Article

Discovery Medicine 2024; 36(180): 199–208
https://doi.org/10.24976/Discov.Med.202436180.19

Curcumol Enhances the Antitumor Effect of Lenvatinib on Hepatocellular Carcinoma Cells

ZhaoLi Wu[1], Jia Wang[2,*]

[1]Department of Integrated Chinese and Western Medicine, Hunan Provincia People's Hospital (The First Affiliated Hospital of Hunan Normal University), 410005 Changsha, Hunan, China
[2]Scientific Research Office, Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University), 410005 Changsha, Hunan, China
*Correspondence: wangjia@hunnu.edu.cn (Jia Wang)
Published: 20 January 2024

Background: Lenvatinib is an important molecular target drug for the treatment of advanced hepatocellular carcinoma (HCC). However, the application of molecular targeted therapies for HCC also faces some challenges. Cumulative evidence has also shown that curcumol is a potential anti-HCC drug. Curcumol can be used as a chemosensitizer to enhance the antitumor effect of chemotherapeutic drugs. The purpose of our study is to explore the effect of curcumol combined with lenvatinib on HCC.
Methods: The antitumor effects of curcumol or/and lenvatinib on Huh 7 cells of the HCC cell line were examined using the cell counting kit-8 (CCK-8) assay, colony formation assay, and transwell assay. For *in vivo* investigation, the effect on subcutaneous growth was also determined in nude mice. Changes in autophagy were determined by transmission electron microscope (TEM). Protein levels of apoptotic-related factors, epithelial mesenchymal transition (EMT)-related factors, autophagy factors, and N-cadherin and janus tyrosine kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) were examined by Western blot.
Results: In this study, we found that curcumol or lenvatinib could promote HCC cell apoptosis *in vitro* and inhibit the growth of HCC tumors *in vivo* (curcumol or lenvatinib group compared with control group, $p < 0.05$). While combination with curcumol treatment could improve the effect of lenvatinib on promoting cell apoptosis of HCC *in vitro* and inhibiting the growth of HCC tumors *in vivo* (combination group compared with lenvatinib group, $p < 0.05$). Curcumol combined with lenvatinib could induce more autolysosome formation detected by TEM. Mechanically, curcumol or lenvatinib could increase the expression of Bcl-2-associated X protein (Bax), E-cadherin, UNC-51-like kinase 1 (ULK), and microtubule-associated protein 1 light chain 3 (LC3B) II/I, whereas it reduced the expression of B-cell lymphoma-2 (Bcl-2), JAK2/STAT3 (curcumol or lenvatinib group compared with control group, $p < 0.05$). Furthermore, combined with curcumol treatment could increase the expression of Bax, E-cadherin, ULK, and LC3B II/I, whereas it reduced the expression of Bcl-2, N-cadherin, and JAK2/STAT3 (combination group compared with lenvatinib group, $p < 0.05$). These findings suggest that curcumol enhances the antitumor effect of lenvatinib on hepatocellular carcinoma cells.
Conclusion: Curcumol enhances the antitumor effect of lenvatinib on hepatocellular carcinoma cells.

Keywords: hepatocellular carcinoma; curcumol; lenvatinib; autophagy; EMT; JAK2/STAT3

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, serious affecting human health. Most HCC patients are initially diagnosed with advanced disease, and only a small percentage of patients have the opportunity for radical surgery for resection [1–3]. Targeted therapy represented by oral multi-kinase inhibitors, such as sorafenib and lenvatinib, is an important strategy for the treatment of unresectable HCC [4]. However, the application of molecular targeted therapies for HCC also faces some challenges. Only 26–43% of patients with advanced HCC are sensitive to sorafenib, and patients who are sensitive to sorafenib may also become resistant to it [5,6]. Currently, the relatively high dose of multi-kinase inhibitors can cause a variety of adverse reactions [7]. Furthermore, patients with advanced HCC are often complicated by cirrhosis, reducing adverse reactions, and increasing drug tolerance is more prominent. Therefore, the development of targeted drug sensitizers is an important research hotspot for improving the therapeutic efficacy of HCC.

The natural products of traditional herbal medicines are a valuable source of new drugs. Curcumol is a dried rhizome derived from Curcuma aromatica from plants of Zingiberaceae [8]. Curcumol has a variety of pharmacological effects, including antioxidant, antiviral, antibacterial, and antitumor effects [9,10]. Cumulative evidence has also shown that curcumol is a potential anti-HCC drug. Re-

search based on network pharmacology clarified that curcumol has a great anti-HCC potential. According to network pharmacology analysis, curcummol significantly affects the function of Sequestosome 1 (SQSTM1) by binding to form the curcumin-SQSTM1 complex [11]. This finding supported the notion that curcumol could play a role in anti-HCC by regulating autophagy. In another study, it is reported that curcumol could inhibit the invasion and metastasis of HCC by histone methylatic modification [12]. Whether curcumol could regulate the epithelial mesenchymal transition (EMT)-related factors E-cadherin and N-cadherin is not clearly. Furthermore, curcumol has also been found to inhibit HepG2 proliferation and induce HepG2 apoptosis through the Parkinson disease (autosomal recessive, early onset) 7 (DJ-1)/ Phosphatase and tensin homolog (PTEN)/ Phosphorylated phosphatidylinositol trikinase (PPI3K)/ Serine/threonine kinase (AKT) pathway [13].

At the same time, some studies have found that curcumol can be used as a chemosensitizer to improve the antitumor effect of chemotherapeutic drugs [14,15]. It increases the sensitivity of doxorubicin in triple negative breast cancer and cisplatin in gastric cancer [14,15]. The clinical specimens of HCC showed that resistance to lenvatinib was related to the STAT3 (signal transducers and activators of transcription 3) pathway [16]. Although curcumol could inhibit STAT3 in human cancers [17,18], it is not clear whether it can increase the sensitivity of lenvatinib in HCC. This study aims to investigate the effects of curcumol and lenvatinib on HCC cell proliferation, apoptosis, invasion, and migration, as well as their relationship with the STAT3 signaling pathway, to determine whether curcumol increases lenvatinib sensitivity in HCC.

Materials and Methods

Agents and Cell Culture

The Huh 7 HCC cell line (#CL-0102) was obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China). The result of the mycoplasma test was negative. The results of the short tandem repeat (STR) identification showed that Amelogenin: X; CSF1PO: 11; D2S1338: 19; D3S1358: 15; D5S818:12; D7S820: 11; D8S1179:14; D13S317: 10, 11; D16S539: 10; D18S51: 15; D19S433: 13, 14; D21S11: 30; FGA: 22,23; PentaE: 11; TH01: 7; TPOX: 8, 11; vMA: 16, 18; D6S1043: 13, 15; D12S391: 20, 21; D2S441: 12, 14. The STR results showed no multiple alleles and no significant cell cross-contamination and matched completely with Huh 7 cells in the German Collection of Microorganisms and Cell Cultures GmbH (DMSZ) database. Huh 7 were cultured in dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin at 37 °C in an atmosphere of 10% CO_2. Curcumol (#S2407) and lenvatinib (#S1164) were obtained from Selleck (Houston, TX, USA). Cell counting kit-8 (CCK-8, #CK18) was purchased from Dojindo (Dongren Chemical Technology, Shanghai, China). The mycoplasma PCR detection kit (#C0130S), the micro-bicinchoninic acid (micro-BCA) protein assay kit (#P0012) and the Annexin V/fluorescein isothiocyanate isomer (FITC) apoptosis detection kit (#C1062S) were purchased from Beyotime (Hangzhou, China). Anti-B-cell lymphoma-2 (anti-Bcl-2, #3498), anti-Bcl-2-associated X protein (anti-Bax, #41162), anti-E-cadherin (#14472), anti-N-cadherin (#13116), anti-UNC-51-like kinase 1 (anti-ULK1, #8054), anti-microtubule-associated protein 1 light chain 3 II/I (anti-LC3B, #3868), anti-phospho-JAK2 (#3771), anti-JAK2 (#3230), anti-phospho-STAT3 (#9145), anti-STAT3 (#12640), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH, #5174) and anti-rabbit IgG (immunoglobulin G), horseradish peroxidase-linked (HRP-linked) antibody (#7074) were obtained from cell signaling technology (Danvers, MA, USA).

CCK-8 Assay for Cell Viability

The CCK-8 assay was applied to analyze cell viability after curcumol and/or lenvatinib treatment. Huh 7 cells were seeded in 96-well plates with 5×10^3 cells each well. The cells were then divided into the control group [0.1% mass fraction of dimethyl sulfoxide (DMSO)], the curcumol group (5, 10, 50, 100 µM), lenvatinib group (5, 10, 25, 50 µM) and combined group (Cur 50 µM + Len 5 µM, Cur 10 µM + Len 10 µM, Cur 5 µM + Len 25 µM). Each group was set with five duplicate wells. 24 h after different treatments, CCK-8 reagent was added with 10 µL per well. And after adding CCK-8 reagent, cells were placed in a 37 °C incubator for another 2 hours of incubation. And the absorbance value of each well at 450 nm was detected using a microplate spectrophotometer (ELx800, BioTek Inc., Santa Clara, CA, USA). The above experiment should be repeated at least three times. The coefficient of drug interaction (CDI) was used to calculate the synergy function of curcumol and lenvatinib with CDI = AB/A×B. AB is the absorbance ratio of 450 nm between the cooperation of two drug groups and the control group, and A or B is the absorbance ratio of 450 nm between each drug alone group and the control group [19].

Clone Formation Assay for Cell Survival Rate

The effect of curcumol, lenvatinib, and the combination of these two drugs on the proliferation of the Huh 7 clone was evaluated by a clone formation experiment. Huh 7 cells were seeded in a 6-well plate with 500 cells in 2 mL of culture medium, consistent with the previous study [20]. Curcumol, lenvatinib, and curcumol/lenvatinib were added to the medium after cell attachment. 24 hours later, each group replaced the drug culture medium with fresh culture medium, allowing the cells to culture in drug-free medium for another 14 days. Next, Huh 7 cells were fixed with an-

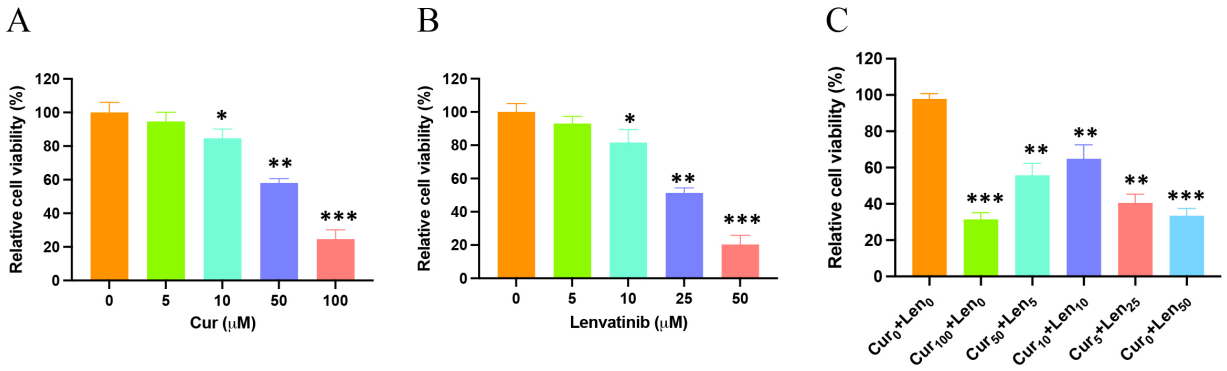

Fig. 1. Curcumol enhances the antiproliferation effect of lenvatinib in Hepatocellular carcinoma (HCC) cells. (A) The cell counting kit-8 (CCK-8) assay is used to determine the effect of curcumol on HCC cells, n = 5. (B) The CCK-8 assay is applied to analyze the effect of lenvatinib on HCC cells, n = 5. (C) The CCK-8 assay is applied to analyze the effect of combined curcumol and lenvatinib on HCC cells, n = 5. Note: compared to the control group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

hydrous ethanol, stained with Giemsa, and counted. Surviving colonies were defined as colonies containing more than 50 cells.

Detection of Apoptosis by Flow Cytometry

Huh 7 cell apoptosis after treatments was determined by the Annexin V/FITC apoptosis kit. Briefly, cells were removed from culture after 24 hours of treatment, and cells and cell supernatant were harvested and cleaned twice to remove culture medium and trypsin with precooled phosphate buffer saline (PBS). Cell precipitation using binding buffer (300 µL) resuspended and dispersed into a single cell suspension. FITC-labelled Annexin V (5 µL) and propidium iodide (PI) (5 µL) were added to the tube and the tube was placed in the dark at room temperature for 15 minutes of incubation. Another binding buffer (200 µL) was added to each tube before analysis. The cell apoptosis rate was then measured using Beckmann flow cytometry Cytoflex analysis software (BE38385, Beckman Coulter, Inc., Bria, CA, USA).

Transwell-Chamber Experiment

Huh 7 cells were seeded in a 6-well plate with 5×10^6 cells in 2 mL fresh medium. After cell adhesion, the same intervention as above was administrated. 24 hours later, the drug-containing medium was removed and the cells were kept cultured for 24 hours. Subsequently, the cells were collected with trypsin (0.25%). Huh 7 cells were seeded in the upper chamber of Transwell with 2.5×10^4 cells. At the same time, complete medium (500 µL) was added to the lower chamber of Transwell. 48 hours later, the chambers were washed twice with PBS. Paraformaldehyde (4%) was applied for 15 min of cell fixation and crystal violet (0.1%) was applied for 10 min of cell staining. Then a microscope was used to take pictures randomly. The number of migrated cells was counted.

Western Blot

Cells treated as above were collected, and whole cell proteins were obtained by adding cell lysis buffer. Subsequently, the micro-BCA protein assay kit was applied to detect protein concentrations in each group. The total protein with 10 µg was loaded into each well. Electrophoresis was performed using sodium dodecyl sulphate-polyacrylamide gel (SDS-PAG) (6%) at 80 v for 30 min, and then electrophoresis was performed at 120 v for 50 min using the different concentrations of SDS-PAG. The concentration was set to 8% for molecular weight more than 70 kDa and 12% for molecular weight less than 70 kDa depending on the molecular weight. After electrophoresis, the protein was transferred to a polyvinylidene fluoride (PVDF) membrane at 200 mA for 50 min–60 min, depending on molecular weight. PVDF membranes were incubated with different primary antibodies in the dilution ratio of 1:1000 overnight on a shaking bed at 4 °C, washing the membrane with phosphate buffered saline with tween 20 (PBST) three times, incubation of secondary antibody in the dilution ratio of 1:2000 on a shaking bed at temperature for 2 hours, washing the membrane with PBST three times, and the membrane was analyzed by chemiluminescence (Bio-Rad ChemDoc high-sensitivity chemiluminescence imaging system, Hercules, CA, USA), and the greyscale analysis of bands was analyzed by ImageJ (version 1.52e, National Institutes of Health, New York, NY, USA). Anti-GAPDH served as a loading control.

Transmission Electron Microscopy (TEM)

The Huh 7 cell were treated with curcumol, lenvatinib, and curcumol/lenvatinib for 24 h as above and cells were collected. The preparation of the cell sample before detection by electron microscopy was carried out as in the previous study [21]. Then each slice was detected and analyzed using a transmission electron microscope (HT7700, HITACHI, Tokyo, Japan).

Tumor Xenograft Assay

4-week-old male nude mice (Shanghai Laboratory Animal Centre, Shanghai, China) were raised in specific pathogen-free (SPF) level animal rooms. 100 µL cell suspension with 1×10^7 Huh cells were used to subcutaneous injection into the upper flank of mice. The tumor-bearing mice were then divided into a control group, a group of curcumol 20 mg/kg (intraperitoneal injected 3 days per week) [22], a group of lenvatinib 5 mg/kg (orally administrated 5 days per week) [23], and a combination group (curcumol 20 mg/kg intraperitoneal injected 3 days per week and lenvatinib 5 mg/kg orally administrated 5 days per week. Each group had 3 mice. The size of the tumor xenograft was recorded every 3 days. All mice were sacrificed at 4 weeks after inoculation. After intraperitoneal injection of 150 mg/kg of sodium pentobarbital, the animal was euthanized. The tumor volume was analyzed as tumor volume (mm^3) = (length $\times$ width^2) / 2. CDI was used to calculate the synergy effect of curcumol and lenvatinib with CDI = AB/A$\times$B *in vivo*. AB is the tumor size ratio between the combination of two drug groups and the control group, and A or B is the tumor size ratio between each drug group alone and the control group. The animal experiment in this study was carried out according to the Institutional Animal Committee of Hunan Provincial People's Hospital (SYXK-Xiang-2014-0006). The animal experiment in this study was also approved by the hospital's ethics committee (Ethical Application Number: 2022-30).

Statistical Analysis

All the data shown in this study represented at least 3 independent experiments. Data were represented as mean plus or minus the standard deviation (SD). Statistical analysis of the data was used by Statistical Product and Service Solutions (SPSS) software (version 22.0, SPSS Inc., Chicago, IL, USA). The statistical significance of differences between the groups was analyzed using a one-way analysis of variance and a Bonferroni post hoc test. And the statistically significant was defined as p-value must be equal to or less than 0.05. The scratch healing area was analyzed using ImageJ software (version 1.52e, National Institutes of Health, New York, NY, USA). GraphPad Prism 5 software (version 9.0, GraphPad Software, Inc., La Jolla, CA, USA) was applied to generate the diagrams.

Result

Curcumol Enhances the Antiproliferation Effect of Lenvatinib in HCC Cells

The proliferation activity of Huh 7 cells was decreased in a dose-depend manner with different concentrations of curcumol treatment (0, 5, 10, 50, 100 µM), shown in Fig. 1A. Compared to the control group, curcumol treatment with 10 µM ($p < 0.05$), 50 µM ($p < 0.01$) and 100 µM ($p < 0.001$) significantly decreased the proliferation activity of Huh 7 cells. Meanwhile, the proliferation activity of Huh 7 cells was decreased in a dose-depend manner with different concentrations of lenvatinib treatment (0, 5, 10, 25, 50 µM) (Fig. 1B). Compared to the control group, treatment with lenvatinib treatment with 10 µM ($p < 0.05$), 25 µM ($p < 0.01$) and 50 µM ($p < 0.001$) significantly decreased the proliferation activity of Huh 7 cells ($p < 0.05$). We selected five different concentrations for the CCK-8 assay alone for each treatment. When designing the combination effect, we combined the concentrations of one drug from low to high and another drug from high to low, in an attempt to find the possible combination concentration for the maximum synergistic effect. The Huh 7 cells were then treated with the curcumol and lenvatinib combination mode at different concentrations. Compared to the blank control, the addition group could inhibit HCC cell proliferation activity (Fig. 1C) ($p < 0.01$). The coefficient of drug interaction (CDI) was analyzed and the result showed that curcumol could have a synergistic effect with lenvatinib at different concentrations for all CDI less than 1 (Table 1). In subsequent experiments, the combined treatment of curcumol 5 µM and lenvatinib 25 µM as the intervention condition of the combination group) was applied for this combination had the most significant synergistic effect.

Table 1. CDI is calculated according to the result of the CCK-8 assay of the different combination concentrations.

Cur (µM)	Len (µM)	CDI
100	0	1.01
50	5	0.82
10	10	0.74
5	25	0.65
0	50	1

Note: CDI, coefficient of drug interaction; Cur, curcumol; Len, lenvatinib.

Curcumol Enhances the Anti-HCC Effect of Lenvatinib

Through the colony formation assay experiment, we found that curcumol ($p < 0.05$) and lenvatinib ($p < 0.01$) had a certain inhibitory effect on colony formation, but the inhibitory effect of the combined group ($p < 0.001$) was the most obvious (Fig. 2A). Compared with lenvatinib group, the combined group had a significant inhibitory effect on colony formation (Fig. 2A) ($p < 0.05$). Next, we found that curcumol ($p < 0.05$) and lenvatinib ($p < 0.01$) could induce more apoptotic cells, and the combination group ($p < 0.001$) induced the most apoptotic cells among all groups, as expected (Fig. 2B). Compared with lenvatinib group, the combined group had a significant promotion effect on cell apoptosis (Fig. 2B) ($p < 0.05$). Furthermore, the protein levels of Bcl-2 and Bax were detected by Western blot. As expected, the combined group could down-regulate Bcl-2 protein levels while up-regulating Bax pro-

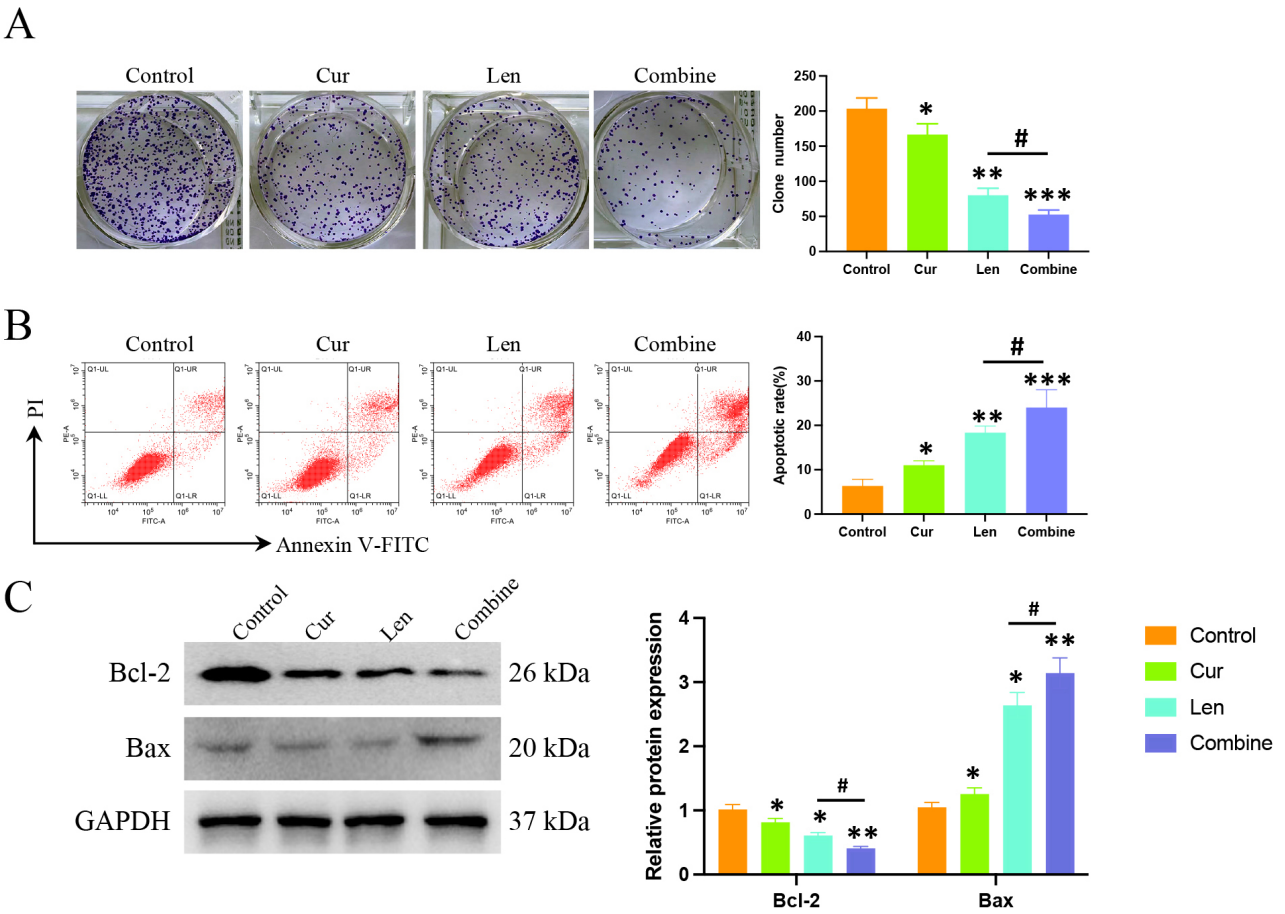

Fig. 2. Curcumol enhances the anti-HCC effect of lenvatinib. (A) The colony formation assay is used to analyze the synergistic effect of curcumol and lenvatinib on colony formation of HCC cells, and it shows that curcumol enhances the inhibition proliferation effect of lenvatinib, n = 3. (B) Annexin V/fluorescein isothiocyanate isomer (FITC) and propidium iodide (PI) staining are used to determine the synergistic effect of curcumol and lenvatinib on the cell apoptosis of HCC cells, and shows that curcumol improves the promotion apoptosis effect of lenvatinib, n = 3. (C) Western blot assay is used to analyze the apoptotic protein levels of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax), n = 3. Cur represents 5 µM curcumol treatment for 24 hours. Len represents 25 µM lenvatinib treatment for 24 hours. Combine represents 5 µM curcumol and 25 µM Lenvatinib co-treatment for 24 hours. GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Note: compared to the control group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; combine group compared to the Len group, $^{\#}p < 0.05$.

tein levels (Fig. 2C) ($p < 0.05$). Compared to the lenvatinib group, the combined group had a significant regulation effect on apoptosis-related proteins (Fig. 2C) ($p < 0.05$).

Next, the result of the transwell assay showed that curcumol ($p < 0.05$) and lenvatinib ($p < 0.05$) inhibited cell invasion, the effect of the combination group ($p < 0.01$) was the most obvious among all groups (Fig. 3A). Compared with lenvatinib group, the combined group had a significant inhibitory effect on cell invasion (Fig. 3A) ($p < 0.01$). The combination group could down-regulated the protein level of the N-cadherin level while upregulating the protein level of E-cadherin by Western blot (Fig. 3B) ($p < 0.05$). Compared with lenvatinib group, the combined group had a significant regulation effect on epithelial mesenchymal transition (EMT) -related proteins (Fig. 3B) ($p < 0.05$).

Curcumol Combined with Lenvatinib Inhibited the STAT3 Pathway during Autophagy Induction

The result of transmission electron microscopy showed that curcumol and lenvatinib could induce cell autophagy, and the effect of the combination group was the most obvious among all groups. In the control image, the overall structure of the cell was normal, the cell membrane was intact, the nucleus was irregularly shaped, and the nuclear membrane was intact. The number of mitochondria was abundant, the membrane of the mitochondria was intact, and the rough endoplasmic reticulum was not dilated. In the Cur image, the overall cells had mild edema, with intact cell membranes and numerous pseudopodia and protrusions visible around them. The number of mitochondria was relatively decreased, with some swelling of the mito-

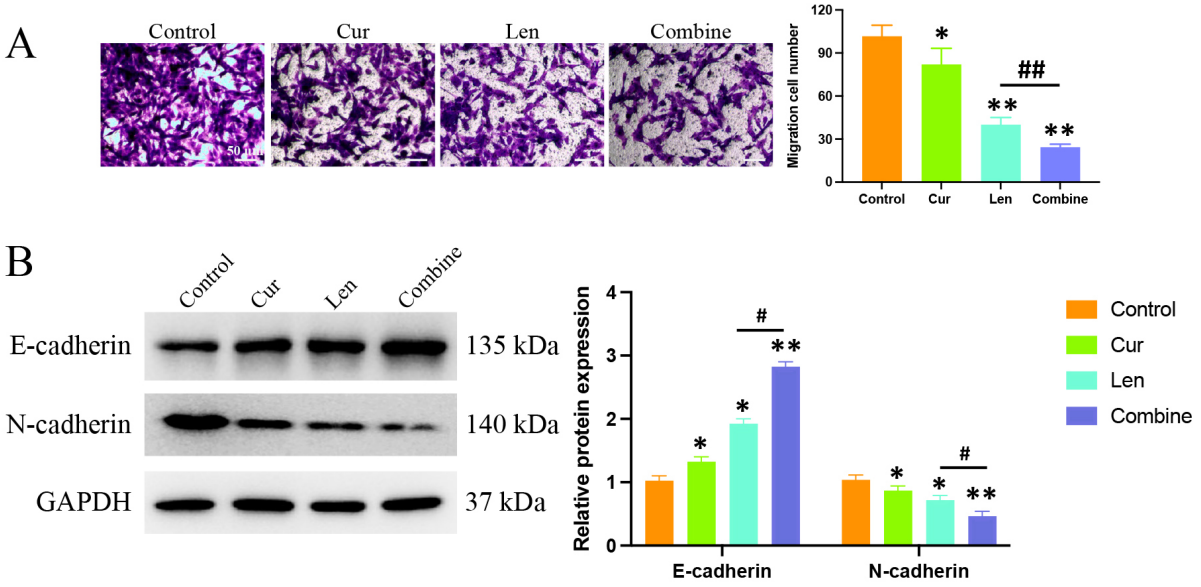

Fig. 3. Curcumol enhances the anti-invasion effect of lenvatinib. (A) The Transwell chamber experiment is applied to determine the synergistic effect of curcumol and lenvatinib on the invasion of HCC cells and shows that curcumol improves the inhibition effect of lenvatinib invasion (scale bar: 50 μm), n = 3. (B) A Western blot assay is used to analyze the epithelial mesenchymal transition (EMT) protein levels of E-cadherin and N-cadherin, n = 3. Cur represents 5 μM curcumol treatment for 24 hours. Len represents 25 μM lenvatinib treatment for 24 hours. Combine represents 5 μM curcumol and 25 μM Lenvatinib co-treatment for 24 hours. Note: compared to the control group, $*p < 0.05$, $**p < 0.01$; combine group compared to the Len group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

chondria and visible autophagic lysosomes. In the Len image, the overall cells had mild edema, with intact cell membranes and numerous pseudopodia and protrusions visible around them. The number of mitochondria decreased significantly, the rough endoplasmic reticulum expanded, and autophagic lysosomes were visible. In the combined image, the overall sign of cell autophagy was obvious, with partial damage and disintegration of the cell membrane, severe vacuolization of organelles, fewer mitochondria, swelling of the mitochondria, expansion of the rough endoplasmic reticulum, and the presence of more autophagic lysosomes (Fig. 4A). The above results indicated that curcumol improved the anti-HCC effect of lenvatinib, including inhibiting proliferation, promoting apoptosis, inhibiting invasion, and inducing autophagy. Furthermore, we detected the autophagy-related proteins ULK1 and LC3B II/I. The results showed that curcumol alone can also promote upregulation of autophagy proteins, and the combined group can significantly increase the expression levels of ULK1 and LC3B II/I (Fig. 4B) ($p < 0.05$). Compared with lenvatinib group, the combined group had the significant regulation effect on autophagy proteins (Fig. 4B) ($p < 0.05$). Meanwhile, we detected the levels of p-JAK2 and p-STAT3, and found that curcumol alone can down-regulate the levels of p-JAK2 and p-STAT3, while lenvatinib alone can also down-regulate the levels of p-JAK2 and p-STAT3, with the combined group having the most significant effect (Fig. 4C) ($p < 0.05$). Compared with lenvatinib group, the combined group had the significant regulation effect on the p-JAK2 and p-STAT3 proteins (Fig. 4C) ($p < 0.05$). Our results suggest that curcumol combined with lenvatinib induces cell autophagy by inhibiting the STAT3 pathway.

Curcumol Enhances the Inhibitory Effect of Lenvatinib on HCC in vivo

To further verify the sensitization effect of curcumol on lenvatinib, we constructed a subcutaneous tumor model in nude mice and treated with curcumol alone, lenvatinib alone, and curcumol combined with lenvatinib *in vivo*. Curcumol, lenvatinib and the combination group were found to reduce tumor volume without affecting mice's weight, indicating that curcumol ($p < 0.01$), lenvatinib ($p < 0.01$), and curcumol combined with lenvatinib ($p < 0.001$) have antitumor activity *in vivo* and the combination group has the most significant antitumor effect (Fig. 5A,B). Meanwhile, all treatments did not affect the body weight of the mice, indicating that the treatment was tolerant (Fig. 5C) ($p > 0.05$). The CDI was calculated and the result showed that curcumol could have a synergistic effect with lenvatinib for CDI less than 1 (Table 2).

Discussion

How to effectively improve the efficacy of the target therapy for HCC remains a major challenge for HCC treatment. Curcumol, as a traditional Chinese medicine monomer with multiple pharmacological effects, has been shown to inhibit HCC activity [11,13]. The results of this

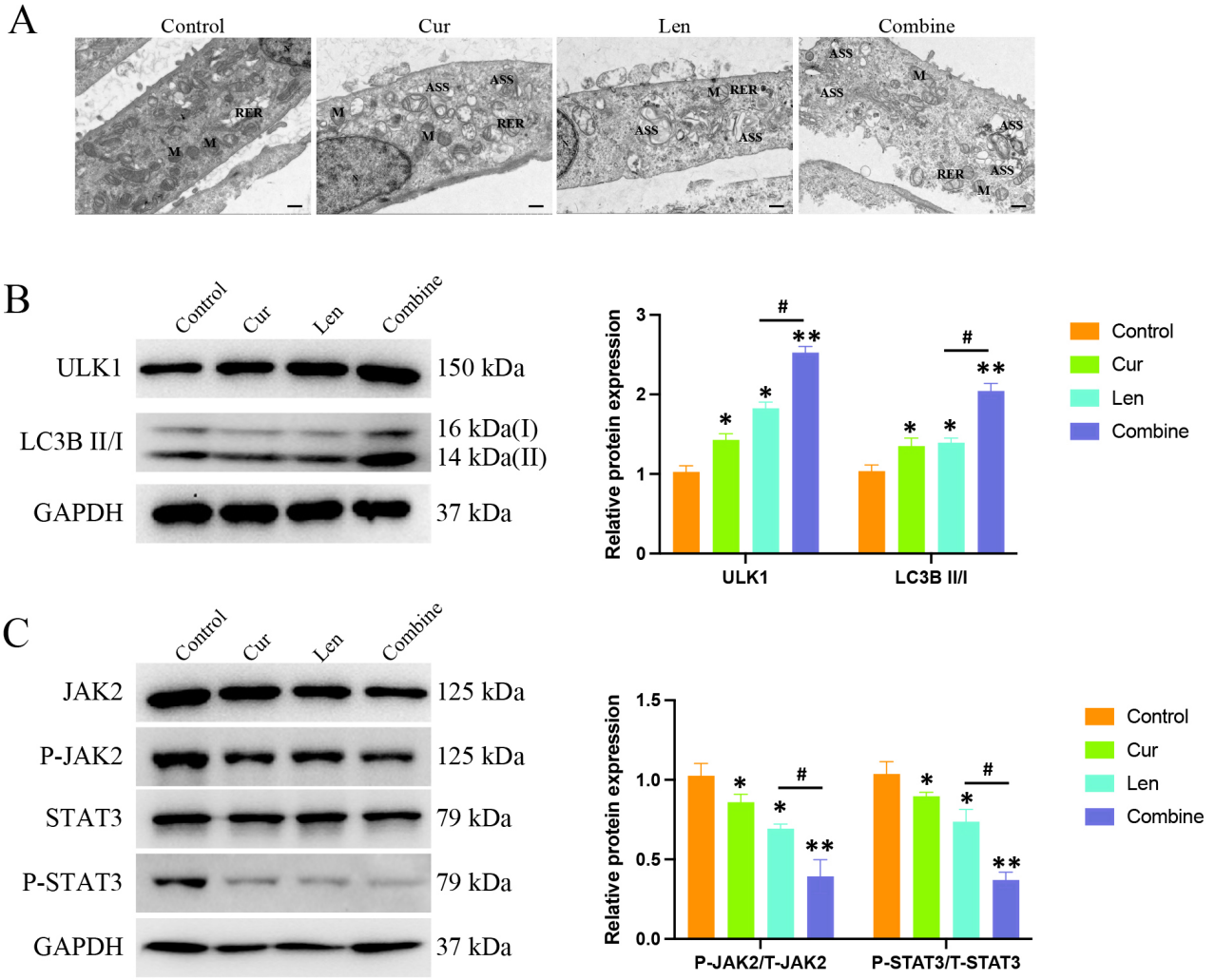

Fig. 4. Curcumol combined with lenvatinib induces cell autophagy by inhibiting the STAT3 (signal transducers and activators of transcription 3) pathway. (A) Huh 7 cells treated with curcumol, lenvatinib, and combination show obvious signs of autophagy by transmission electron microscope (TEM) (scale bar: 50 μm), n = 3. N, nuclear; M, mitochondrion; RER, rough endoplasmic reticulum; ASS, autolysosome. (B) Western blot assay is applied to determine the autophagy protein levels of ULK (UNC-51-like kinase 1) and LC3B (microtubule-associated protein 1 light chain 3), n = 3. (C) Western blot assay is applied to determine the protein levels of JAK2 (janus tyrosine kinase 2) and STAT3, n = 3. Cur represents 5 μM curcumol treatment for 24 hours. Len represents 25 μM lenvatinib treatment for 24 hours. Combine represents 5 μM curcumol and 25 μM Lenvatinib co-treatment for 24 hours. Note: compared to the control group, $*p < 0.05$, $**p < 0.01$; combine group compared to the Len group, $^{\#}p < 0.05$.

study revealed that curcumol could increase the inhibition effect of lenvatinib in HCC, including inhibiting proliferation, promoting apoptosis, inhibiting invasion, inhibiting the JAK2/STAT3 signaling pathway, and promoting autophagy. This provides experimental evidence for the application of curcumol in the sensitization of lenvatinib in HCC.

There is growing evidence for the application of curcumol in antitumor therapy, but the exploration of its mechanism in HCC is still very limited. Based on network pharmacology research, it has been elucidated that curcumol exerts inhibition effects of HCC through various mechanisms, including regulating autophagy in HCC [11]. Abnormal tumor autophagy is a very hot topic, and existing research has found that there are changes in autophagy levels in various tumors [24,25]. Autophagy can play different roles in promoting and inhibiting tumor occurrence and development at different stages. Curcumol can promote apoptosis and autophagy in nasopharyngeal carcinoma cells, as well as ferroptosis and autophagy in prostate cancer [26,27]. However, there has been no research on the effect of curcumol on autophagy in HCC cells. Our results indicate that curcumol can promote autophagy in HCC cells, and with its inhibition effect on HCC, it provides a new kind of the mechanism of curcumol in HCC.

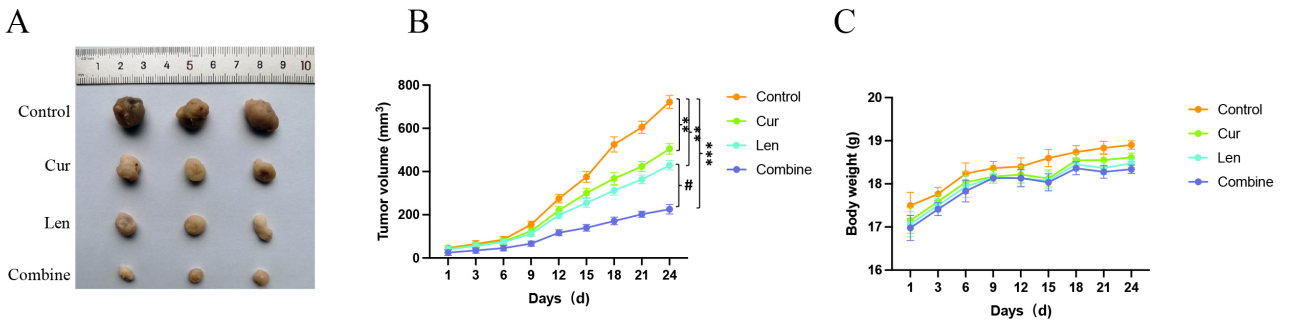

Fig. 5. Curcumol enhances the inhibitory effect of lenvatinib on HCC *in vivo*. (A) Tumor size of mice with different treatment, n = 3. (B) Tumor volume of mice with different treatment, n = 3. (C) Tumor weight of mice with different treatment, n = 3. Note: compared to the control group, $^{**}p < 0.01$, $^{***}p < 0.001$; combine group compared to the Len group, $^{\#}p < 0.05$.

Table 2. CDI is calculated according to the tumor volume of mice with curcumol, lenvatinib and combination treatment.

Control (mm^3)	690	750	725
Cur (mm^3)	480	528	508
Len (mm^3)	407	449	431
Combine (mm^3)	200	240	236
CDI		0.75	

Note: CDI, Coefficient of drug interaction; Cur, Curcumol; Len, Lenvatinib.

Lenvatinib is a new type of multi-kinase inhibitor for HCC treatment. Lenvatinib sensitivity is reported to be regulated by the level of autophagy of tumor cells, and activation of autophagy may be helpful to increase lenvatinib sensitivity [28,29]. Sorafenib could significantly reduce the level of STAT3 phosphorylation in glioma cells, induce autophagy, and thus inhibit tumor cell proliferation [30]. Another study showed that inhibition of STAT3-mediated epidermal growth factor receptor (EGFR) function could enhance autophagy levels and accelerate tumor cell apoptosis in non-small cell lung cancer [31]. Furthermore, SC-59, a sorafenib derivative, could induce autophagy by inhibiting STAT3 activity in all hepatoma cell lines, including PLC5, Sk-Hep1, HepG2, and Hep3B [32]. Otherwise, overexpression of STAT3 in HCC cell lines could inhibit autophagy. After STAT3 dephosphorylation with SC-2001, release of Beclin1 increased and autophagy levels increased significantly [33]. All of these studies indicate that STAT3 is very important in the regulation of tumor autophagy. Our results indicated that curcumol might increase the inhibitory effect of lenvatinib on STAT3 and increase autophagy levels, which may be an important mechanism by which curcumol increased the anti-tumor effect of lenvatinib. At the same time, we also found that curcumol could increase the antitumor effect of lenvatinib *in vivo*, providing strong evidence for curcumol as a sensitizer for lenvatinib.

Conclusion

In summary, curcumol could synergistically inhibit HCC proliferation, promote apoptosis, inhibit invasiveness, and promote autophagy. It has been preliminarily found that curcumol could increase the sensitivity of lenvatinib to HCC and is related to inhibiting the JAK2/STAT3 signaling pathway. Our research indicated that curcumol combined with lenvatinib inhibited the STAT3 pathway during autophagy induction in HCC treatment. In the future, we hope to see the combination of curcumol and lenvatinib applied in clinical practice to provide new treatment strategies to improve the efficacy of HCC.

Availability of Data and Materials

The data sets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

JW: Conceptualization, Methodology, Writing-Original draft preparation, Data curation, Visualization, Investigation; ZLW: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervision, Validation. Both authors have reviewed the final version and approved it to be published. And both authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

The animal experiment in this study was approved by the hunan provincial hospital's ethics committee (Ethical Application Number: 2022-30).

Acknowledgment

Not applicable.

Funding

This work was financially supported by the Center subsidy for Quality of Traditional Chinese Medicine Specialized Medical Treatment (Traditional Chinese Medicine Specialty 2022-0001).

Conflict of Interest

The authors declare no conflict of interest.

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