Title page

Autophagic blockade potentiates anaplastic thyroid cancer ferroptosis

Running title: Anlotinib promotes ferroptosis

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Abstract

Anlotinib-mediated angiogenic remodeling was delineated in various tumors. Meanwhile, we previously showed that anlotinib inhibited tumor angiogenesis in anaplastic thyroid cancer (ATC). However, the potential role of anlotinib on cell lethality in ATC remains an enigma. Herein, we found that anlotinib inhibited the viability, proliferation and migration of KHM-5M, C643 and 8505C cells in a dose-dependently manner. Under anlotinib treatment, PANoptosis (pyroptosis, apoptosis and necroptosis) markers were not changed; however, ferroptosis targets (transferrin, HO-1, FTH1, FTL, GPX4) were significantly downregulated. ROS levels also increased in a concentration-dependent manner after anlotinib treatment in KHM-5M, C643 and 8505C cells. In addition, protective autophagy was activated in response to anlotinib, and autophagic blockade potentiated anlotinib-mediated ferroptosis and antitumour effects in vitro and in vivo. Our new discovery identified autophagy-ferroptosis signalling pathway which provides mechanistic insight into anlotinib-mediated cell death, and synergistic combination therapy may help develop new ATC treatment strategies.

Introduction

Thyroid cancer is one of the most common endocrine malignancies with increasing incidence (Cabanillas et al. 2016, Sung et al. 2021). Although ATC accounts for only 2% of all thyroid cancers, it is responsible for the majority of thyroid cancer deaths due to its high aggressiveness and few available treatment options (Perrier et al. 2018, Bible et al. 2021). Considering the functional role of neovascularization in ATC progression, antiangiogenic therapy has become a mainstay treatment for ATC suppression (Figure 1A-B, Zhu et al. 2010, Ravaud et al. 2017, Feng et al. 2021).

Anlotinib, a multitarget receptor tyrosine kinase (RTK) inhibitor, has already achieved remarkable efficacy in lung cancer and osteosarcoma due to its potent antiangiogenic properties (Han et al. 2018, Wang et al. 2019, Su et al. 2022). In addition, our previous study confirmed that anlotinib could suppress ATC carcinogenesis by inhibiting angiogenesis in vitro and in vivo (Liang et al. 2021). However, the potential role of anlotinib on cell lethality in ATC remains unclear.

Ferroptosis is a novel nonapoptotic regulated cell death process (Jiang et al. 2021, Tang et al. 2021). It is characterized by abnormal cysteine and glutathione metabolism and iron-dependent accumulation of peroxidized lipids. Accumulating evidence suggests the importance of proferroptosis strategies in cancer treatment, especially in drug-resistant malignancies (Hassannia et al. 2019, Chen et al. 2021). This study aims to investigate the regulatory mechanism underlying anlotinib-mediated ferroptosis and identify potential combination therapies for ATC.

Materials and Methods

Cell culture and reagents

Human ATC cell lines KHM-5M, C643, 8505C and PTC cell lines TPC-1 were conserved by the
Institute of Clinical Medicine, Zhejiang Provincial People's Hospital. Cell lines were cultured in RPMI-1640 (Hyclone, China) with 10% fetal bovine serum (FBS, KEL Biotech, China). Cells were cultured at 37 °C in 5% CO₂. All cells were preserved at −80 °C using CELLSAVING (New Cell & Molecular Biotech, China). Anlotinib (AL3818) and Chloroquine (T0194) were respectively purchased from Shanghai Lanmu Chemical Co and Shanghai Taoshu Biotechnology Co, and were dissolved in sterile purified water and diluted with medium to the desired concentration. Recombinant human CXCL11 (rhCXCL11) and recombinant human HB-EGF (rhEGF) were purchased from PeproTech (USA).

**CCK-8 assay**

The cytotoxicity of anlotinib was assessed by the CCK-8 (Beyotime Biotechnology, China) method. ATC cells (4000 per well) were seeded in ninety-six-well plates and were treated with anlotinib at 0, 1, 2, 4, 8, 16 and 32µM for 24h. At the test point, 100 µl CCK-8 was added and then the viability values were detected by spectrometer (BIO TEK, China).

**Colony-formation assay**

ATC cells (1000 per well) were seeded in six-well plates and incubated at 37 °C in 5% CO₂ for 24 h. The 6-well plates were washed 3 times with sterile PBS to remove the exfoliated cells. Then, 0, 1, 2, 4, and 8µM of anlotinib were added. One week later, 500ul paraformaldehyde was added to each well for 30min fixation, then the fixative was discarded and replaced by 500μl of crystalline violet dye per well. After 30 min staining, clone number could be calculated.

**Scratch assay**

ATC cells (300,000 per well) were seeded in twelve-well plates for 48h. After scratching with a gun tip in the center of the adherent cells, the 12-well plates were rinsed 3 times with sterile PBS. Then, 0, 1, 2, 4, and 8µM anlotinib diluted with serum-free RPMI-1640 was added. The migration ability was assessed under a light microscope at 40x and 100x for 0, 6, 12, and 24h.

**Transwell assay**

Migration and invasion assay were performed using Transwell Permeable Plate (LABSELECT, China,6.5mm). Serum-free medium (200µl) containing 5×10⁴ ATC cells was added to the upper chamber, and 700 µl of a series of concentrations of anlotinib (0, 1, 2, 4, 8µM) diluted with serum-containing medium were added to the lower chamber. After incubation of 24 or 48h, cells were stained with 0.1% crystal violet for 30 min. The number of migrating cells was normalized to the number of total cells and was calculated per microscopic field. The mean number was estimated by counting average cells in five visual fields of three independent experiments.
Western blot (WB) analysis

WB was performed as previous described (Jin et al. 2020). All protein samples were lysed in WB and IP (immunoprecipitation) cell lysate and then quantified using the BCA Protein Analysis Kit (Thermo Scientific China). Proteins were separated by 15%-20% SDS-PAGE gel and transferred onto PVDF membranes. After the block with 5% skimmed milk prepared of 20% TBST for 2h, membranes were incubated at 4 °C overnight with primary antibodies. Blots were probed with: rabbit anti-GSDMD (ab209845 1:1000), rabbit anti-caspase1 (24232T 1:1000), rabbit anti-cleaved-caspase1 (4199T 1:1000), rabbit anti-cleaved GSDMD (36425T 1:1000), rabbit anti-GSDME (ab215191 1:3000), rabbit anti-cleaved GSDME (ab215191 1:3000), rabbit anti-PARP (9532T 1:1000), rabbit anti-caspase3 (ab184787 1:2000), rabbit anti-cleaved-PARP (5625T 1:2000), rabbit anti-cleaved-caspase3 (9661T 1:1000), mouse anti-caspase8(9746T 1:5000), rabbit anti-RIP (3493T 1:1000), rabbit anti-P-RIP(65746T 1:1000), mouse anti-cleaved-caspase8(9746T 1:5000)rabbit, anti-ATG7 (ab52472 1:1000), rabbit anti-Beclin1 (ab210498 1:1000), rabbit anti-LC3B (ab192890 1:2000), rabbit anti-P62 (ab207350 1:1000), mouse anti-caspase3 (9661T 1:1000), mouse anti-caspase8(9746T 1:5000), mouse anti-cleaved-PARP (5625T 1:2000), rabbit anti-ATG7 (ab52472 1:1000), rabbit anti-Beclin1 (ab210498 1:1000), rabbit anti-LC3B (ab192890 1:2000), rabbit anti-Beclin1 (ab210498 1:1000), rabbit anti-LC3B (ab192890 1:2000), mouse anti-cleaved caspase3 (9661T 1:1000), mouse anti-cleaved caspase8 (9746T 1:5000), mouse anti-cleaved PARP (5625T 1:2000). Goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG was used as secondary antibody (Santa Cruz Biotechnology). Finally, the protein bands were analyzed using chemiluminescent substrate HRP (Verte Biotechnology, China).

Flow cytometry analysis

Flow cytometry was performed as previous described (Feng et al. 2018). Cells were treated with 0,1,2,4,8,16 and 32 µM anlotinib for 8h, and then were incubated with a H2DCFDA probe for 30 min under dark environment. The residual dye was washed with ice-cold PBS, and suspended in 100 µl serum-free medium. Final measurements were performed on a flow cytometer (Beckman Coulter Ireland Inc, Ireland). The fluorescence of each probe was measured using the FlowJo software program.

Immunofluorescence (IF)

IF was performed as previous described (Feng et al. 2022). Cells were treated with 0,1,2,4,8,16 and 32 µM anlotinib for 8h, and then were incubated with DCFA and Hoechst probes for 30 min and 10 min under dark environment. Finally, images were collected by using confocal microscope.

In vivo Xenograft tumour model and immunohistochemistry

ATC xenograft models in nude mice were established (Liang et al. 2021). Three-week-old female BALB/c nude mice were purchased from Shanghai slac laboratory animal co. ltd. All experiments were performed following the official recommendations of the Chinese Society of Zoology, and animals received humane care according to the standards listed in the Ethical Review Form for
Laboratory Animal Welfare. Suspensions containing 8505C cell were subcutaneously injected into the right flank of the nude mice. After approximately 2 weeks, when the tumor diameter reached approximately 5 mm, all mice were randomly categorized into four different groups, including control, anlotinib (3 mg/kg), chloroquine (60 mg/kg) and combined treatment with anlotinib and chloroquine groups (five mice in each group). Anlotinib and chloroquine were administered by oral gavage and intraperitoneal injection respectively. Tumor size and volume were recorded every two days. Tumor size was measured using vernier calipers and tumor volume was calculated using the following formula: \( V = W^2 \times L / 0.5 \). Three days after the last injection, animals were executed by CO\(_2\) inhalation, and tumors were removed, weighed and fixed in formalin. Immunohistochemistry was performed as previously described (Feng et al. 2021). Immunohistochemistry images were evaluated by the pathologist. Immunohistochemistry scoring was completed according to the percentage of positive cells (0 = 0–5%, 1 = 5–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%) and the staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong). The two scores were multiplied to generate an immunoreactive score ranging from 0 to 12 (Liang et al. 2021).

Statistics

Statistical analyses were processed using GraphPad Prism 8.0. One-way ANOVA and Student’s t test were chosen for comparison among groups. Categorical data were evaluated with the Chi-square test or Fisher’s exact test. P <0.05 were considered significant.

Results

Anlotinib suppressed the malignant behaviours of ATC cells

TPC-1, KHM-5M, C643 and 8505C cells were incubated with a series of concentrations of anlotinib for 24 hours (Figure 1C). The CCK8 results showed that anlotinib hardly decreased cell viability at concentrations less than 1 \( \mu \)M, and cell death was significantly induced at concentrations greater than 4 \( \mu \)M (Figure 1E). The IC50 values of TPC-1, KHM-5M, C643 and 8505C were 9.49, 6.01, 4.85 and 4.46 \( \mu \)M, respectively (Figure 1D). To evaluate the inhibitory effect of anlotinib on the proliferation of ATC cells, we found that the number of clones of KHM-5M, C643 and 8505C cells decreased in a dose-dependent manner by using a colony assay. Moreover, the number of cell clones was markedly reduced at concentrations greater than 2 \( \mu \)M (Figure 1F, G). These in vitro results indicated the inhibitory effect of anlotinib on ATC cell viability.

Scratch and Transwell assays were employed to investigate whether anlotinib could affect the migration and invasion ability of ATC cells. A wider wound healing area and fewer migrating cells were observed in the anlotinib group. When the concentration of anlotinib reached 4 \( \mu \)M, the migration ability of ATC cells was significantly weakened (Figure 2A-D). Furthermore, the results of the Transwell invasion assay indicated that anlotinib could reduce the number of ATC cells in the lower chamber (Figure 2E, F). In order to exclude the impact of EGF and CXCL11 on anlotinib induced anti-migrating ability (Liang et al. 2021), we added rhCXCL11 and rhEGF to tumor cell with or without anlotinib treatment, and found that invasion and migration of ATC cells could hardly
be influenced (Supplemental Figure S1A-D). Therefore, the anlotinib mediated malignant suppression is angiogenesis-independent. Altogether, the migration and invasion ability of ATCs was inhibited upon anlotinib treatment.

**Anlotinib-mediated cell death occurred mainly via ferroptosis but not PANoptosis**

PANoptosis emphasizes the signal crosstalk between various regulated cell death types, including pyroptosis, apoptosis and necroptosis. To identify the precise pathway of cell death under anlotinib treatment, PANoptosis markers were examined by western blotting (Figure 3A). Interestingly, the markers of pyroptosis (GSDMD, GSDME, C-GSDME, Caspase1, C-Caspase1, C-GSDMD), apoptosis (PARP, Caspase 3, C-PARP, C-Caspase 3), and necroptosis (Caspase 8, C-Caspase 8, RIP, P-RIP) were not significantly altered after anlotinib intervention (Figure 3B-G). Therefore, anlotinib-mediated ATC cell death may not occur through PANoptosis.

Then, we further explored whether anlotinib could trigger metabolic dysfunction and ferroptosis. Consequently, five targets (transferrin, HO-1, FTH1, FTL, GPX4) were significantly downregulated after treatment with anlotinib compared with the control group in KHM-5M, C643 and 8505C cells (Figure 3H-J). In addition, after ATC cells were coincubated with anlotinib and a ferroptosis inhibitor (FER-1), the suppressed cell viability was reversed by FER-1 (Figure 3K).

Considering that ROS play a central role during ferroptosis, we hypothesized that anlotinib could induce a ROS homeostasis disorder in ATC. By using flow cytometry, ROS levels increased in a concentration-dependent manner after anlotinib treatment in KHM-5M, C643 and 8505C cells (Figure 4A, B). Furthermore, ROS changes were visualized by immunofluorescence. Compared with the control group, more ROS signal was detected in the anlotinib group (Figure 4C, D). Then, we tried to reverse ROS with the ROS scavenger NAC, and we found that ATC cell viability in the anlotinib + NAC group was significantly higher than that in the anlotinib alone group (Figure 4E).

We further investigated the potential effect of EGF and CXCL11 on ferroptosis (Liang et al. 2021). We added rhCXCL11 and rhEGF to tumor cell with or without anlotinib treatment, and found that anlotinib mediated ferroptosis effect is CXCL11- and EGFR-independent (Supplemental Figure S1I-L). Altogether, these results preliminarily indicated that anlotinib elicited antitumour effects via ferroptosis in ATC and that ROS were dysregulated.

**Autophagic blockade potentiated anlotinib-mediated ferroptosis**

Accumulating studies have shown that autophagy functions importantly during ferroptosis (Dai et al. 2020, Wei et al. 2020). Therefore, autophagic markers were examined in our series (Figure 5A). In KHM-5M, C643 and 8505C cells, the expression of P62 was significantly reduced in response to anlotinib, while the increased expression of LC3B-II, ATG7 and Beclin1 further validated the autophagic effect of anlotinib (Figure 5B, C).

To further explore whether autophagy played a protective role in anlotinib-mediated ferroptosis, we
initially confirmed that autophagy inhibitor (CQ or 3MA) alone could hardly alter the baseline level of ferroptosis under a series of concentration (Supplemental Figure S1E-H). Then, we divided ATC cells into four groups (control group, anlotinib group, autophagy inhibitor group (CQ or 3MA), and anlotinib + autophagy inhibitor group). Consequently, CQ and 3MA hardly influenced the expression level of ferroptosis markers; however, adding CQ and 3MA to anlotinib resulted in lower levels of ferroptosis markers (GPX4, FTH1, FTL, transterrin, and HO-1) than anlotinib treatment alone (Figure 5D-G). In addition, the CCK8 results further validated that inhibition of autophagy could enhance anlotinib-induced ferroptosis in KHM-5M, C643 and 8505C cells (Figure 5H, I).

**CQ improved anlotinib-mediated antitumorigenesis in vivo**

Considering the *in vitro* findings, a xenograft model was used to validate the protective effect of autophagy *in vivo*. Mice with ATC tumours were exposed to anlotinib, CQ and combination treatment groups. Comparison with the control group indicated that treatment with anlotinib decreased tumour volume, and the tumour volumes in the CQ treatment group did not show a significant difference compared with those in the control group. Moreover, the combination of CQ with anlotinib significantly enhanced the inhibitory effect compared with the anlotinib group (Figure 6A, B). Consistent with the results of tumour volume, tumour weight could be suppressed by anlotinib, and a combination of CQ with anlotinib showed a more impressive antitumorigenesis effect (Figure 6C).

Tumorigenic markers were assayed by IHC. A comparison with the control group indicated that the tumours in the anlotinib group had lower expression levels of Ki67 and ferroptosis molecules. Enhanced TUNEL staining was observed in the anlotinib group (Figure 6D-F).

**Discussion**

ATC is a highly aggressive malignancy with treatment resistance (Wendler *et al*. 2016, Prasongsook *et al*. 2017). Despite comprehensive treatment, the prognosis of ATC patients has not improved significantly (Dierks *et al*. 2021, Liang *et al*. 2022). Anlotinib has been seen as a potent angiogenic modulator for treating ATC (Liang *et al*. 2021). However, this study was the first to illuminate the potential regulatory role of anlotinib in ferroptosis. At present, accumulating studies argue for complex crosstalk between various cell death types, such as pyroptosis, apoptosis and necroptosis (Bertheloot *et al*. 2021). These regulated cell death types have gradually led to the concept of PANoptosis, which is triggered by the formation of the inflammatory PANoptosis complex (Zheng & Kanneganti 2020, Lee *et al*. 2021). In addition, the activation of caspase 8 is the key regulator of the inflammatory response and the focal point of converging pathways that mediate PANoptosis (Kesavardhana *et al*. 2020, Jiang *et al*. 2021). Previous studies have shown that anlotinib induces cell lethality mainly through PANoptosis (Ruan *et al*. 2019). However, in our study, the cellular markers and features of PANoptosis were not identified, and c-caspase 8 was not observed. We found that ferroptosis was significantly triggered in an ROS pathway-dependent manner upon anlotinib treatment in ATC. In contrast to the inflammatory process in PANoptosis, ferroptosis is characterized by metabolic dysfunction and iron-dependent accumulation of peroxidized lipids. Considering the resistance to apoptotic inducers...
in various tumours, killing tumour cells by promoting ferroptosis may become a potential gateway to overcome multiple drug resistance in the anticancer therapy (Juchum et al. 2015, Liang et al. 2019). Therefore, illuminating the regulatory mechanism of ferroptosis in ATC may further broaden the therapeutic potential of anlotinib.

During anlotinib-mediated ferroptosis, autophagy was enhanced and exerted a protective impact on cell viability. However, autophagy inhibitor alone can hardly promote ferroptosis, partially because the level of protective autophagy cannot be activated without anlotinib treatment. The interaction between autophagy and ferroptosis has been partly investigated previously (Dai et al. 2020). Specifically, autophagy leads to the degradation of cellular ferritin; thus, the balance of intracellular iron is destroyed (Gupta et al. 2023). The existence of intracellular free iron can increase the concentration of reactive oxygen species, which triggers ferroptosis. In this study, protective autophagy was observed, and autophagic blockade potentiated anlotinib-mediated ferroptosis and antitumour effects.

In summary, this study is the first to demonstrate the effects of anlotinib on ferroptosis. The identified autophagy-ferroptosis signalling pathway may provide a potential combination therapeutic strategy for ATC.

**Ethical Committee Approval**

The animal experiments involved in this study were approved by the Laboratory Animal Management and Ethics Committee of Zhejiang Provincial People's Hospital (Approval number: IACUC-A20220023).

**Author Disclosure Statement**

No competing financial interests exist.

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**AUTHOR CONTRIBUTIONS**

JF.W., MH.G. designed the study. JY.L. analyzed the data and revised the manuscript. JJ.W. wrote the manuscript and performed most of the experiments. RQ.L., WL.M. carried out data curation and
visualization. T.L., Y.P., KY. F. carried out investigation. L.H.J., Z.T. carried out supervision and project administration. Q.L. and WH.Q. designed the revision and performed the part of experiments. All of the authors discussed the results, reviewed and approved the final manuscript.

References


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Figure legend

Figure 1 Anlotinib inhibited the proliferation of ATC cells. A Anti-angiogenic therapeutics is the mainstay treatment for ATC. B The clinical trails of anlotinib in multiple tumors. C Flow chart of anlotinib suppressing the malignant behaviours of ATC cells. D ATC cells (TPC-1, KHM-5M, C643 and 8505C) were treated with control medium or a series of concentrations of anlotinib (0, 1, 2, 4, 8, 16 and 32 µM) for 24 h. Cell viability was assessed by CCK-8 assay. E The IC-50 value of anlotinib treatment in TPC-1, KHM-5M, C643 and 8505C cells for 24 h. F, G Representative images of contact-dependent clone formation and quantification of the clone number in KHM-5M, C643 and 8505C cells. Each experiment was representative of three independent experiments. All data were obtained from three independent experiments. *P < 0.05; **P < 0.01.

Figure 2 Anlotinib suppressed ATC migration and invasion abilities. A, C Compared with the control groups, anlotinib decreased the migration ability of KHM-5M, C643 and 8505C cells in a dose-dependent manner. B, D Histograms show the relative wound healing area and migration number of cells. E, F The invasion ability of KHM-5M, C643 and 8505C cells was inhibited upon anlotinib treatment. Histograms show the invasion number of cells. All data are obtained from three independent experiments. *P < 0.05; **P < 0.01.

Figure 3 Anlotinib-mediated cell death occurred mainly via ferroptosis but not PANoptosis. A Flow chart of anlotinib-mediated cell death in ATC cells. B-G KHM-5M, C643 and 8505C cells were treated with anlotinib for 24 h. PANoptosis marker expression (pyroptosis (GSDMD, GSDME, C-GSDME, Caspase1, C-Caspase1, C-GSDMD), apoptosis (PARP, Caspase 3, C-PARP, C-
Caspase 3), and necroptosis (Caspase 8, C-Caspase 8, RIP, P-RIP) were detected by western blot.

H-J Ferroptosis markers (GPX4, FTH1, FTL, transterrin, and HO-1) were detected by western blotting. K Compared with the anlotinib groups, the cell viability was reversed when ATC cells were co-incubated with anlotinib and ferroptosis inhibitor (FER-1). All data are obtained from three independent experiments. *P < 0.05; **P < 0.01.

**Figure 4** Anlotinib increased ROS levels in ATC cells. A-D The levels of lipid ROS were detected under anlotinib treatment in KHM-5M, C643 and 8505C cells with an H2DCFDA probe via flow cytometry and immunofluorescence. E Compared with the anlotinib groups, the cell viability was reversed by NAC when ATC cells were co-incubated with anlotinib and ROS inhibitor (NAC). All data are obtained from three independent experiments. *P < 0.05; **P < 0.01.

**Figure 5** Autophagic blockade potentiates anlotinib-mediated ferroptosis. A Flow chart of investigating the crosstalk between autophagy and ferroptosis under anlotinib treatment. B, C KHM-5M, C643 and 8505C cells were treated with a series of concentrations of anlotinib for 8 h. The expression levels of autophagy markers (ATG7, P62, Beclin 1, LC3-II) and GAPDH were detected by western blot. D-G KHM-5M, C643 and 8505C cells were treated with anlotinib with or without autophagy inhibitor (CQ 100 μM, or 3MA 10 mM) for 24 h. GPX4, FTH1, FTL, transterrin, and HO-1 were detected by WB. H The CCK8 results of ATC cells treated with the control medium or a series of concentrations of CQ (0, 10, 20, 40, 80, 160 and 320 μM) for 24 h. I Compared with the anlotinib group, cell lethality was potentiated when ATC cells were coincubated with anlotinib and CQ. All data are obtained from three independent experiments. *P < 0.05; **P < 0.01.
Figure 6 CQ improved anlotinib-mediated antitumorigenesis *in vivo*. A, Xenograft models were generated by injecting 8505C. Four groups of mice were treated with the control medium, anlotinib, CQ and a combination of two agents. B, C Quantification of tumor volumes and weights of the four groups. D, E Images and quantifications of TUNEL assay and IHC staining of Ki67, P62, LC3B, HO-1 and GPX4. F Schematic of anlotinib-mediated autophagy-ferroptosis signaling in anaplastic thyroid cancer.
Figure 2

(A) Anlotinib (μM) effects on wound healing in KHM-5M, C643, and 8505C cell lines.

(B) Graph showing wound healing percentage for different concentrations of Anlotinib (μM) in KHM-5M, C643, and 8505C cell lines.

(C) Anlotinib (μM) effects on cell migration in KHM-5M, C643, and 8505C cell lines.

(D) Graph showing cell migration percentage for different concentrations of Anlotinib (μM) in KHM-5M, C643, and 8505C cell lines.

(E) Anlotinib (μM) effects on cell invasion in KHM-5M, C643, and 8505C cell lines.

(F) Graph showing cell invasion percentage for different concentrations of Anlotinib (μM) in KHM-5M, C643, and 8505C cell lines.

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**Figure 5**

A) **CQ—Autophagic Ferroptosis**

- **vitro**
- **vivo**

- **CCK-8 assay**
- **WB assay**
- **CDX model**

B) **KHM-5M C643 8505C**

**Control**, **Anlotinib**, **CQ**, **Anlotinib+CQ**

C) **Relative grey value/GAPDH**

- **ATG7**
- **P62**
- **Beclin1**
- **LC3B I**
- **LC3B II**
- **GAPDH**

D) **Relative TFR expression (%)**

- **KHM-5M**
- **C643**
- **8505C**

- **Control**, **Anlotinib**, **CQ**, **Anlotinib+CQ**

E) **Relative FTH1 expression (%)**

- **KHM-5M**
- **C643**
- **8505C**

- **Control**, **Anlotinib**, **3MA**, **Anlotinib+3MA**

F) **Relative FTL expression (%)**

- **KHM-5M**
- **C643**
- **8505C**

- **Control**, **Anlotinib**, **3MA**, **Anlotinib+3MA**

G) **Relative GPX4 expression (%)**

- **KHM-5M**
- **C643**
- **8505C**

- **Control**, **Anlotinib**, **3MA**, **Anlotinib+3MA**

H) **Cell viability (%)**

- **CQ (µM)**

- **Control**, **Anlotinib**, **CQ**, **Anlotinib+CQ**

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Autophagic blockade potentiates anaplastic thyroid cancer ferroptosis

Supplementary Figure legends

Supplemental Figure 1 A-D The impact of rhCXCL11 (100ng/ml) and rhEGF (10nmol/L) with or without anlotinib on invasion and migration were investigated in KHM-5M, C643 and 8505C cells. Histograms show the migration number and invasion number of cells. E-H KHM-5M, C643 and 8505C cells were treated with control medium or a series of concentrations of CQ (0, 50 and 100µM) or 3MA (0, 5 and 10mM) for 24 h. GPX4, FTH1, FTL, transterrin, and HO-1 were detected by WB.

I-L KHM-5M, C643 and 8505C cells were treated with anlotinib with or without rhCXCL11 and rhEGF for 24 h. The expression levels of ferroptosis markers (GPX4, FTH1, FTL, transterrin, and HO-1) and GAPDH were detected by western blot. All data are obtained from three independent experiments. *P < 0.05; **P < 0.01.