Engineering 19 (2022) 128–138

Contents lists available at ScienceDirect

Engineering

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Research
Traditional Chinese Medicine—Article

Tanshinone IIA Suppresses Non-Small Cell Lung Cancer Through Beclin-1-Mediated Autophagic Apoptosis

Shasha Bai [a,#], Sainan Cui [a,#], Wenhao Wen [a,#], Elaine Lai-Han Leung [b], Jing Bai [a], Huiyuan Lin [a], Yongfei Cui [a], Lei Yang [a], Zhongqiu Liu [a], Yuan Zheng [c,*], Rong Zhang [a,*]

[a] School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou 510006, China
[b] State Key Laboratory of Quality Research in Chinese Medicine/Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology, Macau 999078, China
[c] Cardiothoracic Surgery Department, The Second Clinical College of Guangzhou University of Chinese Medicine, Guangdong Provincial Hospital of Chinese Medicine, Guangzhou 510006, China

ARTICLE INFO

Article history:
Received 6 May 2021
Revised 28 June 2021
Accepted 6 July 2021
Available online 9 September 2021

Keywords:
Tan IIA
Autophagy
Apoptosis
Beclin-1

ABSTRACT

It is necessary to develop a new strategy for treatment of lung cancer since it is the main cause of cancer death. Tanshinone IIA (Tan IIA), an active ingredient of a commonly used traditional Chinese herb *Salvia miltiorrhiza*, provides a new direction to develop a new strategy for the treatment. It has been found that Tan IIA could inhibit lung cancer *in vitro* and *in vivo* by inducing autophagic apoptosis. Tan IIA increased apoptotic cells and the expression of cleaved caspase 3 and cleaved caspase 9, decreased B-cell lymphoma-2 (Bcl-2)/Bcl-2 associated X protein (Bax) ratio in human non-small cell lung cancer (NSCLC) cell lines, which was promoted by an autophagy activator Rapamycin, and weaken by autophagy inhibitor 3-methyladenine (3-MA). In addition, Tan IIA induced more autophagosomes, up-regulated light chain 3β (LC-3B) I and LC-3B II conversion and less sequestosome 1 (SQSTM1/p62) expression, which cannot be weakened by the caspase 3 antagonist. Moreover, overexpression of LC-3B gene (*LC3B*) and downregulation of autophagic gene 5 (*ATG5*) further confirmed that Tan IIA induced autophagic apoptosis in NSCLC cell lines. Beclin-1 gene (*BECN1*) overexpression and silence attenuated the effects of Tan IIA, suggesting autophagic apoptosis that Tan IIA induced was dependent on Beclin-1. Overall, our study demonstrated a new treatment mechanism of Tan IIA and suggested that Tan IIA is a potential new anti-cancer therapeutic option.

1. Introduction

According to the 2020 International Agency for Research on Cancer (IRAC) *World Cancer Report*, cancer accounted for approximately 9.6 million deaths, the second largest cause of death in 2018. Among these deaths, 1.69 million (approximately 20%) were caused by lung cancer [1,2]. Research and development of new effective anti-cancer therapeutic strategy has become an important solution. As a treasure over thousands of years in China, traditional Chinese medicine (TCM) has become a new research direction for cancer treatment. Several major principles for TCM treatment of tumors were proposed based on abundant syndrome differentiation and treatments for patients with tumors [3,4]. Patients with tumor had deficiency of vital energy or Qi and blood, leading to Qi stagnation and blood stasis, heat accumulation, condensed phlegm, and damp obstruction [5]. Invigorating blood circulation and eliminating stasis is one of principles of cancer treatment [6–9]. Hence, many scientific research workers have conducted deep studies on the internal scientific mechanism of TCM [10,11]. Studies revealed that many TCMs and active ingredients could kill various tumors, thus proving their anti-tumor effects. However, in order to meet the demand of personalized treatment and precision medicine, in-depth studies on the pharmacological mechanism of TCM is indeed necessary, which can also better promote the internationalization of TCM.

Salvia miltiorrhiza is a classical Chinese herb that invigorates blood and eliminates stasis. Various modern preparations of this

* Corresponding authors.
E-mail addresses: 1737180@qq.com (Y. Zheng), zhangrong@gzucm.edu.cn (R. Zhang).
[#] These authors contributed equally to this work.

https://doi.org/10.1016/j.eng.2021.07.014

drug have been applied for cardiovascular and cerebrovascular diseases for years [12,13]. The tumor resistance of *Salvia miltiorrhiza* has been widely studied [14,15]. *Salvia miltiorrhiza* and its various extracts all show extensive anti-tumor activities. Salvianolic acid inhibits the proliferation and promotes the apoptosis of tumor cells [16–19]. Tanshinone IIA (Tan IIA) not only exhibits the above functions, but also induces autophagy in tumor cells [20–24].

Autophagy, which mainly refers to macro-autophagy, is a programmed process that separating parts of the cells. This process can avoid cell deaths turning into adverse environment. However excessive autophagy can induce cell death. Autophagy also can inhibit cell mutation or proliferation of mutated cells during tumor initiation, thus inhibiting tumor growth [25]. Moreover, autophagy also can help mutant cells to adapt to low blood supply and hypoxia in tumor microenvironment and promote tumor development [26]. When chemotherapeutic kills tumor cells, autophagy can help tumor cells to escape from chemotherapeutic and participate in drug resistance and tumor relapse [27]. Thus, whether autophagy should be induced or inhibited for cancer therapy is still controversial [28,29]. Drugs which induce autophagic cell death (ACD) of tumor, may shed light on this direction [30,31].

In a coincidence, the TCM theory invigorating blood circulation and eliminating stasis faces similar controversies in the treatment of tumors. It has been reported that the anti-tumor effects of Tan IIA can be antagonized by autophagy inhibitors. Is autophagy the key to Tan IIA's anti-tumor effects? How does it work? Conversely, does the special treatment mechanism of Tan IIA help to explain the TCM theory of invigorating blood circulation and eliminating stasis? Further research on this mechanism may be helpful for the interpretation of the TCM anti-cancer treatment mechanism. Thus, the treatment effect of Tan IIA in lung cancer cells was explored in this study.

2. Materials and methods

2.1. Reagents

Tan IIA (> 99% purity) and cisplatin (DDP) (Aldrich Chemical Co., USA) were dissolved in dimethyl sulfoxide. 3-methyladenine (3-MA), rapamycin, procaspase activating compound (PAC)-1, and antagonist of caspases (Z-DEVD-FMK) were obtained from Selleck Chemicals (USA). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Bestbio, China) was purchased for cell apoptosis assay. Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin, light chain 3β (LC-3B), sequestosome 1 (SQSTM1/p62), Beclin-1, cleaved caspases (c-caspases), B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax) (Cell Signaling Technology, USA), and horseradish peroxidase (HRP) conjugated secondary antibodies (Multisciences, China) were obtained for proteins test.

2.2. Cell culture

The cell lines A549, PC-9, HCC827, and H1975 (China Center for Type Culture Collection, China) were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS), 100 units penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin as human lung cancer cells.

2.3. Cell viability assay

Lung cancer cells were seeded at 5×10^4 cells$\cdot mL^{-1}$ and incubated in appropriate environment (5% CO_2, 37 °C). The viability of cells was tested with cell counting kit-8 (CCK-8) (DOJINDO, Japan) 12, 24, and 48 h after Tan IIA and vehicle (0.1% dimethyl sulfoxide (DMSO)) added. The optical density at 450 nm (OD_{450}) was measured with 630 nm as reference. The cell viability index was calculated by normalizing the data by setting the untreated vehicle group as 100%.

2.4. Cell apoptosis assay

Cells (1×10^5) were centrifuged in phosphate buffer saline (PBS). Then staining buffer with Annexin V-FITC and PI were added. The samples were incubated at 4 °C for 10 min, and then quickly detected on FASCanto II (Becton Dickinson, USA), and FlowJo X 10.0.7 (Becton Dickinson, USA).

2.5. Immunoblotting

Cells were collected and lysed on ice. The supernatant was obtained for protein quantification. Samples were predegenerated and then separated and transferred to the membranes. The pre-blocked membranes were stayed with antibodies overnight and incubated with the second antibody after necessary cleaning. The membranes were analyzed with multifunctional gel imager (ChemiDoc XRS+; Bio-Rad, USA) following color development. The levels of proteins were displayed as gray value ratio using the Image Lab™ software (for PC Version 6.1; Bio-Rad, USA).

2.6. Transmission electron microscopy assay

Cells were digested with 0.5% trypsin and centrifuged to the apical of the Eppendorf tubes. 2.5% precooled glutaraldehyde solution was added to fix the dense cell masses. After 1 h at room temperature and 3 h at 4 °C, fixed cells were replaced to PBS and examined with transmission electron microscopy (TEM) (HT7800; HITACHI, Japan) according to the standard operating procedure.

2.7. Monodansylcadaverine staining assay

Cells were cultured on slips for the following treatment. All the culture medium was removed before monodansylcadaverine (MDC) staining. Wash buffer (1×) was prepared and added to the chambers twice. Cell slips were incubated in MDC staining work solution (1:9 diluted) for 30 min in dark. The slips were washed with 1× wash buffer and detected using fluorescence microscope (Eclipse 80i; Nikon, Japan).

2.8. In vivo *xenograft model*

This study was approved by the ethics committee of Guangzhou University of Chinese Medicine and performed in accordance with the guidance recommended by the National Academy of Sciences. The nude BALB/c mice (18–20 g) were inoculated with A549 cells for the xenograft model. In total, 5×10^6 cells in 200 μL PBS was subcutaneously injected into right armpit. Tumor volume was metered and calculated every other day (tumor volume = $(\text{width})^2 \times$ length ÷ 2). Mice were randomly grouped when tumor exceeded 200 mm^3. The administration of each group was described as follows, 0.02 $mL \cdot g^{-1}$ PBS for vehicle; 5, 10, and 20 $mg \cdot kg^{-1}$ for Tan IIA; 2 $mg \cdot kg^{-1}$ cisplatin for DDP. Tan IIA were injected intraperitoneally every day, and DDP was given every other day. The whole treatment lasted for three weeks, then mice were sacrificed.

2.9. Hematoxylin and eosin staining

Tumor tissue were removed and fixed in 10% formalin, paraffinized and sliced into 4 μm, then the sections were stained with hematoxylin and eosin (H&E) according to the protocol.

2.10. Network pharmacology analyses

The active ingredients of *Salvia miltiorrhiza, Ligusticum wallichii, Carthamus tinctorius L., Persicae Semen, Curcumae rhizoma, Sparganii Rhizoma, Angelicae sinensis radix,* and *Herba Leonur* were obtained from the database of Traditional Chinese Medicine Systems Pharmacology (TCMSP)[†]. PharmMapper server, which can predict the possible drug targets based on the structure of compounds, obtained a score value for the potential targets, and a certain number of targets were selected for follow-up research. Using autophagy as a key word, disease-related targets were searched in GeneCard database[‡]. Gene ontology (GO) enrichment was carried out using David database, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was performed using KEGG Orthology Based Annotation System (KOBAS) database.

2.11. Statistical analyses

All data was derived from at least two independent repetitions and were statistically analyzed by statistical product and service solutions (SPSS) software and displayed in the form of mean ± standard deviation (SD). $p < 0.05$ was defined as statistically significance.

3. Results

3.1. Tan IIA displays anti-neoplastic effects on lung cancer

Tan IIA can inhibit the proliferation of four different lung cancer cell lines including A549, PC-9, HCC827, and H1975 (Fig. 1(a)), however, of A549 and PC-9 were much more sensitive than the other two cell lines. Hence, A549 and PC-9 were selected for the research in the follow-up experiments. Tan IIA can inhibit the proliferation of A549 and PC-9 in time- and dosage-dependence manner. The 50% inhibitory concentration (IC_{50}) values after 12, 24, and 48 h of Tan IIA applied onto A549 were (10.04 ± 2.80), (2.58 ± 0.91), and (0.95 ± 0.40) $\mu mol \cdot L^{-1}$, respectively (Fig. S1(a) in Appendix A), and those of Tan IIA applied onto PC-9 were (5.26 ± 1.27), (1.94 ± 0.57), and (0.96 ± 0.40) $\mu mol \cdot L^{-1}$ (Fig. S1(b) in Appendix A), respectively. In these experiments, Tan IIA showed no significant toxic effects on normal alveolar epithelial cells until the concentration was raised above 4 $\mu mol \cdot L^{-1}$. Therefore, for safety purposes, 2 $\mu mol \cdot L^{-1}$ is the preferred concentration of Tan IIA to treat the cells in subsequent studies, except for verifying the dose–effect relationship (Fig. 1(b)).

According to data of Annexin V-FITC/PI staining, Tan IIA can induce the apoptosis of A549 and PC-9 in dose-dependent manner (Fig. 1(c)). Apoptosis-related proteins were analyzed through Western blot. C-caspase 3 and 9 increased significantly after Tan IIA treatment (Fig. 1(d)). C-caspase 8 showed no evident changes (data not shown). This finding revealed that Tan IIA induced mitochondrial apoptosis of lung cancer cells. Other related proteins, such as Bcl-2 and Bax, showed a consistent change.

More autophagosomes were observed using TEM in the lung cancer cells after Tan IIA treatments (Fig. 2(a)) than the vehicle group. Compared to the vehicle group, Tan IIA up-regulated the LC-3B II/LC-3B I ratio and down-regulated SQSTM1/p62 expression, indicating that autophagy was induced. This compound also increased the expression of Beclin-1 (Fig. 2(b)).

3.2. Tan IIA diminishes the tumor in tumor-bearing mice

The tumor-bearing mouse model established by A549 achieved a significant reduction of tumor volume and weight after Tan IIA injection (Fig. 3(a) and Fig. S2(a) in Appendix A). Test results of multiple organ indexes showed no toxicity of the agent (Figs. S2(b) and (c) in Appendix A). Tumor tissue slices of tumor-bearing mice were stained with H&E and observed with microscope. The tumor tissues of vehicle group were in sharpen alignment, whereas the cells in tumor tissues of Tan IIA group and DDP group were in loose and disordered arrangement, and pyknotic nucleus (Fig. 3(b)), suggesting induction of apoptosis by both drugs.

Western blot analysis on apoptosis related proteins was performed after obtaining the tumor tissues and the proteins were extracted. The results showed the expression of the apoptosis related proteins increased (c-caspase 3, Bcl-2, and Bax) (Fig. 3(c)). Autophagy-related proteins LC-3B I, LC-3B II, and SQSTM1/p62 were also induced after Tan IIA treatment in the tumor tissues of tumor-bearing mice, indicating the occurrence of autophagy (Fig. 3(d)).

3.3. Interaction between autophagy and apoptosis induced by Tan IIA in lung cancer cells

The relationship between autophagy and apoptosis was further studied. Whether the autophagy was the protective reaction of lung cancer cells from apoptosis was examined. At first, lung cancer cells treated by Tan IIA were collected at different time-points for protein detection. The results showed that after 4 h treatment of Tan IIA, the LC-3B II expression in A549 was up-regulated significantly. C-caspase 3 showed no significant change until 8 h. This finding revealed that autophagy might occur before apoptosis (Fig. 4(a)). PC-9 cells have developed autophagy slightly later but still before apoptosis (Fig. 4(b)). Whether autophagy has participated in Tan IIA-induced apoptosis, lung cancer cells were pretreated by autophagy agonist rapamycin (RAPA) and antagonist 3-MA. Result showed that RAPA could increase c-caspase 3 level by Tan IIA. On the contrary, 3-MA weakened the above effects of Tan IIA (Fig. 5(a)). The results preliminarily proved that autophagy was not induced by apoptosis in lung cancer cells but was induced by Tan IIA. Meanwhile, lung cancer cells were pre-processed using the agonist and antagonist of caspases. The autophagy induced by Tan IIA did not change significantly by the antagonist of caspases (Z-DEVD-FMK) but was aggravated by the agonist of caspase 3 (PAC-1) (Fig. 5(b)). This finding proved that Tan IIA induced autophagy is participated mediating apoptosis of lung cancer.

For further verification, custom small hairpin RNA (shRNA) for autophagic gene 5 (*ATG5)* were designed and synthesized, lentivirus interference vectors were constructed and transfected into A549 cells (marked as $ATG5^{lo/lo}$) for efficiency testing and the following studies. MDC staining and LC-3B assay results suggested that autophagy was already knocked down (Figs. 6(a) and (b)). In this event, A549 cells showed less sensitivity to Tan IIA (Fig. 6(b)).

A549 cell strain with stable overexpression of autophagic gene, LC-3B gene (*LC3B)*, was established for further determination. MDC staining and immunoblot tests suggested autophagy was up-regulated in $LC3B^{hi/hi}$ cell strain (Fig. 6(c)). Tan IIA increased LC-3B and c-caspase 3 levels, and decreased Beclin-1 expression in $LC3B^{hi/hi}$ cell strain (Fig. 6(d)). These data suggested Tan IIA induced autophagic apoptosis is possibly through Beclin-1.

3.4. Tan IIA induces autophagic apoptosis of lung cancer cells through Beclin-1

In wild-type A549 cells and PC-9 cells, the expression level of Beclin-1 increased significantly after Tan IIA treatment (Figs. 2(b)

[†] https://tcmspw.com/tcmsp.php.
[‡] https://www.genecards.org.

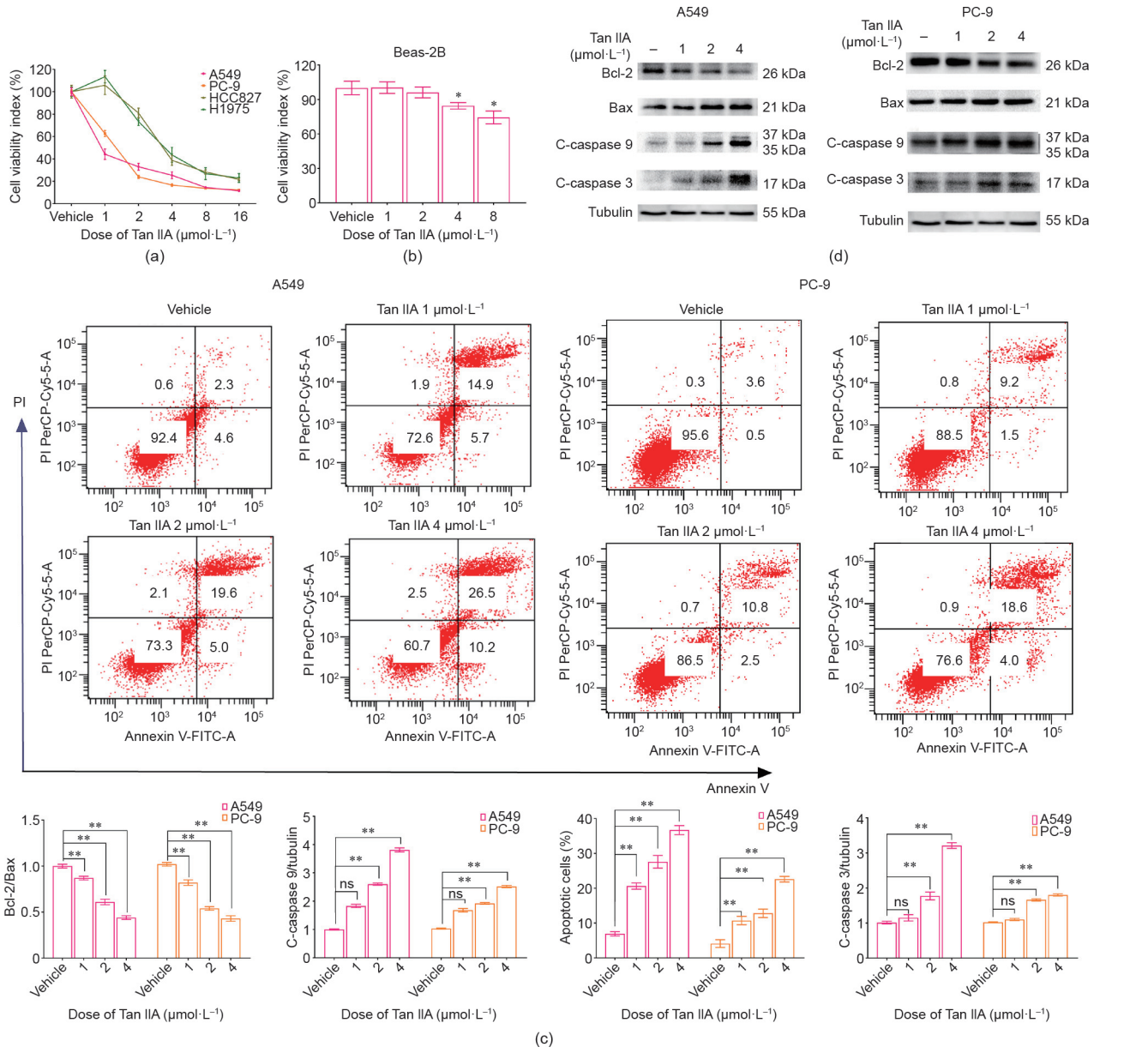

Fig. 1. Effects of Tan IIA on NSCLC cells. (a) Four NSCLC cell lines (A549, PC-9, H1975, and HCC827) were cultured in medium with and without Tan IIA. Cell viability was detected with CCK-8. (b) Normal bronchial epithelial cells beas-2B was cultured for cytotoxicity evaluation. (c) Cell apoptosis was studied with flow cytometry. Each group had normal distribution. (d) Cleaved caspases (c-caspases) 3 and 9, Bax, and Bcl-2 expression was detected by immunoblot assay. For the data of CCK-8, repeated measurement analysis of variance (ANOVA) was used to meet the spherical hypothesis. In-subject effect test and pairwise comparison were conducted along with homogeneity of variance. For the data of apoptosis that satisfies homogeneity of variance, univariate analysis was used, and multiple comparisons were pairwise compared by least significance difference test (LSD-T). $^{*}p < 0.05$, $^{**}p < 0.01$; $n = 4$. ns: no significance; PI: propidium iodide.

and 3(d)). Observed in *LC3B*$^{hi/hi}$ cells treated by Tan IIA, Tan IIA also could enhance Beclin-1 expression in *ATG5*$^{lo/lo}$ A549 cells but weakened Beclin-1 expression (Fig. 6(d)). As following, *BECN1*$^{hi/hi}$ and cell *BECN1*$^{lo/lo}$ strain was established. The results showed that LC-3B and c-caspase 3 levels were rised in *BECN1*$^{hi/hi}$ cells and decreased in *BECN1*$^{lo/lo}$ cells. Tan IIA did not increase LC-3B and c-caspase 3 levels in both *BECN1*$^{hi/hi}$ and *BECN1*$^{lo/lo}$ cells (Figs. 6(b) and (d)).

4. Discussion

In 2018, lung cancer has the highest morbidity and fatality among 18 million new diagnosed cancer cases in the world, and lower incidence (4.3 million new cases) but higher mortality (2.9 million cancer deaths) in China, which is due to poor prognosis [1,32,33]. Nowadays, cancer becomes the leading cause of death without good treatment. TCM is a treasure of China and plays important role in maintaining people's health over thousands of years. Therefore, more attention has been applied to TCM in tumor treatment.

Among the early study on the effects of Chinese herbs and components on lung cancer, which was done with network pharmacological analysis, more than 20 compounds were screened for efficacy verification. Of which, Tan IIA is one of the components with good efficacy and safety. There are many reports on the anti-tumor mechanism of Tan IIA, but one of them aroused our

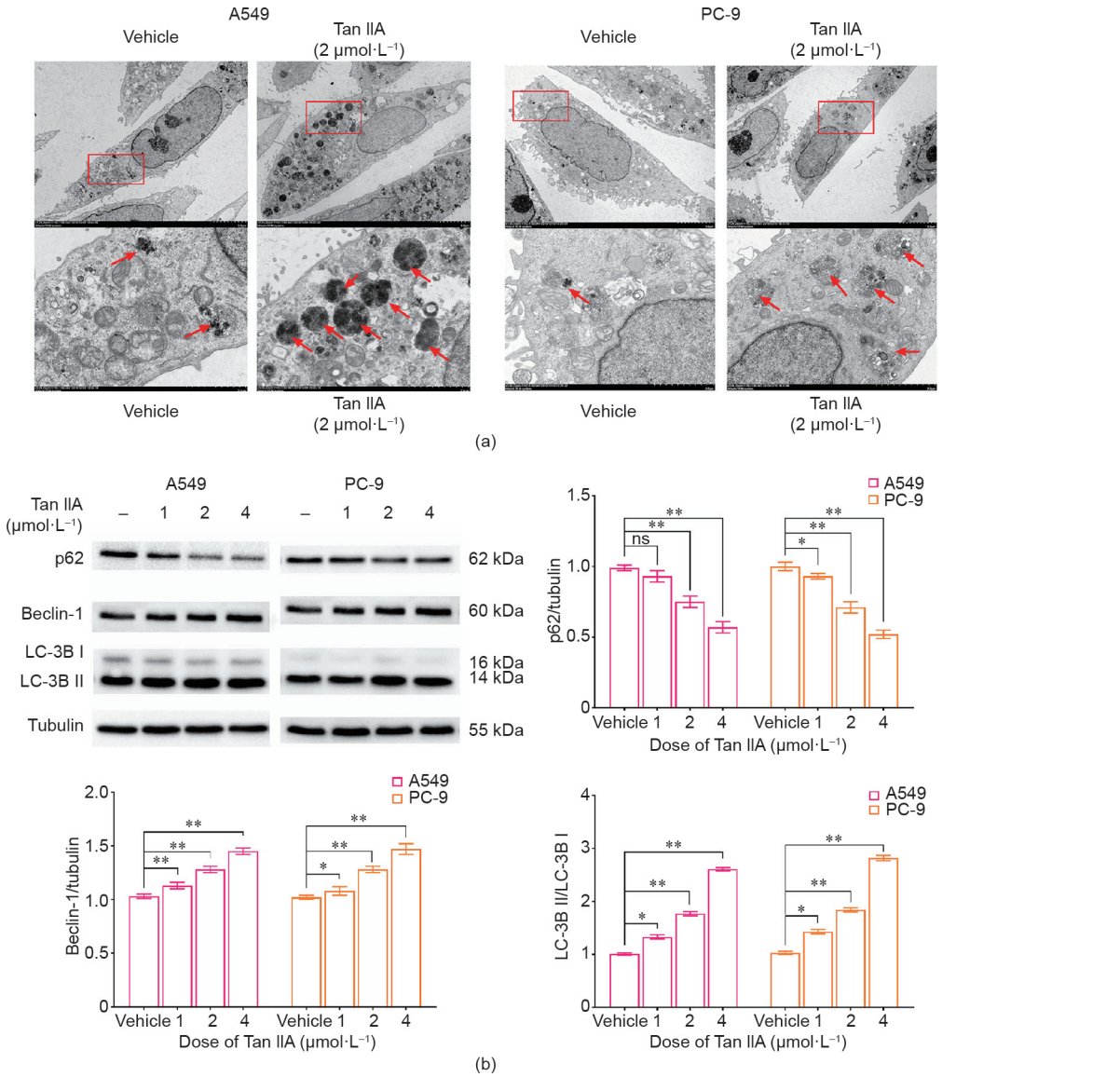

Fig. 2. Tan IIA induced cell autophagy in NSCLC cells. (a) Cells in presence of Tan IIA were fixed with 2.5% glutaraldehyde/0.1 mol·L^{-1} PBS for TEM detecting. The red arrows in the figure refer to the autophagosomes. (b) LC-3B I, LC-3B II, Beclin-1, SQSTM1/p62, and tubulin protein expression was analyzed by immunoblot assay in A549 and PC-9 cells with Tan IIA. Each group had normal distribution. Satisfying homogeneity of variance, univariate analysis was used, and multiple comparisons were pairwise compared by LSD-T test. $^{*}p < 0.05$, $^{**}p < 0.01$; $n = 3$. ns: no significance.

attention that Tan IIA may cause ACD [30,31]. Unfortunately, there was no further proof to confirm Tan IIA treatment mechanism.

ACD was described by Richard Lockshin more than half a century ago, but there is indeed intense controversy about it [34–39]. Someone believes that ACD is a third cell death pathway without morphological symptoms of necrosis and apoptosis [40,41]. On the other hand, someone attempts to define ACD by three characters: autophagy is induced, autophagy is parallel or precede cell death, and cell death can be blocked by autophagy inhibition [34,36,42,43]. No matter which way is defined, autophagy is the "key player."

Autophagy is a well-known "double-edged sword" in tumorigenesis [27,29,38,44]. When treating a tumor, whether autophagy should be induced or suppressed has been a controversial issue. In this study, Tan IIA induced autophagy to promote cell death and played an anti-tumor role. However, there are more reports that autophagy can protect tumor cells from the destruction of chemotherapy or mediate the immune escape, and the autophagy inhibitor chloroquine has also been reported to have the potential to become an anti-tumor drug [45–47]. A similar debate exists in the TCM treatment of tumors. It is whether invigorating blood and eliminating stasis can help tumor form better blood supply and promote tumor growth or promote the bloody metastasis of tumors, while treating the tumors. It has been reported that single-flavor Chinese herb for invigorating blood and eliminating stasis, promoted the angiogenesis of Lewis lung cancer cells in mice, or impaired the function of immune cells, leading to the release of cancer cells into the blood and accelerating the blood metastasis of tumors [48,49]. However, also found that, compared to the control group patients of radiotherapy of nasopharyngeal carcinoma who accepted invigorating blood and eliminating stasis treatment had no statistical significance in the five-year survival rate patients and the recurrence rate [50,51]. Recently, more and

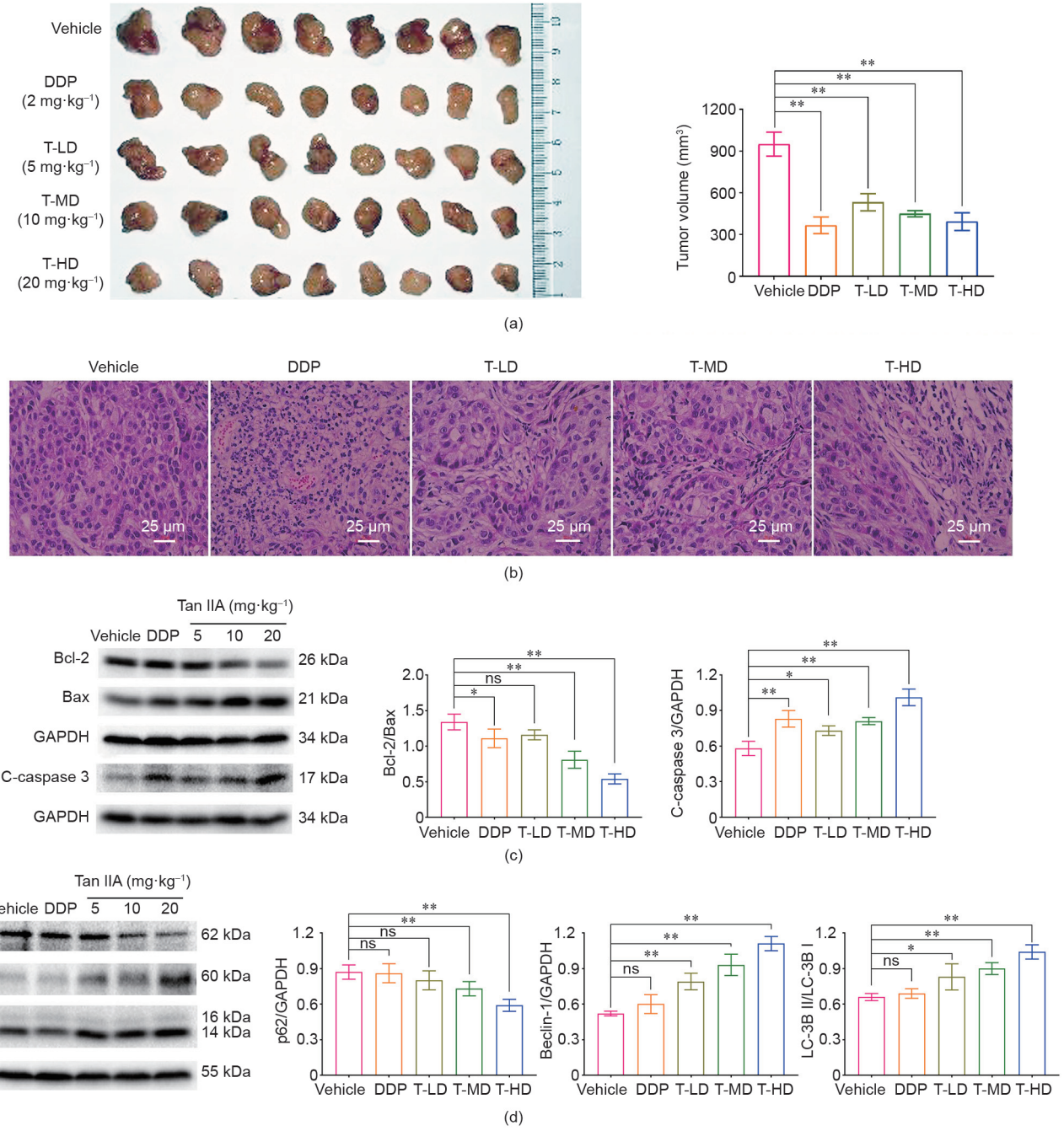

Fig. 3. Tan IIA induced autophagy and apoptosis in tumor-bearing mice. (a) The photograph and measured volumes of tumors human lung cancer xenograft model mice tanshinone. (b) Tumor tissue sections was observed by H&E staining. Proteins for (c) apoptosis (Bcl-2, Bax, and c-caspase 3) and for (d) autophagy (LC-3B I, LC-3B II, SQSTM1/p62, and Beclin-1) were analyzed by immunoblot assay. Each group had normal distribution. Satisfying homogeneity of variance, univariate analysis was used, and multiple comparisons were pairwise compared by LSD-T test. $^{*}p < 0.05$, $^{**}p < 0.01$; $n = 8$. ns: no significance; T-HD: Tan IIA high-dose group; T-MD: Tan IIA medium-dose group; T-LD: Tan IIA low-dose group.

more scholars put forward invigorating blood and eliminating stasis can normalize tumor angiogenesis and microenvironment, thus play a role of cancer treatment [52–54]. In this study, we found that Tan IIA which is the active components of *Salvia miltiorrhiza* showed significant anti-tumor effects, and no significant increase in *in situ* metastasis was found in Tan IIA treated mice. To sum up, such a similar situation leads us to believe that there must be a relationship between autophagy and the mechanism of anti-tumor effect of Tan IIA, which can invigorate blood and eliminate stasis. Will autophagy cell death be the key? Indeed, according to our research results, Tan IIA did induce autophagy cell death, more specifically, autophagy apoptosis, in human NSCLC cells. The results showed that Tan IIA induced both autophagy and apoptosis in lung cancer cells. However, autophagy occurred earlier, and inhibition of autophagy can block cell apoptosis. However, inhibition of apoptosis did not prevent autophagy, although induction of apoptosis enhanced autophagy.

For further confirmation, the key signal molecules in ACD were selected for intervention [55,56]. Beclin-1, known as a key molecule in the process of autophagy, could bond with Bcl-2 and become inactivated [57–59]. It could also be phosphorylated by unc-51 like autophagy activating kinase 1 (ULK1) once separated

from Bcl-2 to increase the activity of vacuolar protein sorting 34 (VPS34) complex [57,60,61]. This step was crucial in the occurrence of autophagy. After autophagy signals are being released, the up-regulated Beclin-1 could bind with many Bcl-2 molecules to release a set of apoptosis-promoting molecules (e.g. Bax and Bad) that bonded with mitochondrial membrane, thus initiating endogenous apoptosis. The results both in *BECN1*$^{hi/hi}$ cells and *BECN1*$^{lo/lo}$ cells showed that Tan IIA-induced apoptosis was weakened, because Tan IIA could not induce lung cancer cells to express more Beclin-1 to bind Bcl-2, the effect of Tan IIA-induced autophagic apoptosis was significantly reduced.

Overall, we have interpreted and elucidated the molecular mechanism of Tan IIA in inhibition of lung cancer. This report has deeply investigated for the first time the role of ACD in Tan IIA against lung cancer in a more systematic and in-depth way, and lays foundations for follow-up studies on the anti-tumor mechanism of other ingredients of *Salvia miltiorrhiza* (Fig. 7). ACD and its link to the theory of invigorating blood and eliminating stasis in TCM. According to the results of network pharmacological analysis on the correlation between traditional Chinese medicines of invigorating blood and eliminating stasis and autophagy, the active components of traditional Chinese medicine form an awfully close interactive network with multiple target molecules related to autophagy (Fig. S3 in Appendix A), and after GO enrichment, multiple pathways related to apoptosis and its regulatory mechanism can be seen (Fig. S4 in Appendix A). Signal pathways were enriched in KEGG database, it was also found that multiple signal mechanisms related to apoptosis and tumor genesis and development were focused (Fig. S5 in Appendix A). Therefore, ACD also suggested that autophagy is likely to be a "key player" of invigorating blood and eliminating stasis in the treatment of tumors. However, the rule of autophagy apoptosis still needs more systematic verification.

"Invigorating blood and eliminating stasis" is one of major principles of TCM treatment for tumors. Interpreting the modern scientific molecular mechanism of this principle is vital for the research and development of new methods and strategies for tumor treatment.

5. Conclusions

Tan IIA, the main ingredient of *Salvia miltiorrhiza,* can time- and dose-dependently induce the ACD in A549 and PC-9 cells and decrease the tumor volume of tumor-bearing mouse without evident toxicity. Tan IIA exerts pharmacological effect on lung cancer by inducing autophagic apoptosis.

Acknowledgments

The study was supported by grants from the National Natural Science Foundation of China (81903943 and 82074219), the Natural Science Foundation of Guangdong Province (2017A030310502). The study was also supported by the Key Projects of Educational Commission of Guangdong Province (2018KZDXM020) and the Science and Technology Planning Project of Guangzhou (202002030009).

Compliance with ethics guidelines

Shasha Bai, Sainan Cui, Wenhao Wen, Elaine Lai-Han Leung, Jing Bai, Huiyuan Lin, Yongfei Cui, Lei Yang, Zhongqiu Liu, Yuan Zheng, and Rong Zhang declare that they have no conflict of interest or financial conflicts to disclose.

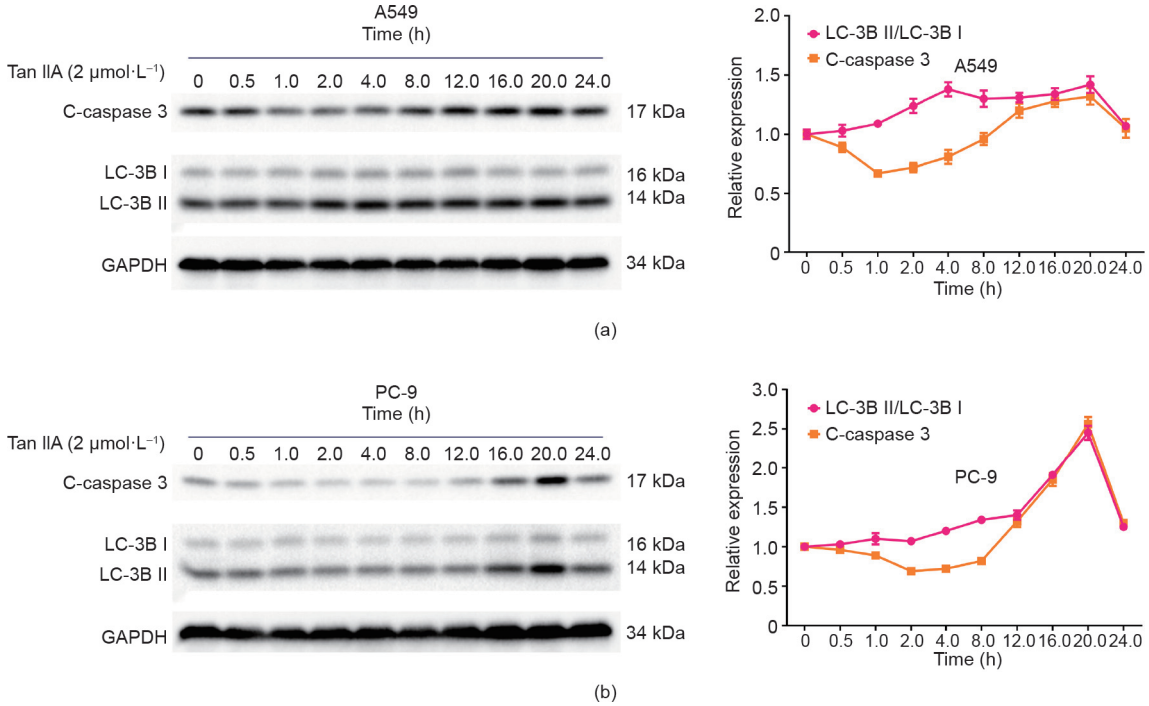

Fig. 4. The relationship of Tan IIA induced autophagy and apoptosis in NSCLC cells. (a) Immunoblot assay of autophagy and apoptosis markers (LC-3B I, LC-3B II, and c-caspase 3) in A549 cells treated with Tan IIA (2 μmol·L^{-1}) for different time. (b) Immunoblot assay of autophagy and apoptosis markers (LC-3B I, LC-3B II, and c-caspase 3) in PC-9 cells treated with Tan IIA (2 μmol·L^{-1}) for different time.

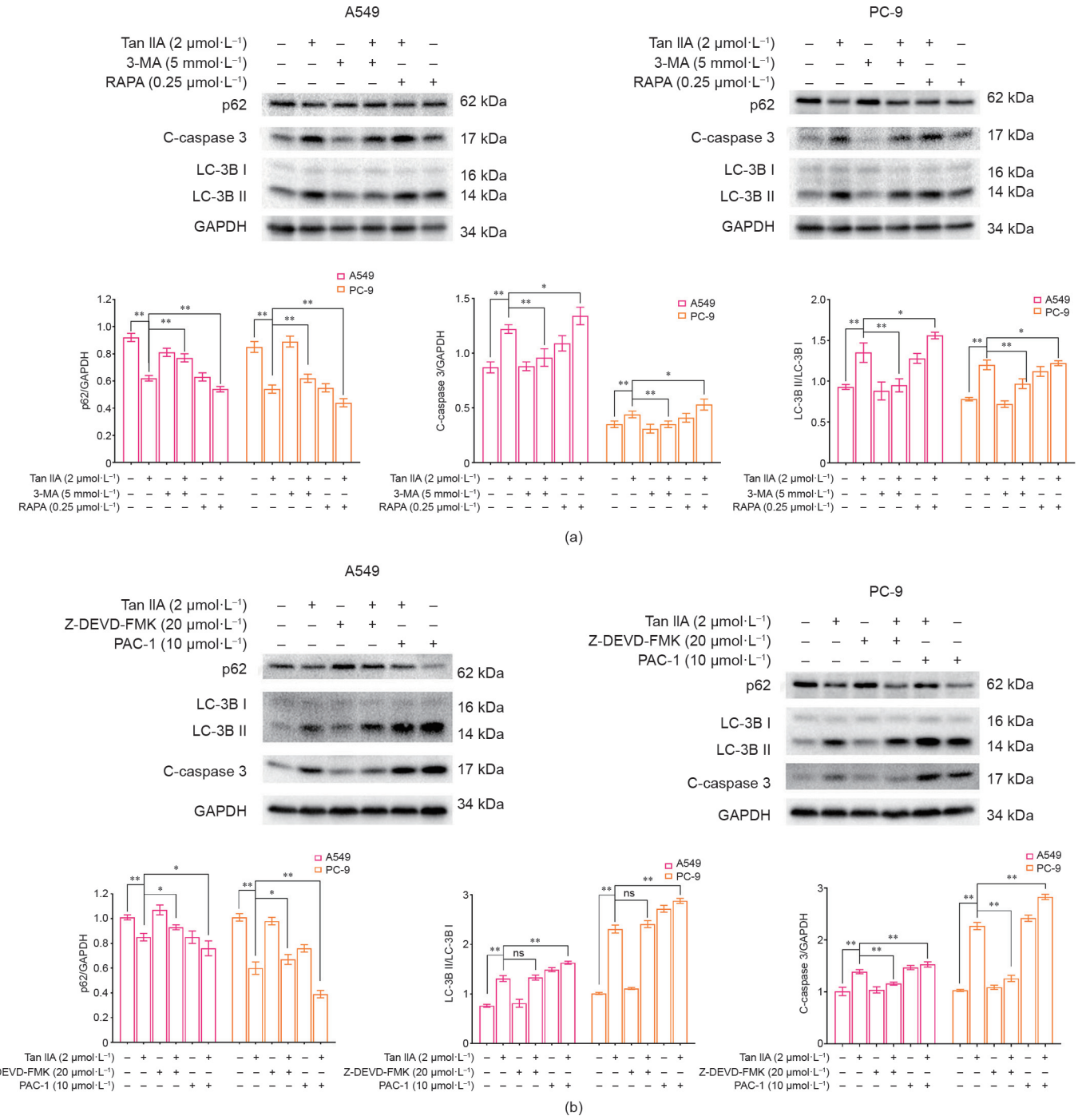

Fig. 5. The interaction of Tan IIA induced autophagy and apoptosis in NSCLC cells. (a) Autophagy agonist (rapamycin (RAPA)) and antagonist (3-MA) were added 1 h before Tan IIA, and then the autophagy and apoptosis markers were detected in A549 and PC-9 cells. (b) Apoptosis agonist (PAC-1) and antagonist (Z-DEVD-FMK) were pretreated for 1 h, then the same operation was done. Each group had normal distribution. Satisfying homogeneity of variance, univariate analysis was used, and multiple comparisons were pairwise compared by LSD-T test. $^{*}p < 0.05$, $^{**}p < 0.01$; $n = 3$. ns: no significance.

Authors' contribution

Shasha Bai designed and performed the experiments. Sainan Cui wrote the initial version of the manuscript, and Elaine Lai-Han Leung revised it. Wenhao Wen assisted with the conduct of the experiments and analyzed the data. Huiyuan Lin assisted with animal experiments. Jing Bai and Yongfei Cui contributed to manuscript preparation and assisted with animal experiments. Lei Yang contributed reagents, materials, analysis tools. Yuan Zheng and Rong Zhang formulated the hypotheses. Zhongqiu Liu supervised the study. All authors reviewed the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2021.07.014.

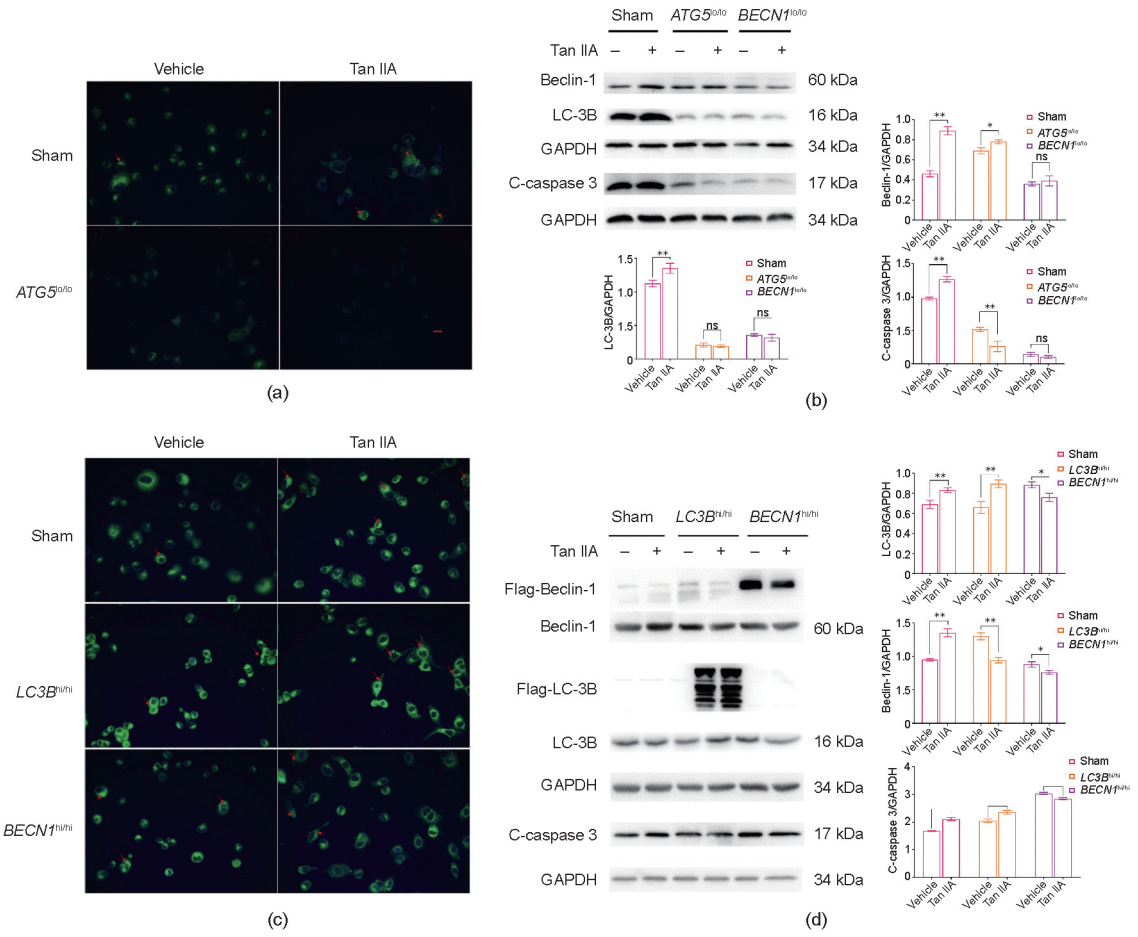

Fig. 6. Tan IIA induced autophagic apoptosis in NSCLC cells. (a) Scramble small interfering RNA (siRNA) (for sham) and *ATG5* siRNA (for $ATG5^{lo/lo}$) were transfected into A549 cells for MDR staining after a Tan IIA treatment at 2 μmol·L^{-1} for 24 h. (b) Scramble shRNA (for sham), *LC3B* shRNA (for $LC3B^{hi/hi}$), and *BECN1* shRNA (for $BECN1^{hi/hi}$) transfected A549 cells were treated in the same way for MDR staining. (c, d) The level of c-caspase 3, LC-3B, and Beclin-1 in $ATG5^{lo/lo}$, $BECN1^{lo/lo}$, $LC3B^{hi/hi}$, and $BECN1^{hi/hi}$ cells was measured by immunoblot assay. Each group had normal distribution. Satisfying homogeneity of variance, univariate analysis was used, and multiple comparisons were pairwise compared by LSD-T test. $^{*}p < 0.05$, $^{**}p < 0.01$; $n = 3$. ns: no significance.

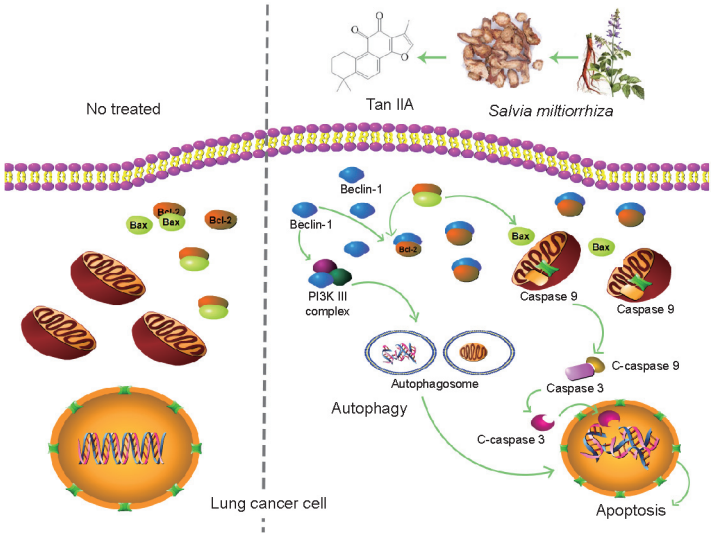

Fig. 7. The molecular mechanism of autophagic apoptosis induced by Tan IIA. PI3K: phosphatidylinositol 3-kinase.

References

[1] Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, et al. Colorectal cancer statistics, 2020. CA Cancer J Clin 2020;70(3):145–64.
[2] Forouzanfar MH, Afshin A, Alexander LT, Anderson HR, Bhutta ZA, Biryukov S, et al.; 2015 Risk Factors Collaborators. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2015: a systematic analysis for the global burden of disease study 2015. Lancet 2016;388(10053):1659–724.
[3] Liao X, Bu Y, Jia Q. Traditional Chinese medicine as supportive care for the management of liver cancer: past, present, and future. Genes Dis 2020;7(3):370–9.
[4] Li Z, Zhang F, Li G. Traditional Chinese medicine and lung cancer—from theory to practice. Biomed Pharmacother 2021;137:111381.
[5] Smith ME, Bauer-Wu S. Traditional Chinese medicine for cancer-related symptoms. Semin Oncol Nurs 2012;28(1):64–74.
[6] Zhang Z, Zhao X, Gao Q, Zhao Y, Zhai L, Jia S, et al. Investigation and study on the TCM syndrome distribution of malignant tumor. World J Integr Tradit West Med 2019;14(2):261–7. Chinese.
[7] Yang XB, Long SQ, Deng H, Liu W, He WF, Pan ZQ, et al. Distribution characteristics of syndrome types in TCM in patients with advanced non-small cell lung cancer (NSCLC). J Liaoning Univ TCM 2013;15(3):188–90. Chinese.
[8] Zhu P, Li ZG, Dong CW. Progress on lung cancer with syndrome differentiation in traditional Chinese medicine. J Pract Tradit Chin Intern Med 2017;31(3):91–3. Chinese.
[9] Xiang Y, Guo Z, Zhu P, Chen J, Huang Y. Traditional Chinese medicine as a cancer treatment: modern perspectives of ancient but advanced science. Cancer Med 2019;8(5):1958–75.
[10] Zhao LN, Yang YQ, Wang WW, Li Q, Xiao H. The effects of traditional Chinese medicine combined with chemotherapy on immune function and quality of life in patients with non-small cell lung cancer: a protocol for systematic review and meta-analysis. Medicine 2020;99(45):e22859.
[11] Lee YW, Chen TL, Shih YR, Tsai CL, Chang CC, Liang HH, et al. Adjunctive traditional Chinese medicine therapy improves survival in patients with advanced breast cancer: a population-based study. Cancer 2014;120(9):1338–44.
[12] Wan AKS, Leung SWS, Zhu DY, Man RYK. Vascular effects of different lipophilic components of "Danshen", a traditional Chinese medicine, in the isolated porcine coronary artery. J Nat Prod 2008;71(11):1825–8.
[13] Ren J, Fu L, Nile SH, Zhang J, Kai GY. *Salvia miltiorrhiza* in treating cardiovascular diseases: a review on its pharmacological and clinical applications. Front Pharmacol 2019;10:753.
[14] Liao S, Han L, Zheng X, Wang X, Zhang P, Wu J, et al. Tanshinol borneol ester, a novel synthetic small molecule angiogenesis stimulator inspired by botanical formulations for angina pectoris. Brit J Pharmacol 2019;176(17):3143–60.
[15] Gao H, Sun W, Zhao J, Wu X, Lu JJ, Chen X, et al. Tanshinones and diethyl blechnics with anti-inflammatory and anti-cancer activities from *Salvia miltiorrhiza* Bunge (Danshen). Sci Rep 2016;6:33720.
[16] Wang LQ, Hua DF, Li XM, Li J, Guan Y. Salvianolic acid-A induces apoptosis, mitochondrial membrane potential loss and DNA damage in small cell lung cancer cell lines. Trop J Pharm Res 2015;14(8):1399–404.
[17] Tang XL, Yan L, Zhu L, Jiao DM, Chen J, Chen QY. Salvianolic acid A reverses cisplatin resistance in lung cancer a549 cells by targeting c-met and attenuating Akt/mTOR pathway. J Pharmacol Sci 2017;135(1):1–7.
[18] Guan Y, Zhu JP, Shen J, Jia YL, Jin YC, Dong XW, et al. Salvianolic acid B improves airway hyperresponsiveness by inhibiting MUC5AC overproduction associated with Erk1/2/P38 signaling. Eur J Pharmacol 2018;824:30–9.
[19] Ye YT, Zhong W, Sun P, Wang D, Wang C, Hu LM, et al. Apoptosis induced by the methanol extract of *Salvia miltiorrhiza* Bunge in non-small cell lung cancer through PTEN-mediated inhibition of PI3K/Akt pathway. J Ethnopharmacol 2017;200:107–16.
[20] Guo R, Li L, Su J, Li S, Duncan SE, Liu ZH, et al. Pharmacological activity and mechanism of Tanshinone IIA in related diseases. Drug Des Dev Ther 2020;14:4735–48.
[21] Zhang Y, Jiang P, Ye M, Kim SH, Jiang C, Lu JX, et al. Tanshinones: sources, pharmacokinetics and anti-cancer activities. Int J Mol Sci 2012;13(10):13621–66.
[22] Zhang Y, Geng Y, He JT, Wu D, Zhang T, Xue L, et al. Tanshinone IIA induces apoptosis and autophagy in acute monocytic leukemia via downregulation of PI3K/Akt pathway. Am J Transl Res 2019;11(5):2995–3006.
[23] Wang X, Shao M, Wang Y. GW29-e0760 Tanshinone IIA protects H9c2 cells from reactive oxygen species through inhabiting excessive autophagy via activation of PI3K/Akt/mTOR signaling pathway. J Am Coll Cardiol 2018;72(16):C24.
[24] Ding L, Wang S, Qu X, Wang J. Tanshinone IIA sensitizes oral squamous cell carcinoma to radiation due to an enhanced autophagy. Environ Toxicol Pharmacol 2016;46:264–9.
[25] Onorati AV, Dyczynski M, Ojha R, Amaravadi RK. Targeting autophagy in cancer. Cancer 2018;124(16):3307–18.
[26] White E, Mehnert JM, Chan CS. Autophagy, metabolism, and cancer. Clin Cancer Res 2015;21(22):5037–46.
[27] Mulcahy Levy JM, Thorburn A. Autophagy in cancer: moving from understanding mechanism to improving therapy responses in patients. Cell Death Differ 2020;27(3):843–57.
[28] Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. Nat Rev Cancer 2017;17(9):528–42.
[29] Amaravadi RK, Kimmelman AC, Debnath J. Targeting autophagy in cancer: recent advances and future directions. Cancer Discov 2019;9(9):1167–81.
[30] Qiu Y, Li C, Wang Q, Zeng X, Ji P. Tanshinone IIA induces cell death via Beclin-1-dependent autophagy in oral squamous cell carcinoma SCC-9 cell line. Cancer Med 2018;7(2):397–407.
[31] Yun SM, Jung JH, Jeong SJ, Sohn EJ, Kim B, Kim SH. Tanshinone IIA induces autophagic cell death via activation of AMPK and ERK and inhibition of mTOR and p70 S6K in KBM-5 leukemia cells. Phytother Res 2014;28(3):458–64.
[32] Mattiuzzi C, Lippi G. Current cancer epidemiology. J Epidemiol Glob Health 2019;9(4):217–22.
[33] Feng RM, Zong YN, Cao SM, Xu RH. Current cancer situation in China: good or bad news from the 2018 global cancer statistics? Cancer Commun 2019;39(1):1–12.
[34] Denton D, Kumar S. Autophagy-dependent cell death. Cell Death Differ 2019;26:605–16.
[35] Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol 2008;9:1004–10.
[36] Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 2007;8:741–52.
[37] Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the nomenclature committee on cell death 2009. Cell Death Differ 2009;16:3–11.
[38] Longo DL, Mizushima N, Levine B. Autophagy in human diseases. N Engl J Med 2020;383:1564–76.
[39] Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. Science 2004;306(5698):990–5.
[40] Shen S, Kepp O, Michaud M, Martins I, Minoux H, Métivier D, et al. Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. Oncogene 2011;30:4544–56.
[41] Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, et al. Molecular definitions of cell death subroutines: recommendations of the nomenclature committee on cell death 2012. Cell Death Differ 2012;19:107–20.
[42] Luciani MF, Giusti C, Harms B, Oshima Y, Kikuchi H, Kubohara Y, et al. Atg1 allows second-signaled autophagic cell death in *Dictyostelium*. Autophagy 2011;7(5):501–8.
[43] Tasdemir E, Galluzzi L, Maiuri MC, Criollo A, Vitale I, Hangen E, et al. Methods for assessing autophagy and autophagic cell death. Methods Mol Biol 2008;445:29–76.
[44] Kocaturk NM, Akkoc Y, Kig C, Bayraktar O, Gozuacik D, Kutlu O. Autophagy as a molecular target for cancer treatment. Eur J Pharm Sci 2019;134:116–37.
[45] Burikhanov R, Hebbar N, Noothi SK, Shukla N, Sledziona J, Araujo N, et al. Chloroquine-inducible Par-4 secretion is essential for tumor cell apoptosis and inhibition of metastasis. Cell Rep 2017;18(2):508–19.
[46] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
[47] Yamamoto K, Venida A, Yano J, Biancur DE, Kakiuchi M, Gupta S, et al. Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. Nature 2020;581:100–5.
[48] Sun M, Hu K, Yang X, Chen Q, Liu Y, Zhou T, et al. Effect and metabolism of high-dose Danshen and Chuanxiong on tumor invasive and metastasis in Lewis lung cancer mouse. World J Integr Tradit West Med 2018;13:4. Chinese.
[49] Ding G, Song MZ, Yu EX. Study on the mechanism of Danshen, Chishao affecting walker 256 liver metastasis in experimental rats. Chin Oncol 2001;11(4):364–6. Chinese.
[50] Cai W, Xu G, Hu Y, Gu X, Yan J, Qin D, et al. Long-term results of combination of radiation with destagnation in the treatment of nasopharyngeal carcinoma (NPC)—a prospective randomized trial on 188 cases. Chin J Radiat Oncol 1992;1(1):8–10. Chinese.
[51] Wang HZ, Liang XY, Leng X, An J, Wu DL, Qiu SJ. The effect of blood-activating and stasis-removing Chinese medicine on the changes of white matter microstructure of patients with nasopharyngeal carcinoma after radiation therapy: an DTI-TBSS study. J Clin Radiol 2020;39(7):1273–7. Chinese.
[52] Zang W, Bian H, Huang X, Yin G, Zhang C, Han LI, et al. Traditional Chinese medicine (TCM) *Astragalus membranaceus* and *Curcuma wenyujin* promote vascular normalization in tumor-derived endothelial cells of human hepatocellular carcinoma. Anticancer Res 2019;39(6):2739–47.
[53] Wang WQ, Liu L, Sun HC, Fu YL, Xu HX, Chai ZT, et al. Tanshinone IIA inhibits metastasis after palliative resection of hepatocellular carcinoma and prolongs survival in part via vascular normalization. J Hematol Oncol 2012;5:69.
[54] Zhang Y, Tie MH, Bi F, Wang K. Tanshinone II improves distribution and anti-tumor efficacy of pegylated liposomal doxorubicin via normalizing the structure and function of tumor vasculature in Hepa1-6 hepatoma mice model. J Tradit Chin Med 2018;38(6):815–22.
[55] Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, et al. Regulation of an ATG7-Beclin 1 program of autophagic cell death by caspase-8. Science 2004;304(5676):1500–2.
[56] Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ 2011;18:571–80.

[57] Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 2005;122 (6):927–39.
[58] Xu HD, Qin ZH. Beclin 1, Bcl-2 and autophagy. Adv Exp Med Biol 2019;1206:109–26.
[59] Li P, Shi M, Maique J, Shaffer J, Yan S, Moe OW, et al. Beclin 1/Bcl-2 complex-dependent autophagy activity modulates renal susceptibility to ischemia-reperfusion injury and mediates renoprotection by klotho. Am J Physiol Renal Physiol 2020;318(3):F772–92.
[60] Toton E, Lisiak N, Sawicka P, Rybczynska M. Beclin-1 and its role as a target for anticancer therapy. J Physiol Pharmacol 2014;65(4):459–67.
[61] Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat Cell Biol 2007;9:1142–51.