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ORIGINAL ARTICLE

Influence of Tanshinone IIA on apoptosis of human esophageal carcinoma Eca-109 cells and its molecular mechanism

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Abstract

Background: Previous studies have shown that Tanshinone (Tan) IIA exerts obvious antitumor efficacy; however, its molecular mechanism remains unclear. This study was conducted to identify the influence of Tan IIA on Eca-109 cell apoptosis, explore its molecular mechanism, and provide a theoretical basis for clinical application.

Methods: Eca-109 cells were cultured in vitro and treated with different concentrations of Tan IIA. Morphologic changes were viewed under inverted fluorescence microscope with dual acridine orange/ethidium bromide staining assay. Methyl-thiazolyl-tetrazolium and Annexin V propidium iodide assays were respectively used to measure cell viability and apoptosis rate. The protein and messenger (m)RNA expression of binding immunoglobulin protein (BIP), mitochondrial cytochrome c (CytC), and caspase-9 were detected by Western blot and quantitative real-time PCR, respectively.

Results: Cell viability decreased and the apoptosis rate significantly increased with increasing concentrations of Tan IIA (0, 20, 40, 60 μg/mL), which indicated that Tan IIA inhibited the proliferation and induced the apoptosis of Eca-109 cells in a dose-dependent manner. Eca-109 cells treated with 60 μg/mLTan IIA showed typical morphological changes of apoptosis under the inverted microscope. Moreover, compared with the negative control group, protein and mRNA expression of BIP decreased significantly ($P < 0.05$), whereas protein and mRNA expression of CytC and caspase-9 increased significantly ($P < 0.05$).

Conclusion: Tan IIA can induce apoptosis in human esophageal carcinoma Eca-109 cells by regulating BIP, CytC, and caspase-9 expression. Endoplasmic reticulum stress and mitochondrial-dependent may be involved in Tan IIA-induced Eca-109 cell apoptosis.

Introduction

Esophageal carcinoma, which is characterized by high morbidity, invasion, metastasis, and poor prognosis, is a great threat to human health and life.[1] Esophageal squamous cell carcinoma in particular, the fourth most commonly diagnosed cancer, can resist a combination of surgery, chemotherapy, and radiotherapy, and is the third leading cause of cancer death in China.[2] Thus, determining a new alternative targeted at esophageal carcinoma to achieve the desired clinical therapeutic effect is urgently required. Traditional Chinese medicine, especially some Chinese herb extracts, can induce tumor cell apoptosis, exert remarkable anti-tumor effects, and offer low toxicity, high efficacy, and low cost and have thus become an advanced research hotspot.

Tanshinone (Tan) IIA, an active ingredient extracted from Danshen, a traditional Chinese medicine for the treatment of cardiovascular diseases and chronic hepatitis, has been reported to exert anti-neoplastic, anti-oxidative, anti-cancer, and anti-inflammatory functions and has proven effective for the prevention of angina pectoris and

myocardial infarction.[3,4] Although Tan IIA has been documented to inhibit the proliferation and induce apoptosis of gastric cancer SGC7901 cells and human breast cancer MDA-MB-231 cells, its effect on human esophageal carcinoma Eca-109 cells remains largely unknown.[5,6] The present study is designed to observe the proliferation and apoptosis effect of Tan IIA on Eca-109 cells, analyze the expression of binding immunoglobulin protein (BIP), mitochondrial cytochrome c (CytC), and caspase-9, and to further investigate the anti-cancer effect and molecular mechanisms of Tan IIA.

Methods

Cell culture and reagents

Human esophageal carcinoma Eca-109 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin under standard conditions (37°C, 5% CO_2).

Methyl-thiazolyl-tetrazolium assay of cell proliferation

Eca-109 cells were plated onto 96-well plates at a density of 2 × 103 cells/well. After incubation overnight, cells were treated with fluorouracil (5-FU, 15 mmol/L) (Xudong Haipu Pharmaceutical Co., Ltd., Shanghai, China) and various concentrations of Tan IIA (20, 40, 60 μg/mL) (Institute for Drug and Biological Product Control, Beijing, China) for 24, 48, and 72 hours. The medium was then exchanged with serum-free medium containing 0.5 mg/mL of methyl-thiazolyl-tetrazolium (MTT) (Sigma-Aldrich, St. Louis, MO, USA), and removed after four hours at 37°C. Then, 150 μL dimethyl sulfoxide was added to each well and shaken for 15 minutes. The optical density (OD) value was determined at 490 nm using an enzyme micro-plate reader. The relative percentage of cell viability was calculated using the following formula: Proliferation rate (%) = (OD test – OD blank) × 100, where OD test and OD blank are the optical density of the test substances and the blank control, respectively.

Morphological observation of cell apoptosis

After treatment with 60 μg/mL Tan IIA for 72 hours, the morphological changes in Eca-109 cells were observed under an inverted microscope (Toshiba, Tokyo, Japan) with dual acridine orange/ethidium bromide double staining.

Detection of cell apoptosis by Annexin V propidium iodide

The operations were carried out according to manufacturer instructions (BD Biosciences, San Diego, CA, USA). The cells were washed with phosphate buffered saline, trypsinized for 2–3 minutes, and then moved to a centrifugal tube with the medium added. After thorough mixing the cells were then centrifuged (1000 r/minutes, 4 minutes) and washed twice with phosphate buffered saline. The cell concentration was then adjusted to 10^6/mL. Further, 0.5 mL of the cell suspension was taken out and centrifuged (1000 r/minutes, 4 minutes). After removing the supernatant, 0.5 mL binding buffer and 1 μL fluorescein isothiocyanate-labeled Annexin V (Roche Applied Science, Basel, Switzerland) was added and the cells were incubated for 20 minutes at room temperature. After incubation, the cells were stained with 5 μL propidium iodide for 5 minutes in the dark at 4°C. Finally, the cell apoptotic rate was measured by flow cytometry and the results were analyzed using the Cell Quest program (Becton Dickinson, Bellport, NY, USA).

Binding immunoglobulin protein (BIP), cytochrome c (CytC), and caspase-9 expression

Total protein lysate containing protease and phosphatase inhibitors was used to extract the total protein in Eca-109 cells. The protein concentration of the supernatant was determined using a bicinchoninic acid protein concentration assay kit (Pierce, Thermo Scientific, Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by loading 30 μg protein per lane. The gels were then transferred to polyvinylidene fluoride membrane and blocked with 5% nonfat milk for 2 hours at room temperature. The proteins were incubated at 4°C overnight with rabbit anti-human BIP, CytC, and caspase-9 antibodies (1:2000 dilutions, Abcam, Cambridge, UK). The secondary antibodies conjugated with horseradish peroxidase goat anti-rabbit immunoglobulin G (1:1000 dilutions, Cell Signaling Technologies, Danvers, MA, USA) were then added and incubated for one hour at room temperature, with gentle shaking. Finally, chemiluminescence was adopted to detect all polyvinylidene fluoride membranes and the results were quantified using Image-Pro Plus 6.0 software (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative real-time-PCR analysis of BIP, CytC and caspase-9 expression

Total RNA was extracted following the manufacturer's instructions (Ambio, Foster, CA, USA), and single-strand

cDNA was synthesized via reverse transcription. The primer sequences for PCR amplification were as follows: BIP (sense: 5′-GAACACAGTGGTGCCTACCAAGAA-3′, antisense: 5′-TCCAGTCAGATCAAATGTACCCAGA-3′, product length 142bp); CytC (sense: 5′-TTGCACTTA-CACCGGTACTTAAGC-3′, antisense: 5′-ACGTCCCCAC TCTCTAAGTCCAA- 3′, product length 62 bp); caspase-9 (sense: 5′- CTGCGAACTAACAGGCAAGC -3′, antisense: 5′- CTAGATATGGCGTCCAGCTG - 3′, product length 286 bp); andβ-actin (sense: 5′-CCGTCTTCCCCTCCA TCG-3′, antisense: 5′-GTCCCAGTTGGTGACGATGC-3′, product length 155 bp). The PCR reaction system was 20 μL: 2 × UltraSYBR mixture 10 μL, forward primer 0.5 μL, reverse primer 0.5 μL, RNase-Free Water 7 μL, and cDNA 2 μL. The reaction conditions were: 95°Cinitial denaturation 10 minutes, 95°Cdenaturation 15 s, and 60°Cannealing/extension one minute. The whole reaction consisted of 40 cycles. Finally, relative quantification of BIP, CytC and caspase-9 messenger (m)RNA expression was analyzed according to standardized β-actin content adopting the method of $2^{-\Delta\Delta CT}$.

Statistical analysis

Data were expressed as mean ± standard deviation (SD), and were analyzed with one-way analysis of variance. Differences between groups were considered to be statistically significant at $P < 0.05$. Statistical analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Proliferation inhibition effect of Tan IIA on Eca-109 cells

The MTT method was adopted to detect the influence of Tan IIA on cell proliferation. After Eca-109 cells were treated with different concentrations of Tan IIA (20, 40, 60 μg/mL) for 72 hours, cell viability gradually decreased: (39.98 ± 2.60)%, (25.41 ± 1.12)%, and (11.55 ± 3.36)%, respectively. Moreover, when the cells were treated with 60 μg/mL Tan IIA for different time lengths (24, 48, 72 hours), cell viability also gradually decreased: (35.61 ± 2.37)%, (23.47 ± 5.28)%, and (11.55 ± 3.36)%, respectively. The differences between groups were statistically significant ($P < 0.05$) (Fig 1). These results revealed that Tan-IIA inhibited Eca-109 cell proliferation in a time and dose-dependent manner. Meanwhile, cell viability in the 60 μg/mL Tan IIA group was lower than in the positive control group (5-FU group), indicating that 60 μg/mL Tan IIA exerted a better proliferation inhibition effect on Eca-109 cells (Fig 1).

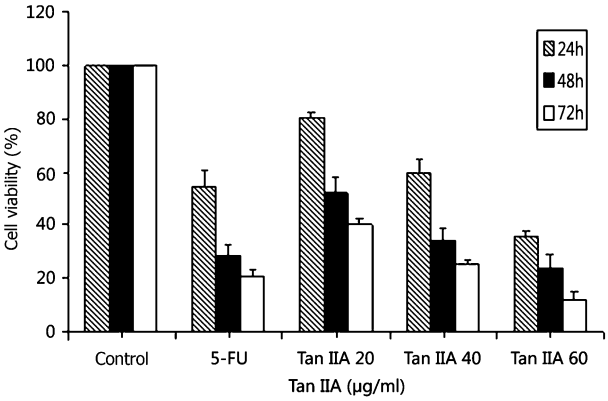


Figure 1 Effect of Tanshinone (Tan) IIA on the inhibition of Eca-109 cell proliferation. Methyl-thiazolyl-tetrazolium assay was used to determine cell viability. Data are represented as the mean ± standard deviation of three independent experiments. Differences between groups were statistically significant ($P < 0.05$).

Morphological observation of cell apoptosis

Untreated Eca-109 cells were dyed green and had a smooth and intact membrane, while Eca-109 cells treated with Tan IIA underwent a range of apoptosis, with characteristic membrane blebbing (bubble formation) in dying cells (Fig 2).

Tan IIA induction of apoptosis in Eca-109 cells

To confirm the effect of Tan IIA on apoptosis, we analyzed Eca-109 cell apoptosis with a flow cytometer. Obvious cell apoptosis was observed in Eca-109 cells treated with Tan IIA for 72 hours, and the apoptosis rate was significantly elevated with the increase of Tan IIA concentration. The apoptosis rates were (4.61 ± 1.13)%, (9.51 ± 1.04)%, and (13.40 ± 2.06)% after treatment with Tan IIA at 20, 40, and 60 μg/mL for 72 hours, respectively (Fig 3), and the differences between groups were statistically significant ($P < 0.05$). These results indicated that Tan IIA can induce Eca-109 cell apoptosis in a dose-dependent manner. Moreover, there was no significant difference between the 5-FU and 60 μg/mL Tan IIA groups (Fig 3).

BIP, CytC, and caspase-9 protein expression

To explore the molecular mechanism of Tan IIA-induced Eca-109 cell apoptosis, we analyzed BIP, CytC, and caspase-9 protein expression involved in cell apoptosis. Western blot analysis showed that with an increase in

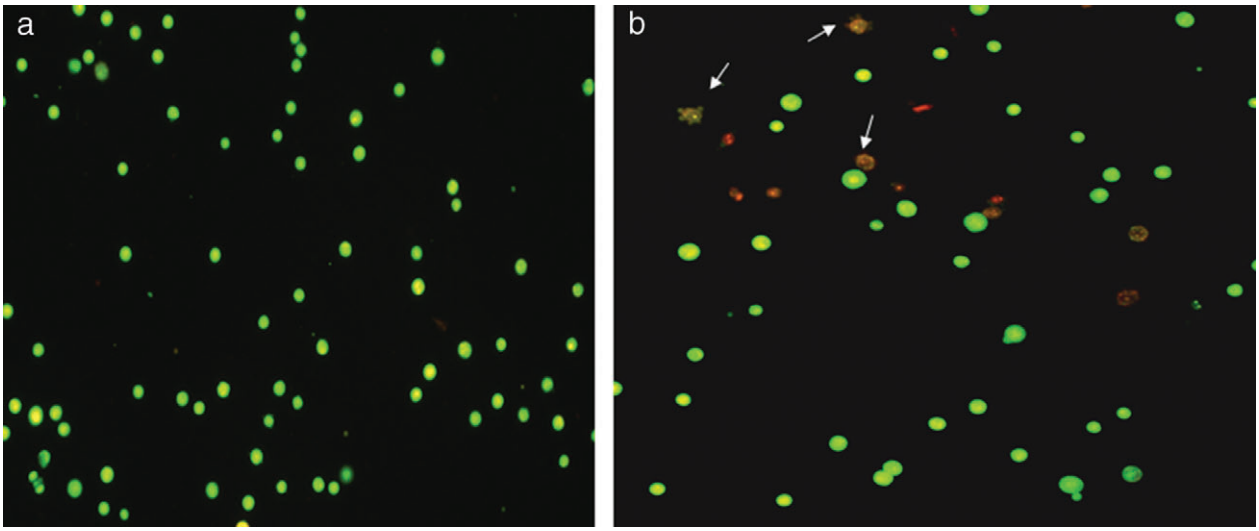


Figure 2 Morphological observation of Eca-109 cell apoptosis induced by Tanshinone (Tan) IIA (×200). (**a**) Untreated, (**b**) 60 μg/mL Tan IIA treatment for 72 hours. The arrows indicate thatEca-109 cells underwent a range of apoptosis, with characteristic membrane blebbing (bubble formation).

concentration of Tan IIA, the expression levels of CytC and caspase-9 gradually increased, while the expression levels of BIP gradually decreased, compared with the negative control group ($P < 0.05$) (Fig 4). This suggested that Tan IIA regulates Eca-109 cell apoptosis via upregulating CytC and caspase-9 expression, and downregulating BIP expression. Furthermore, there was no significant difference between 5-FU and different concentrations of Tan IIA (20, 40, 60 μg/mL) in term of BIP and CytC protein expression ($P > 0.05$). Nevertheless, there was a significant difference in caspase-9 expression between Tan IIA and 5-FU groups ($P < 0.05$) (Fig 4). This implies that Tan IIA and 5-FU had the same regulatory effect on BIP and CytC protein expression.

Messenger RNA expression of BIP, CytC, and caspase-9

To further confirm the effect of Tan IIA on the induction of Eca-109 cell apoptosis, we also analyzed BIP, CytC, and caspase-9 mRNA expression. As expected, compared with the negative control group, Tan IIA upregulated mRNA levels of CytC and caspase-9 and downregulated mRNA levels of BIP ($P < 0.05$) (Fig 5). These results are consistent with our Western blot results, revealing that Tan IIA can induce Eca-109 cell apoptosis via the upregulation of CytC expression and downregulation of BIP expression. However, no significant differences were observed between the 5-FU and Tan IIA groups (20, 40, 60 μg/mL) in terms of BIP and CytC mRNA expression ($P < 0.05$) (Fig 5).

Discussion

Apoptosis, or programmed cell death, is a physiological process that plays a pivotal role in maintaining tissue homeostasis, killing unwanted cells without inducing immune responses or inflammatory reactions.[7] This process is precisely regulated by multiple intrinsic and extrinsic aspects, such as immune response, gene regulation, and signal transduction.[8] Disorders of apoptosis regulation may result in apoptosis escape, tumorigenesis, and other pathological changes.[9] Therefore, inducing tumor cell apoptosis and inhibiting its proliferation represents a new anti-tumor therapy for clinical application.

Tan IIA, a traditional Chinese medicine extract, has shown unique advantages by improving symptoms, enhancing life quality, and prolonging survival period in patients with tumors. Most importantly, it can induce tumor cell apoptosis and, thus, exert a remarkable anti-tumor efficacy.[10] In our study, MTT assay and flow cytometry analysis displayed that after treatment with 20, 40, and 60 μg/mL Tan IIA for 72 hours, cell viability decreased by $(39.98 \pm 2.60)\%$, $(25.41 \pm 1.12)\%$, and $(11.55 \pm 3.36)\%$, respectively, while the cell apoptosis rate increased by $(4.61 \pm 1.13)\%$, $(9.51 \pm 1.04)\%$, and $(13.40 \pm 2.06)\%$, respectively. The differences between groups were statistically significant ($P < 0.05$) (Figs 1,3). These results revealed that Tan IIA can inhibit proliferation and induce apoptosis of Eca-109 cells in a dose-dependent manner. To confirm that apoptotic cells were emerging in the experiments, we observed the morphological changes of these cells under an inverted microscope. The results displayed characteristic changes of apoptosis, such as a reduction in cell numbers, membrane blebbing (bubble formation), and the

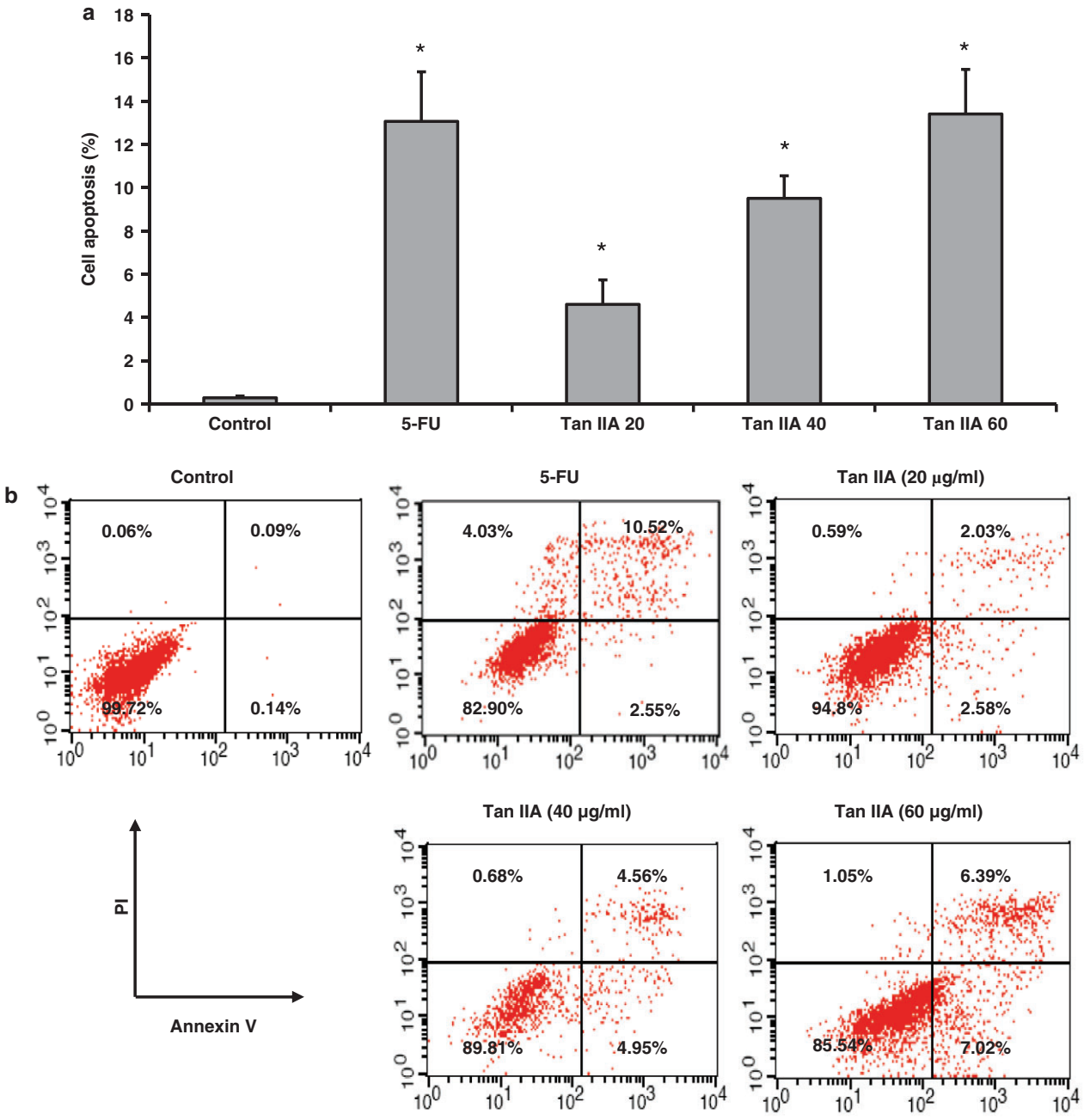


Figure 3 Flow cytometry assay of Eca-109 cell apoptosis. (**a**) Cell apoptosis rate, (**b**) Eca-109 cell apoptosis rate displayed by flow cytometer. The 5-fluorouracil (FU) group was used as the positive control. The cell apoptosis rate gradually elevated with an increase in Tan IIA concentration (20, 40, 60 μg/mL). Differences between groups were statistically significant. *$P < 0.05$, compared with the negative control. PI, propidium iodide.

occurrence of dyed orange (Fig 2), indicating that Tan IIA induces Eca-109 cell apoptosis.

The molecular mechanisms and signaling pathways involved in cell apoptosis are very complicated, including endoplasmic reticulum stress (ERS), mitochondria-dependent, and death receptor pathways. The mitochondria-dependent and ERS pathways are most widely studied. The related genes and proteins, such as CytC and BIP are of vital importance.

The mitochondria-independent pathway plays an important role in the regulation of cell apoptosis. Apoptosis-inducing factors, such as oxidative stress, DNA damage, X-ray, and chemotherapy drugs can either directly or through the B-cell lymphoma family open the mitochondrial

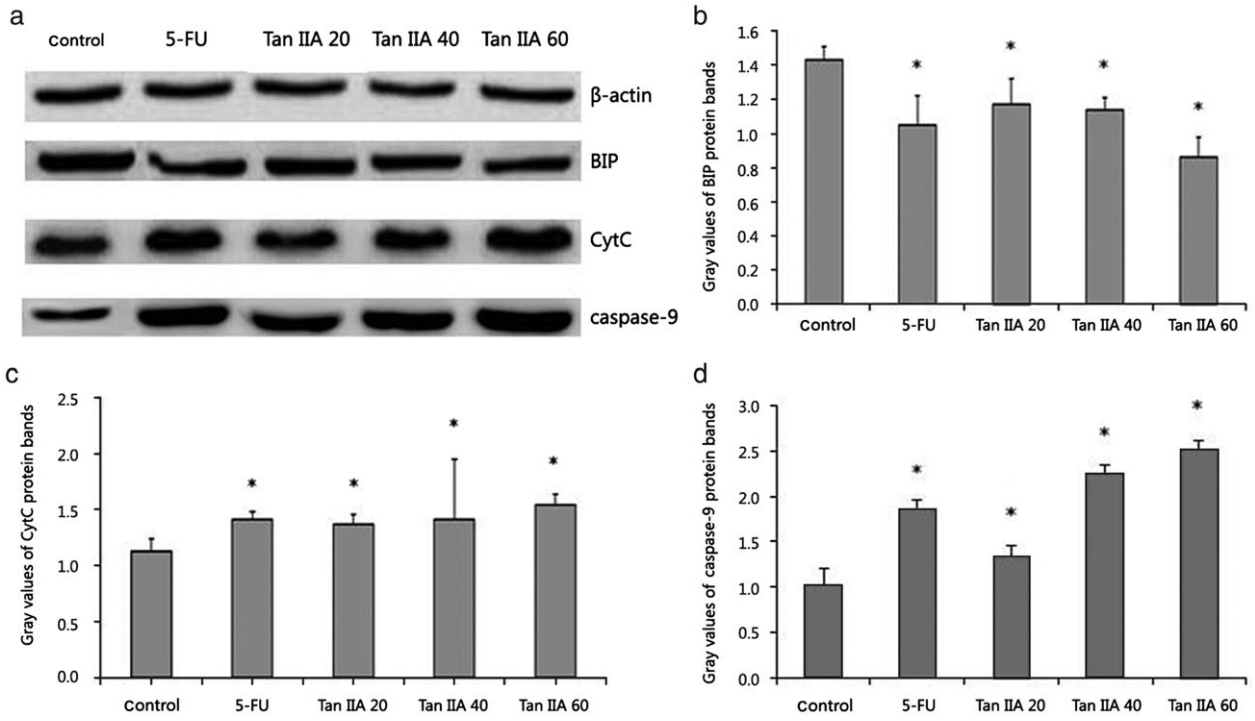
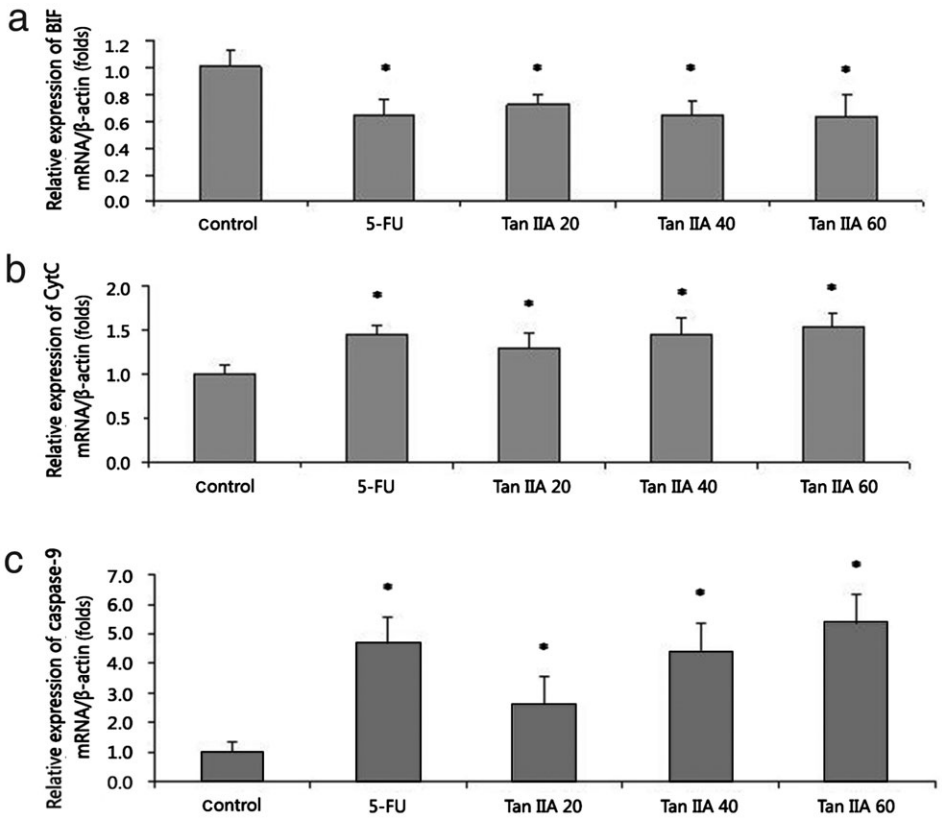


Figure 4 Western blot analysis. (**a**) Protein bands. Gray values of: (**b**) binding immunoglobulin protein (BIP), (**c**) cytochrome c (CytC), and (**d**) caspase-9 protein bands. β-actin was used as the internal standard and the 5-fluorouracil (FU) group was used as the positive control. Bar graphs were constructed according to the gray values of the protein bands. $*P < 0.05$, compared with the negative control.

Figure 5 Quantitative real-time PCR analysis of messenger (m) RNA levels. Relative expression of: (**a**) binding immunoglobulin protein (BIP) mRNA, (**b**) cytochrome c (CytC) mRNA, and (**c**) caspase-9 mRNA. mRNA levels were analyzed by $2^{-\bigtriangledown\bigtriangledown Ct}$ method. β-actin was used as the internal standard and the 5-fluorouracil (FU) group was used as the positive control. $*P < 0.05$, compared with the negative control.



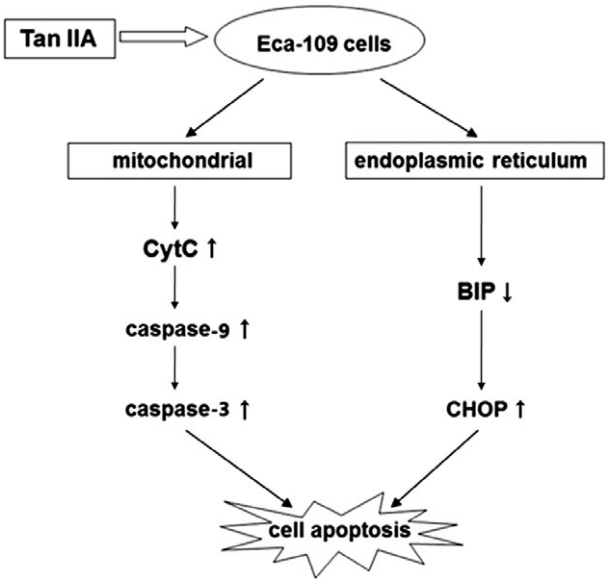


Figure 6 Proposed model of mitochondria-dependent and endoplasmic reticulum stress pathways in Tanshinone (Tan) IIA-induced Eca-109 cell apoptosis. BIP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; CytC cytochrome c.

permeability transition pore. This results in CytC releasing into the cytoplasm where CytC binds to Apaf-1 and procaspase-9, thereby activating caspases and inducing cell apoptosis.[11] CytC release and overexpression has been investigated by various studies, as tumor cell apoptosis is induced by different drugs.[12] Our study also displayed CytC overexpression after Eca cells were treated with Tan IIA (20, 40, 60 μg/mL) for 72 hours (Figs 4–5). These results are consistent with findings by other researchers, implying that CytC overexpression most probably participates in Tan IIA-induced Eca-109 cell apoptosis.[13] It is well known that caspase activation is generally considered a key characteristic of apoptosis. Because caspase-9 activation is regarded as the earliest event in the caspase activation cascade, we also analyzed caspase-9 expression.[14] Our results showed that caspase-9 expression also increased (Figs 4–5). Active caspase-9 in turn activates caspase-3, which can cleave poly (ADP-ribose) polymerase, finally leading to apoptosis.[15,16] Therefore, it seems likely that mitochondrial CytC release induced by Tan IIA results in caspase-9 and caspase-3 activation, and a classical mitochondria-independent pathway participates in Tan IIA-induced Eca-109 cell apoptosis (Fig 6).

The endoplasmic reticulum (ER) is a eukaryotic organelle that has many functions, including protein synthesis formation and transport, cell calcium storage, post-translational modifications, membrane synthesis and fold, and metabolism regulation.[17] Various ERS stimuli, such as oxidative stress, the accumulation of unfolded or misfolded proteins, and viral infection, can disturb cell homeostasis. BIP, the main molecular chaperone in ER, can trigger and activate an unfolded protein response (UPR) to restore cell homeostasis and enhance cell survival. However, if ERS is so severe that cell homeostasis is hardly restored, UPR will induce cell apoptosis by activating downstream signaling pathway molecules, such as C/EBP homologous protein (CHOP), caspase-12, and so on.[18] It has been reported that CHOP overexpression is involved in the molecular mechanism of Tan IIA-induced Eca-109 cell apoptosis.[19] In our study, BIP expression decreased after Eca-109 cells were treated with Tan IIA (20, 40, 60 μg/mL) for 72 hours (Figs 4–5). Increasing studies have demonstrated that BIP overexpression is linked with the occurrence, invasion, and metastases of many tumors, including esophageal carcinoma, which suggests that a decrease in BIP expression may lead to the inhibition of tumor cell proliferation and the induction of tumor cell apoptosis.[20,21] Accordingly, Tan-IIA can induce Eca-109 cell apoptosis by decreasing BIP expression and then activating CHOP, and the ERS pathways may play an important role in Tan-IIA-induced Eca-109 cell apoptosis (Fig 6).[22,23]

In summary, our results suggest that Tan IIA can significantly induce apoptosis and inhibit the proliferation of human esophageal carcinoma Eca-109 cells in-vitro via the upregulation of CytC and caspase-9 expression and down-regulation of BIP expression. Both mitochondria-independent and ERS pathways may participate in the molecular mechanism of Eca-109 cell apoptosis, thus providing support for the application of Tan IIA for treatment of esophageal carcinoma. In addition, some studies have reported that there might be cross-talk between these two pathways;[24,25] however, the detailed mechanism still requires further study. Our study provides a basis for further clarification of the apoptosis mechanism and exploration of the anti-tumor therapy effects of traditional Chinese medicine.

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Disclosure

No authors report any conflict of interest.

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