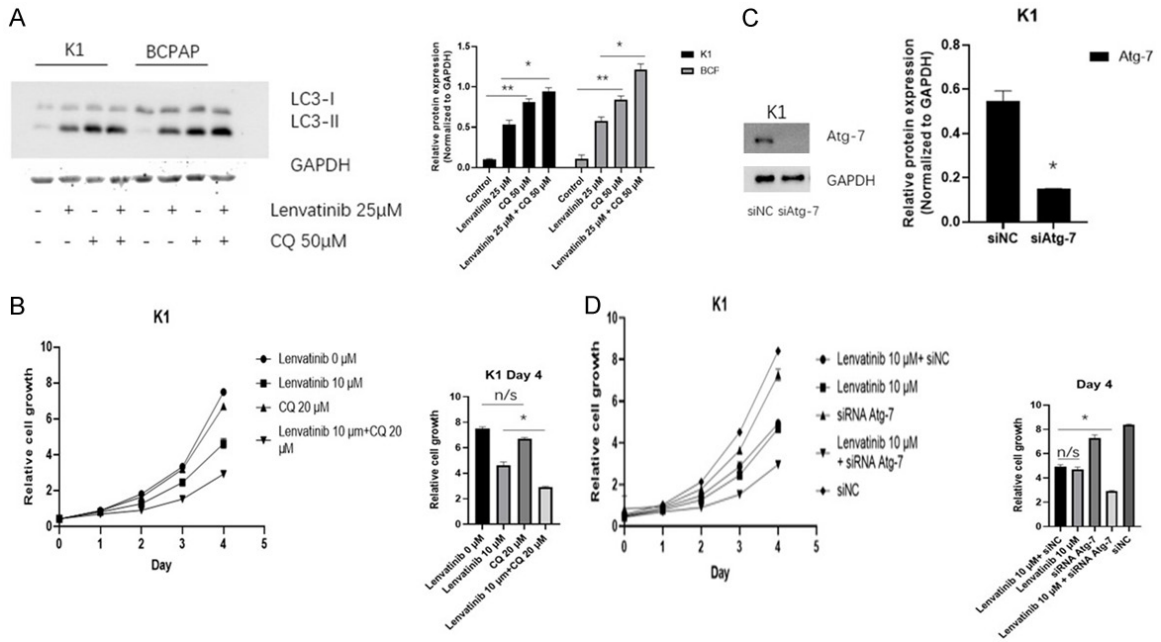

Figure 3. CQ inhibited autophagy and enhanced the inhibitory effect of lenvatinib in human thyroid cancer cells. A. After 24 h of treatment with lenvatinib 25 μM with or without CQ 50 μM, the expression of LC3-II was detected by Western blotting. B. Autophagy inhibition with CQ 20 μM resulted in a decrease in the viability of lenvatinib-treated cells measured by by MTT assay. C. Atg-7 knockdown or control siRNA (siNC) were established in K1 cells. D. MTT assay revealed that inhibiting autophagy with Atg-7 siRNA increased inhibitory effects in K1 cells treated with lenvatinib 10 μM. n/s no significant difference, $^{*}P < 0.05$, $^{**}P < 0.01$. Each experiment was performed in triplicate.

An ELISA kit was used to determine the VEGFA concentration in the supernatant of cell cultures (serum-free) collected from K1 and BCPAP cells following 24 h treatment with lenvatinib 25 μM and/or CQ 50 μM. For K1 cells, lenvatinib in combination with CQ resulted in a greater reduction of VEGFA than either lenvatinib (25 μM) or CQ (50 μM) treatment alone ($P < 0.05$) (**Figure 4A**). A similar result was found for BCPAP cells ($P < 0.05$). We then discovered that the supernatant of K1 cells treated with lenvatinib for 48 h inhibited the wound healing capacity of HUVEC cells. In contrast, the supernatant of K1 cells that had been treated with CQ enhanced the ability of lenvatinib to inhibit the migratory capacity of HUVEC cells by the scratch-wound healing assay (**Figure 4B**). A similar trend was found in the migratory ability of HUVECs by the transwell migration assay (**Figure 4C**). Tube formation assay was used to detect the effect of lenvatinib in combination with or without CQ on angiogenesis. Lenvatinib-treated K1 cell supernatant inhibited tube length formation, and the therapeutic effect was enhanced when it was combined with CQ (**Figure 4D**). These findings indicated that lenvatinib could decrease VEGFA production or secretion and inhibit the angiogenic properties in HUVECs cells. CQ could intensify this action.

Inhibiting autophagy boosted lenvatinib's therapeutic effectiveness on PTC development in vivo

To investigate the therapeutic potential of lenvatinib *in vivo*, we subcutaneously injected nude mice with K1 cells into their right flanks to form xenograft tumors. Treatment started from Day 10 after injection when solid tumors were formed (minimal diameter greater than 5 mm). lenvatinib or lenvatinib co-administered with CQ significantly decreased the tumor size compared with the control group ($P < 0.05$) (**Figure 5A**), and it was noted that coadministration of lenvatinib with CQ resulted in the greatest tumor suppression. Substantial weight loss (**Figure 5B**) or treatment-related fatalities were not seen during the *in vivo* therapy. For tumor tissues, IHC was performed to assess the VEGF makers VEGFA and CD31, and the proliferation marker c-Myc. Lenvatinib plus CQ treatment significantly reduced the levels of all three pro-

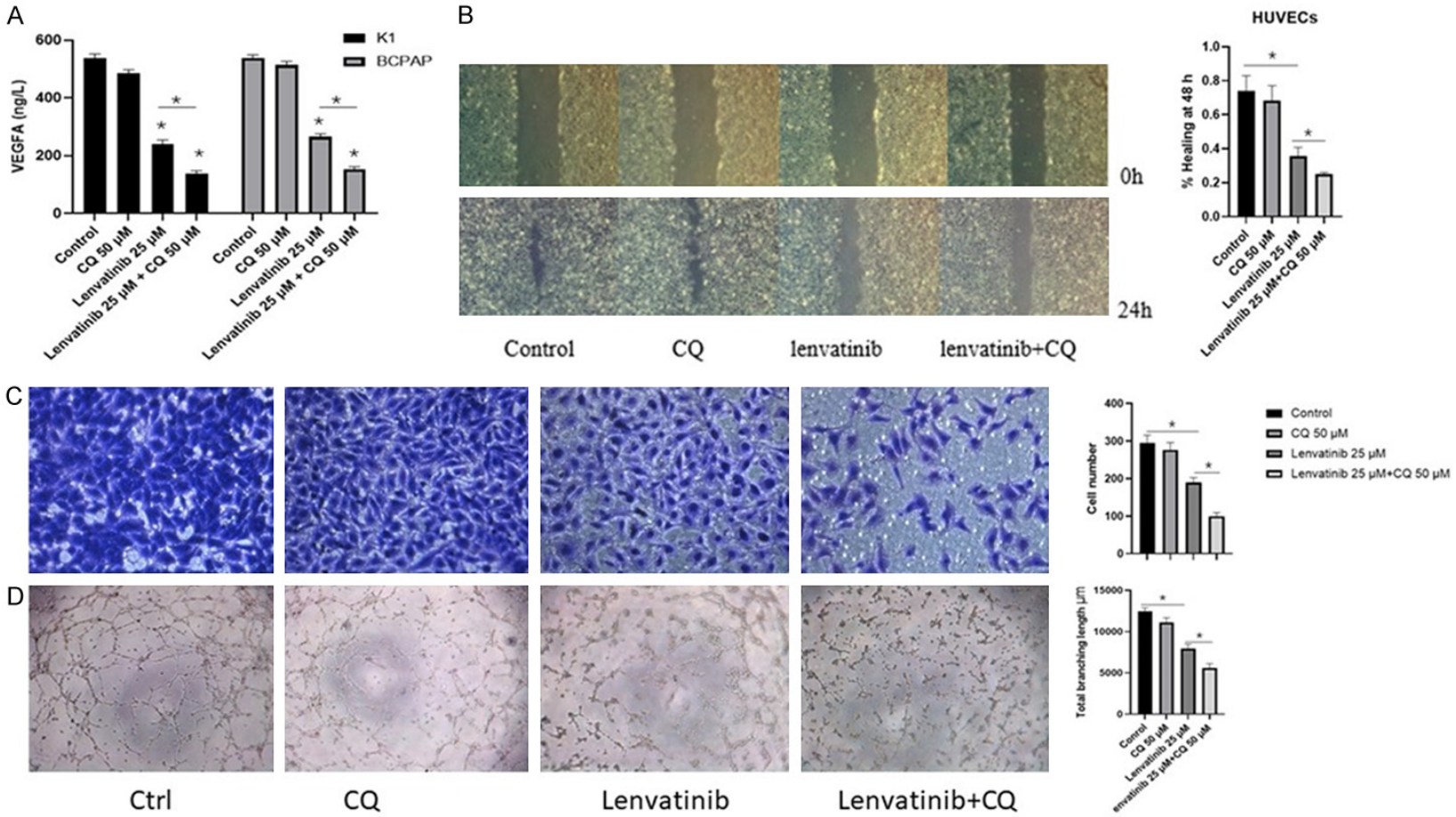

Figure 4. Autophagy inhibition potentiated the anti-angiogenic property of lenvatinib in thyroid cancer cells. A. VEGFA levels in the thyroid cell supernatant were determined by ELISA. B. HUVECs were treated with conditioning K1 cells supernatant for the scratch-wound healing assay. Magnification 100×. C. HUVECs were treated with conditioning K1 cells supernatant for the Transwell assay. Magnification 50×. D. HUVECs were treated with conditioning K1 cell supernatants for tubular formation assay. Magnification 50×. n/s no significant difference, $^{*}P < 0.05$, $^{**}P < 0.01$. Each experiment was performed in triplicate.

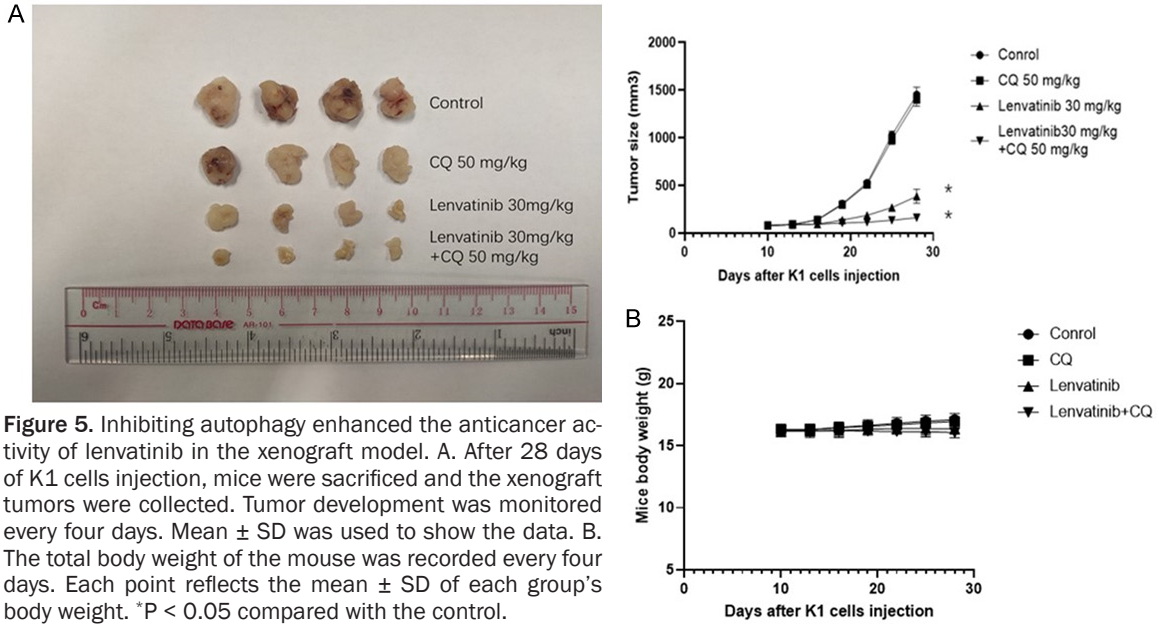

Figure 5. Inhibiting autophagy enhanced the anticancer activity of lenvatinib in the xenograft model. A. After 28 days of K1 cells injection, mice were sacrificed and the xenograft tumors were collected. Tumor development was monitored every four days. Mean ± SD was used to show the data. B. The total body weight of the mouse was recorded every four days. Each point reflects the mean ± SD of each group's body weight. *P < 0.05 compared with the control.

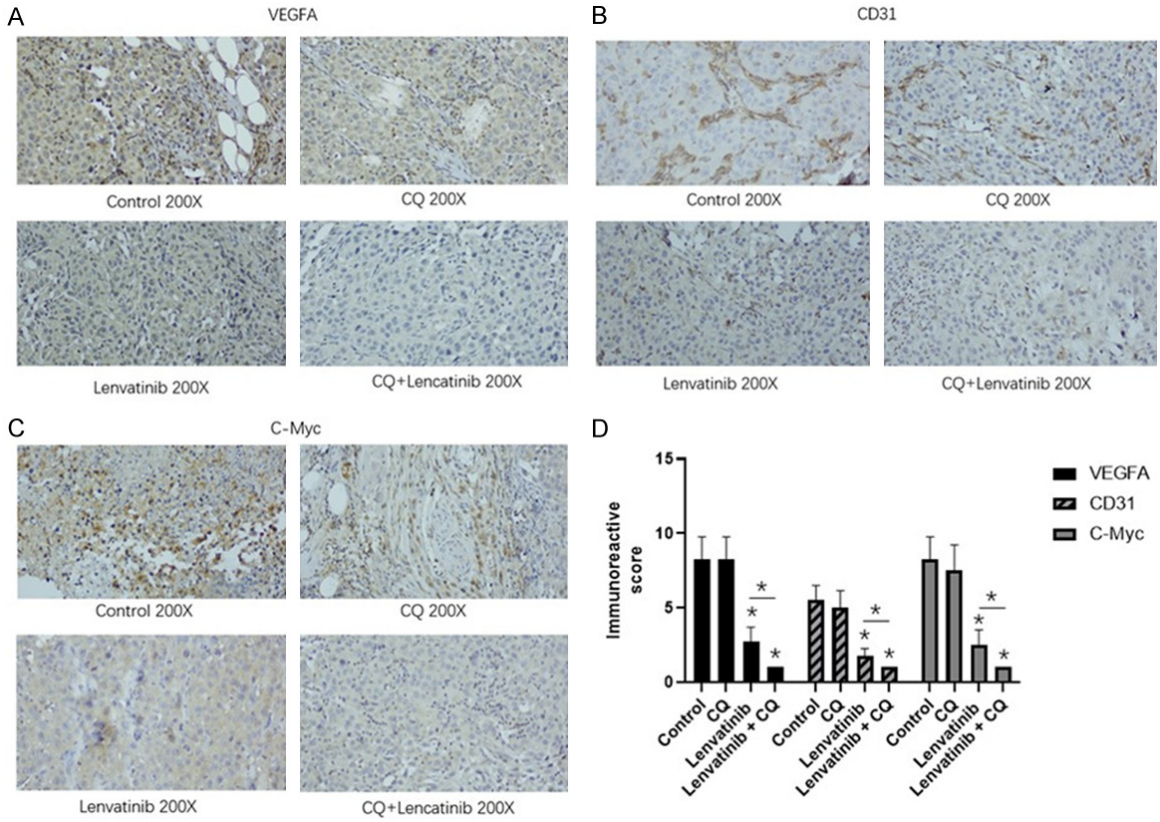

Figure 6. Representative IHC images of VEGFA (A), CD31 (B), and c-Myc (C) of the tumor tissues. Antibody dilution: VEGFA 1:200; CD31 1:200; c-Myc1:500. (D) The immune score of these three molecules in tumor formed in nude mice. Treatment with lenvatinib + CQ significantly reduced the levels of all three proteins compared to the other groups.

teins compared to the other groups (**Figure 6**). These results indicated that the inhibition of autophagy by CQ enhanced lenvatinib's therapeutic effectiveness in K1 xenograft growth.

Discussion

In recent years, the incidence of thyroid cancer has increased, which has understandably attracted attention. The conventional treatment for thyroid cancer has been reasonably stable and is generally reliable and effective [24]. Molecular targeted therapy for thyroid cancer on the other hand has been rapidly developed over the past few years [25]. With the continuous discovery of new molecular targeted therapy pathways, new drugs, and combination medications, the targeted therapy of RR-DTC and undifferentiated thyroid cancer has also progressed [26, 27]. Lenvatinib, a multi-target tyrosine kinase inhibitor (TKI), can prolong the PFS of many tumors [16]. However, congenital or acquired drug resistance limits its efficacy. Autophagy, as a way of intracellular waste degradation, is related to the drug resistance of TKIs. Tumor drug resistance is a complex problem involving multiple genes, factors, and systems. It is widely divided into pharmacokinetic resistance, tumor cell self-resistance, and tumor microenvironment-related factors [28]. At present, 90% of solid tumors develop hypoxic areas because the vascular system cannot provide the oxygen required for tumor growth [28]. Therefore, there is a close relationship between the level of autophagy and angiogenesis. TKIs can induce autophagy, but the effect is bidirectional. Autophagy can weaken the cytotoxic effect of TKIs, mediate tumor drug resistance, promote cell death and reverse tumor drug resistance in cases of excessive autophagy. Therefore, regulating the level of autophagy may provide a key to overcoming TKIs drug resistance in tumor patients. In this study, we demonstrated that lenvatinib could inhibit the survival rate of human PTC cell lines, induce apoptosis, and activate autophagy. Additionally, we showed that the anti-tumor growth and anti-angiogenic activity of lenvatinib could be enhanced if autophagy was inhibited synergistically with other treatments. In this regards, CQ was used as a pharmacological autophagy inhibitor to enhance the effect of lenvatinib. The expression of Atg-7 and LC3-II was determined to evaluate the autophagy. As the data demonstrated, lenvatinib not only increased the level of autophagosomes but also stimulated autophagic flux. In line with our finding, lenvatinib has been shown to induce autophagy in liver cancer cells [29]. Anlotinib, another TKI also targeting VEGFR, has been shown to induce autophagy in human lung cancer cells [26].

It is well established that PI3K/Akt/mTOR and MEK/ERK pathways are central to autophagy. The Akt/mTOR/mTOR pathway inhibits autophagy, whereas the MEK/ERK pathway promotes it [30, 31]. In this study, we showed that lenvatinib regulated the phosphorylation of ERK1/2 and mTOR in a dose-dependent manner, inducing autophagy in K1 and BCPAP cells. Studies have shown that the increase in autophagy levels in tumor cells is directly related to treatment with TKIs and causes drug resistance [32, 33]. A better understanding of autophagy's bidirectional roles in cancer biology may aid in the identification and development of a more effective antitumor treatment that either induces or inhibits autophagic flux. In human thyroid cancer cells, lenvatinib-induced autophagy appears to be protective and may represent a new mechanism of drug resistance.

Additionally, we showed that both chemically blocking autophagy with CQ and genetically knocking it down by Atg-7 by its si-RNA might effectively suppress *in vitro* proliferation of PTC cells. These *in vitro* results were substantiated by our *in vivo* mouse work that showed that the co-administration of lenvatinib and CQ had the greatest tumor-suppressive capacity compared to treatment with lenvatinib or CQ alone. Studies have also shown that inhibiting autophagy increased the cytotoxicity of chemotherapeutic agents such as cisplatin and 5-fluorouracil in certain types of malignant tumors [34].

While more studies are required to fully understand the correlation between autophagy and apoptosis in cancer cells, it is hypothesized that the cytotoxicity brought about by lenvatinib treatment induces cell stress, resulting in cell death. At the same time, this cellular stress also leads to a level of protective autophagy in cancer cells by degrading harmful, damged and unnecessary cellular proteins to maintain cell survival and resist to anti-tumor treatments. CQ can effectively inhibit the autophagy in cancer cells, thus reversing the potential drug resistance caused by lenvatinib-induced protective autophagy.

The research of anti-angiogenic agents primarily focuses on preventing tumor neo-angiogene-

sis. However, relatively few studies have examined the relationship between autophagy and the effect of TKIs. We have also provided data to show that lenvatinib inhibits angiogenesis effectively, as determined by HUVEC migration, wound healing formation, and tube formation assay. Further, we showed that the inhibition of autophagy via CQ or Atg-7 knockdown enhanced lenvatinib's therapeutic effectiveness, implying that autophagy may be a promoting factor of angiogenesis. Consistent with our findings, Feng *et al.* [35] found that apatinib induced autophagy and cell death in human anaplastic thyroid cancer (ATC) cells by inhibiting the AKT/mTOR pathway. Additionally, inhibiting apatinib-induced autophagy by CQ boosted the death in ATC cells and increased tumor suppression *in vivo* and *in vitro*. Abdel-Aziz *et al.* [36] reported that CQ enhanced sunitinib's anti-angiogenic activity by blocking autophagic and angiogenic machinery.

We have observed that in comparison to either lenvatinib or CQ alone, co-treatment with lenvatinib and CQ resulted in a larger reduction in VEGFA levels in the tumor cell supernatants, and *in vivo* animal studies confirmed these findings. Our data have shown that lenvatinib can control tumor growth and angiogenesis, thus playing an anti-tumor role in PTC. However, lenvatinib can also cause protective autophagy in tumor cells, which promotes the survival and stimulates angiogenesis. Importantly, our data have demonstrated that blocking autophagy can further enhance the therapeutic effect of lenvatinib by inhibiting tumor formation and angiogenesis. Therefore, the combination of lenvatinib and CQ may become a new treatment for RR-DTC. Although the inhibition of autophagy can enhance tumor treatment of lenvatinib *in vitro* and *in vivo* as shown in this study, further research is needed to assess this strategy in better *in vivo* models with different types of thyroid cancers.

In conclusion, the data of this study confirms that lenvatinib induces autophagy in PTC. This is an important finding since lenvatinib is wildly used to treat thyroid cancer since the 2018 NCCN guidelines were published, although the emergence of drug resistance will remain a challenge. Further, the data of this study support the concept that lenvatinib, in combination with autophagy inhibition, may represent a novel and effective treatment option for advanced thyroid cancer, and it is likely to overcome the tumor drug resistance. These findings lay the groundwork for future clinical trials to determine whether chloroquine can be used in combination with lenvatinib to increase the efficacy.

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Disclosure of conflict of interest

None.

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