Tanshinone IIA inhibits proliferation and induces apoptosis of human nasopharyngeal carcinoma cells via p53-cyclin B1/CDC2

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Abstract. Tanshinone IIA exhibits natural antioxidative and antineoplastic activity. However, to the best of our knowledge, the effects of tanshinone IIA on human nasopharyngeal carcinoma cells remains unknown. The present study aimed to investigate whether tanshinone IIA inhibits proliferation and induces apoptosis of human nasopharyngeal carcinoma cells via p53-cyclin B1/cell division cycle gene 2 (CDC2). Cell proliferation, cytotoxicity and apoptosis of 13-9B cells were evaluated by an MTT assay, lactate dehydrogenase assay and flow cytometry, respectively. ELISA and western blot analysis were used to analyze caspase-3 activity and poly (ADP-ribose) polymerase (PARP), p53, cyclin B1 and CDC2 protein expression in 13-9B cells. Treatment of 13-9B cells with tanshinone IIA significantly suppressed cell proliferation and significantly induced cytotoxicity and apoptosis of 13-9B cells. Furthermore, tanshinone IIA significantly increased caspase-3 activity, and significantly increased the protein expression levels of PARP, p53, cyclin B1 and CDC2 in 13-9B cells. In summary, the current results indicate that tanshinone IIA inhibits proliferation and induces apoptosis of human nasopharyngeal carcinoma cells via PARP, p53, cyclin B1/CDC2 and caspase-3-mediated signaling.

Introduction

The basic etiology and pathogenesis of nasopharyngeal carcinoma is a low hemoglobin level, exhaustion and coagulation of phlegm, and the incidence rate is 10-30 cases per 100,000 in the general population (1,2). The key to the recurrence and metastasis of cancer is that immune cells fail to suppress cancer cells (3). NPC is curable when diagnosed early and a 5-year survival as high as 90% may be achieved (4). Once cancer cells are generated, the body struggles to eliminate them (4). During pre-surgical resection of cancer or during the early stage of the disease, there may be micrometastasis (5). Following surgical resection and radiotherapy, the micrometastasis can spread and survive in the lymph system, blood circulation, bone marrow, liver, lungs and other tissues and organs (6). Micrometastasis often has no obvious clinical symptoms; however, the residual cancer cells can be the key prerequisites for the recurrence and metastasis of tumors in the clinic (6).

The p53 gene has been identified to be closely associated with several types of human cancer, such as liver and lung cancer, as well as nasopharyngeal carcinoma (7). The p53 gene was first discovered in 1979 and it has since been demonstrated to serve a number of different roles, including as an oncogene and a tumor suppressor gene (8). The p53 gene and the protein it encodes are associated with cell cycle regulation, cell growth and apoptosis, which are regulators of cell division (8). Overexpression of p53 can induce apoptosis of human cancer cells (9). Following specific inhibition of caspase-8 and caspase-9, p53 can also be inhibited, which indicates that p53 serves a role in death-receptor-mediated and mitochondria-mediated apoptosis (10). Stable expression of the p53 protein is crucial for the completion of its various functions.

Cyclin Bl serves an important regulatory role in the G2/M stage of the cell cycle (11). It has been reported that cell division cycle gene 2 (CDC2) and cyclin B1 function together in eukaryotes. CDC2/cyclin B1 serves a role at the G2/M phase of the cell cycle (12). Furthermore, CDC2/cyclin B1 can accelerate the mitosis of cells, which is a process that is mediated by phosphorylation and requires numerous different factors (13). The kinase activity of CDC2/cyclin B1 promotes mitosis via the G2/M phase (13).

Tanshinone IIA is a composition of active monomers extracted from the traditional Chinese plant, *Salvia miltiorrhiza*, which is commonly used for the treatment of patients with

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cardiovascular disease, cerebrovascular disease or hepatitis (14). Modern medicine has demonstrated that the main effects of *S. miltiorrhiza* include dilation of blood vessels and the improvement of microcirculation (15). In recent years, a number of studies have focused on the use of traditional Chinese medicines for the treatment of tumors (15-17). Tanshinone IIA has been studied due to its potential antineoplastic activity. Studies have demonstrated that tanshinone IIA exhibits a specific cytotoxic effect on leukemia, hepatocellular carcinoma and breast cancer cells (16,17). However, to the best of our knowledge, the effects of tanshinone IIA on human nasopharyngeal carcinoma cells remain unclear. The present study was designed to investigate the anti-cancer effects of tanshinone IIA, particularly the potential inhibition of proliferation and promotion of apoptosis of human nasopharyngeal carcinoma cells. In addition, the possible underlying mechanism was discussed.

Materials and methods

Cell culture and reagents. The human nasopharyngeal carcinoma cell line 13-9B was purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 complete medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO_2 at 37˚C. Tanshinone IIA [≥97% (HPLC); Fig. 1] was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and dissolved in DMSO to a concentration of 0, 5, 10, 20 or 25 μg/ml.

MTT assay. 13-9B cells ($1x10^3$ cells/well) were seeded in 96-well culture plates containing 0, 5, 10, 20 or 25 μg/ml tanshinone IIA with 100 μl growth medium and cultured for 24, 48 and 72 h at 37˚C. Subsequently, 20 μl MTT (Sigma-Aldrich; Merck KGaA) was added to each well and incubated at 37˚C for 4 h. DMSO (20 μl) was added to terminate the reaction and the absorbance was measured at 490 nm using an automatic microplate reader.

Lactate dehydrogenase (LDH) assay. 13-9B cells ($1x10^3$ cells/well) were seeded in 96-well culture plates containing 0, 5, 10, 20 and 25 μg/ml tanshinone IIA with 100 μl growth medium and cultured for 24, 48 and 72 h at 37˚C. Subsequently, 100 μl LDH assay substrate (cat. no. C0017; Beyotime Institute of Biotechnology, Haimen, China) was added to each well and the cells were further incubated for 30 min. The absorbance was then measured at 490 nm using an automatic microplate reader.

Flow cytometry. 13-9B cells ($1x10^6$ cells/well) were seeded in 6-well culture plates containing 0, 5, 10 or 20 μg/ml tanshinone IIA with 2 ml growth medium and cultured for 24 and 48 h at 37˚C. Subsequently, 13-9B cells were stained with an Annexin V/propidium iodide apoptosis detection kit (Thermo Fisher Scientific, Inc.) for 15 min at room temperature. Apoptotic cells were then analyzed using a flow cytometer (FACScalibur; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and FlowJo version 7.6.1 software (FlowJo, LLC, Ashland, OR, USA).

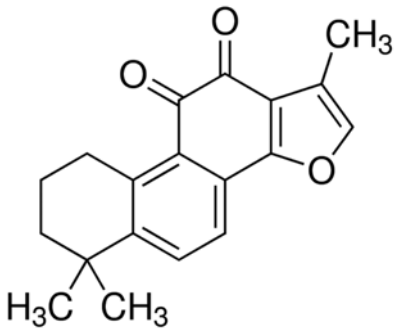

Figure 1. Chemical structure of tanshinone IIA.

Measurement of caspase-3 activity. 13-9B cells ($1x10^6$ cells/well) were seeded in 6-well culture plates containing 0, 5, 10 or 20 μg/ml tanshinone IIA with 2 ml growth medium and cultured for 48 h at 37˚C. Subsequently, 50 μg protein extract from 13-9B cells was incubated and added to a reaction buffer containing Ac-dEVd-pNA (cat. no. C1116; Beyotime Institute of Biotechnology) at 37˚C for 4 h. The absorbance was measured at 405 nm using an automatic microplate reader.

Western blot analysis. 13-9B cells ($1x10^6$ cells/well) were seeded in 6-well culture plates containing 0, 5, 10 or 20 μg/ml tanshinone IIA with 2 ml growth medium and cultured for 48 h at 37˚C. Subsequently, 13-9B cells were harvested and lysed in ice-cold RIPA buffer (Beyotime Institute of Biotechnology) containing 20 mM Tris-HCl (pH 7.5) for 5-10 min. The supernatants were collected following centrifugation at 12,000 x g for 10 minutes at 4˚C and the protein concentration was determined using a Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amount of proteins (50 μg/lane) were subjected to 10% SDS-PAGE and then electronically transferred onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). The blot was blocked with TBS and 0.1% Tween-20 containing 10% non-fat milk at room temperature for 1 h. The membranes were then incubated with diluted primary antibodies against PARP (cat. no. sc-136208; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p53 (cat. no. sc-47698; 1:500; Santa Cruz Biotechnology, Inc.), CDC2 (cat. no. sc-8395; 1:3,000; Santa Cruz Biotechnology, Inc.), cyclin B1 (cat. no. sc-245; 1:500; Santa Cruz Biotechnology, Inc.) and β-actin (cat. no. sc-8432; 1:5,000; Santa Cruz Biotechnology, Inc.) at 4˚C overnight with gentle agitation. The membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The proteins were then visualized using chemiluminescent detection reagents (Eastman Kodak Co., Rochester, NY, USA).

Statistical analysis. Data are presented as the mean ± standard deviation (N=3). Data were analyzed using SPSS v.17.0 (SPSS, Inc., Chicago, IL, USA). Student's t-test was used for the pair-wise comparisons and one-way ANOVA with Tukey's post hoc test was used for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Tanshinone IIA inhibits proliferation of human nasopharyngeal carcinoma cells. To evaluate the anti-cancer effects of tanshinone IIA on the proliferation of human nasopharyngeal carcinoma cells, an MTT assay was performed to evaluate the proliferation of 13-9B cells. The dose- and time-dependent anti-cancer effects of tanshinone IIA on the proliferation of 13-9B cells were observed (Fig. 2). Following treatment with 10, 20 and 25 μg/ml tanshinone IIA for 24, 48 and 72 h, the proliferation was significantly inhibited compared with cells treated with 0 μl/ml tanshinone IIA (P<0.01; Fig. 2).

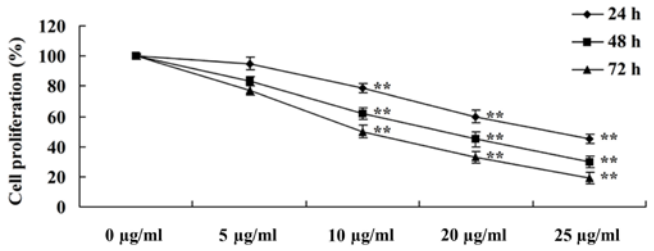

Figure 2. Tanshinone IIA inhibits cell proliferation of human nasopharyngeal carcinoma cells. A MTT assay demonstrated that treatment with 10, 20 and 25 μg/ml tanshinone IIA for 24, 48 and 72 h significantly inhibited he proliferation of 13-9B cells compared with the control cells. **P<0.01 vs. 0 μg/ml.

Tanshinone IIA increases the percentage of cytotoxic human nasopharyngeal carcinoma cells. To evaluate the cytotoxic effects of tanshinone IIA on human nasopharyngeal carcinoma cells, cytotoxic 13-9B cells were measured using an LDH assay. Following treatment with tanshinone IIA (10, 20 and 25 μg/ml) for 24, 48 and 78 h, the percentages of cytotoxic 13-9B cells on days 3, 5 and 7 were significantly increased compared with untreated cells (P<0.01; Fig. 3).

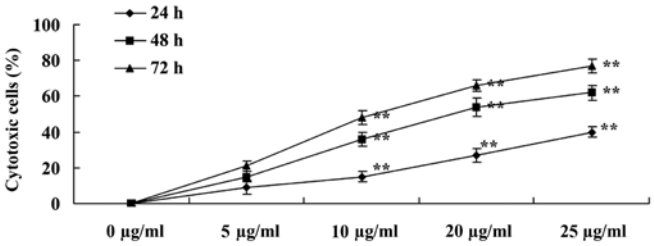

Figure 3. Tanshinone IIA increases the percentage of cytotoxic human nasopharyngeal carcinoma cells. A lactate dehydrogenase assay revealed that treatment of 13-9B cells with 0, 5, 10, 20 and 25 μg/ml tanshinone IIA for 24, 48 and 72 h significantly increased the percentage of cytotoxic cells on days 3, 5 and 7 compared with the untreated cells. **P<0.01 vs. 0 μg/ml.

Tanshinone IIA induces apoptosis of human nasopharyngeal carcinoma cells. Furthermore, to detect the anti-cancer effects of tanshinone IIA on the apoptosis of human nasopharyngeal carcinoma cells, the apoptotic rate of 13-9B cells was measured using flow cytometry. As presented in Fig. 4, the apoptotic rate of 13-9B cells was significantly increased following treatment with tanshinone IIA (10 and 20 μg/ml) for 24 and 48 h, compared with cells treated with 0 μg/ml tanshinone IIA (P<0.01).

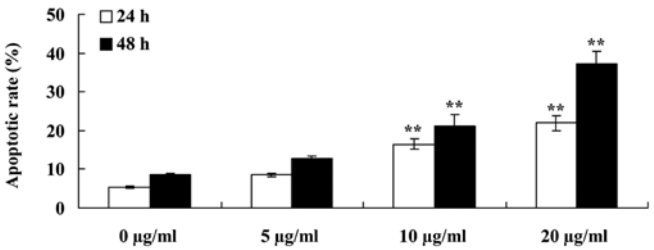

Figure 4. Tanshinone IIA inhibits apoptosis of human nasopharyngeal carcinoma cells. Flow cytometry demonstrated that the apoptotic rate of 13-9B cells significantly increased following treatment with 10 and 20 μg/ml tanshinone IIA for 24 and 48 h compared with the control untreated cells. **P<0.01 vs. 0 μg/ml.

Tanshinone IIA induces PARP and p53 protein expression in human nasopharyngeal carcinoma cells. In the present study, western blot analysis was used to determine the anti-cancer effects of tanshinone IIA on PARP and p53 protein expression in human nasopharyngeal carcinoma cells. Following exposure to different concentrations of tanshinone IIA (10 and 20 μg/ml) for 48 h, PARP and p53 protein expression in 13-9B cells was significantly increased compared with cells treated with 0 μg/ml tanshinone IIA (P<0.01; Fig. 5). However, 5 μg/ml tanshinone IIA did not significantly increase PARP and p53 protein expression levels in 13-9B cells.

Tanshinone IIA induces CDC2 and cyclin B1 protein expression in human nasopharyngeal carcinoma cells. Subsequently, the anti-cancer effects of tanshinone IIA on CDC2 and cyclin B1 protein expression in human nasopharyngeal carcinoma cells were evaluated by western blot analysis (Fig. 6). When 13-9B cells were treated with tanshinone IIA (10 and 20 μg/ml) for 48 h the CDC2 and cyclin B1 protein expression levels were significantly increased compared with cells treated with 0 μg/ml of tanshinone IIA (P<0.01; Fig. 6). However, 5 μg/ml tanshinone IIA did not significantly increase CDC2 and cyclin B1 protein expression in 13-9B cells.

Tanshinone IIA induces caspase-3 activity in human nasopharyngeal carcinoma cells. To investigate the anti-cancer effects of tanshinone IIA on caspase-3 activity in human nasopharyngeal carcinoma cells, caspase-3 activity in 13-9B cells was measured using an ELISA kit. The results indicated that caspase-3 activity in 13-9B cells was significantly increased following treatment with 10 and 20 μg/ml tanshinone IIA compared with 0 μg/ml tanshinone IIA (P<0.01; Fig. 7); the results of 5 μg/ml tanshinone IIA treatment were not statistically significant.

Discussion

Currently, the predominant clinical treatments for nasopharyngeal carcinoma are surgery, radiotherapy and chemotherapy (18). Patients who are eligible for receiving surgery typically choose surgical treatment; however, certain patients may lose pronunciation function (19). Following surgery, radiotherapy and chemotherapy can be performed according to the condition of the disease (20). Patients with advanced tumor, who lose the opportunity of receiving surgery, can select radiotherapy

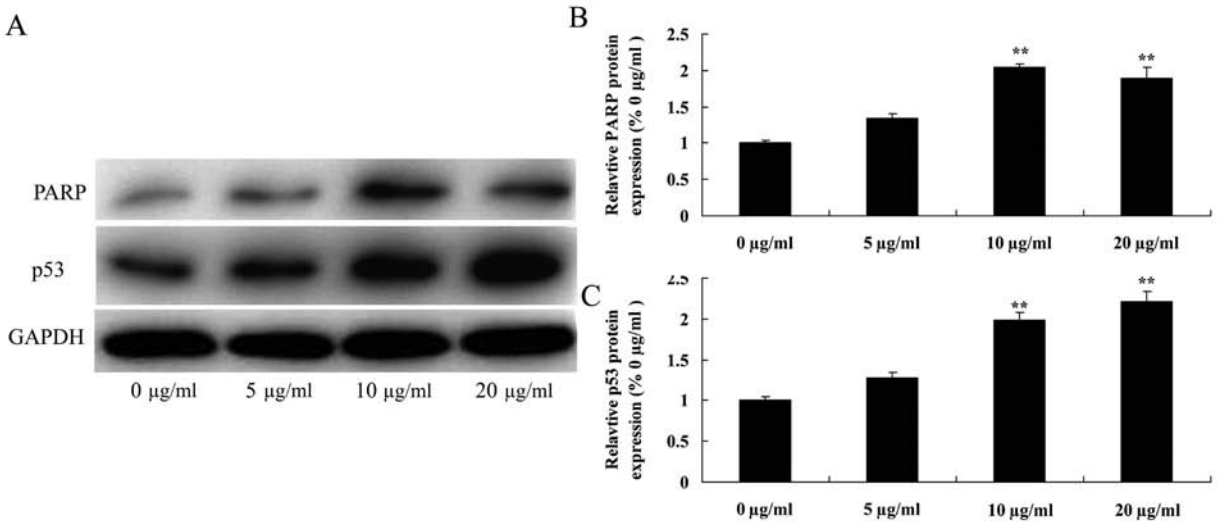

Figure 5. Tanshinone IIA inhibits PARP and p53 protein expression in human nasopharyngeal carcinoma cells. (A) The protein expression levels of PARP and p53 were measured in 13-9B cells following treatment with tanshinone IIA by western blot analysis. (B) Statistical analyses revealed that the protein expression levels of (B) PARP and (C) p53 were significantly increased in cells treated with 10 and 20 μg/ml tanshinone IIA compared with the control cells. **P<0.01 vs. 0 μg/ml. PARP, poly (ADP-ribose) polymerase.

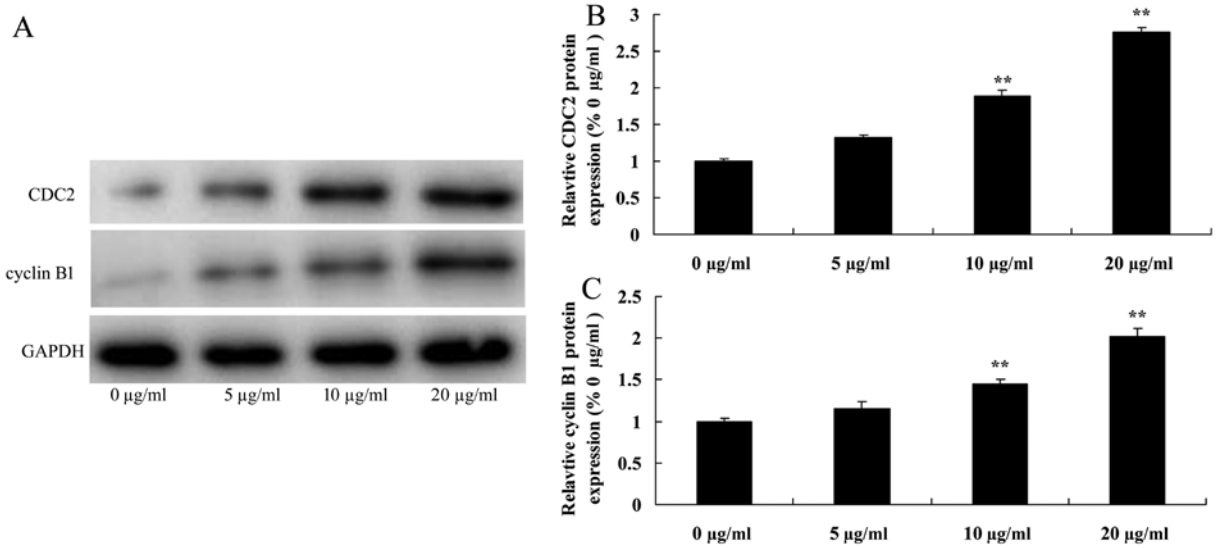

Figure 6. Tanshinone IIA inhibits CDC2 and cyclin B1 protein expression in human nasopharyngeal carcinoma cells. (A) The protein expression levels of CDC2 and cyclin B1 were measured in 13-9B cells following treatment with tanshinone IIA by western blot analysis. (B) Statistical analyses revealed that the protein expression levels of (B) CDC2 and (C) cyclin B1 were significantly increased in cells treated with 10 and 20 μg/ml tanshinone IIA compared with the control cells. **P<0.01 vs. 0 μg/ml. CDC2, cell division cycle gene 2.

and chemotherapy directly; however, a tracheostomy is often required to relieve laryngeal obstruction (19). In summary, a combined application of surgery, radiotherapy and chemotherapy demonstrates an improved treatment effect; however, these treatments may cause different degrees of throat injury, hyperemia and edema (21). Considering the present treatment options, there is as requirement to investigate Chinese medicines that may be administered to patients following surgery, radiotherapy and chemotherapy to reduce postoperative recurrence and metastasis, decrease the side effects of chemotherapy, and improve the quality of life and the survival rate (22). The present results demonstrated that tanshinone IIA could inhibit cell proliferation and induce apoptosis of human nasopharyngeal carcinoma cells.

The p53 gene and the protein it encodes are associated with cell growth and apoptosis in the regulation of cell cycle (9). Following the damage of DNA in cells, p53 induces arrest of the cell cycle and activates repair of the DNA damage in order to maintain genomic stability (23). The C-terminal of the p53 protein can detect and bind to regions of DNA damage, and regulate and activate a gene cluster that participates in repairing the DNA (24). At the same time, the p53 protein itself also exhibits exonuclease activity, which can directly serve a role in the process of DNA repair (25). If DNA damage cannot be repaired, p53 will activate the transcription of apoptotic genes, which initiates programmed cell death, also termed apoptosis. p53 protein can promote the expression of numerous apoptosis-associated genes (25). The present

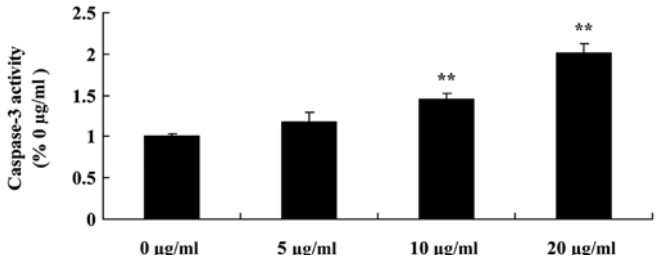

Figure 7. Tanshinone IIA inhibits caspase-3 activity in human nasopharyngeal carcinoma cells. Caspase-3 activity in 13-9B cells was significantly increased following treatment with 10 and 20 μg/ml tanshinone IIA compared with the control cells. **P<0.01 vs. 0 μg/ml.

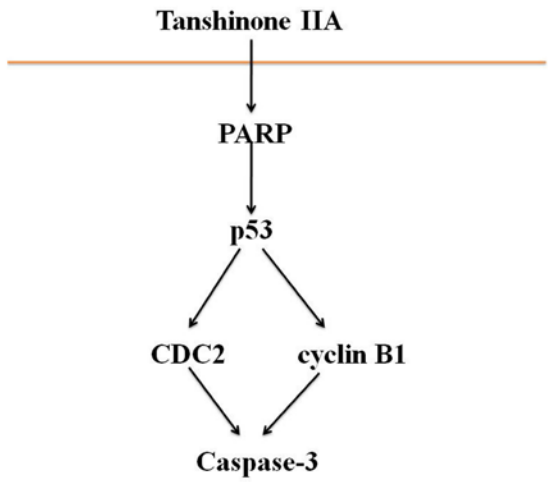

Figure 8. Tanshinone IIA may inhibit proliferation and induce apoptosis of human nasopharyngeal carcinoma cells by activation of PARP, p53, cyclin B1/CDC2 and caspase-3-mediate signaling. PARP, poly (ADP-ribose) polymerase; CDC2, cell division cycle gene 2.

results indicate that tanshinone IIA induces PARP and p53 protein expression in human nasopharyngeal carcinoma cells. Similarly, Munagala *et al* (26) suggested that tanshinone IIA induces apoptosis of cervical cancer cells via p53 and PARP.

There are six subtypes of cyclin B, of which cyclin B1 is most widely studied. Cyclin B1 is the most closely associated with tumors and has therefore received the most attention (27). Previous studies have used *in situ* hybridization and polymerase chain reaction to demonstrated that cyclin B1 serves key role in the process of yeast mitosis (28,29). Cyclin B1 can combine with CDC2 to form a complex. Once activated, this complex can initiate cells to progress from the G1/S phase to the G2/M phase (29). A previous study has reported that drugs can act on and inhibit the cyclin B1/CDC2 complex, which delays the G2/M phase and inhibits cell growth (30). In the present study, tanshinone IIA was demonstrated to induce CDC2 and cyclin B1 protein expression, and increase the activity of caspase-3 in human nasopharyngeal carcinoma cells. Similarly, Su (31) reported that tanshinone IIA inhibits gastric carcinoma AGS cells via CDC2 and cyclin B1 expression. The present study only used 13-9B cells as a model of nasopharyngeal carcinoma, which was a limitation to the study; a wider range of models needs to be used in future research.

In conclusion, the results of the present study indicate that tanshinone IIA inhibits proliferation and induces apoptosis of human nasopharyngeal carcinoma cells via activation of PARP, p53, cyclin B1/CDC2 and caspase-3-mediated signaling (Fig. 8). The present study provides experimental evidence that supports the use of tanshinone IIA in the clinical treatment of human nasopharyngeal carcinoma.

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Availability of data and materials

The data sets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LL designed the experiments. BL, AZ, ZS, HY, ZW, YS, TM and YZ performed the experiments. LL and BL analyzed the data. LL wrote the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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