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*Original Article*

# Daidzein induces apoptosis of non-small cell lung cancer cells by restoring STK4/YAP1 signaling

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**Abstract:** Aim: It was reported that daidzein (DAZ) exerted anti-cancer effects on many kinds of human cancer cells by inducing apoptosis. However, the molecular mechanisms are still vague and unclear. This study was designed to observe the inhibitory activity of DAZ on NSCLC cells and to further investigate possible mechanisms. Methods: Two NSCLC cell lines, namely A549 and H1299 were incubated with serially diluted DAZ solutions. The proliferation inhibition was assessed by MTT assay. The RNA interference technique was utilized to knockdown STK3 and STK4 respectively. Cell apoptosis was evaluated by TUNEL assay. Real-time PCR and western blotting were used to assess the expression levels of STK3, STK4, YAP1, phosphorylated YAP1 and cleaved caspase3. Results: DAZ incubation inhibited proliferation of NSCLC cells in a concentration-dependent manner by inducing apoptosis. Moreover, DAZ incubation significantly restored the expression of STK4 and further increased the phosphorylation of YAP1 which induced cell apoptosis, evidenced by elevated expression level of cleaved caspase3. However, the expression level of STK3 was unchanged by DAZ incubation. STK3 and STK4 were knocked-down by specific shRNAs. After DAZ incubation, silence of STK4 instead of STK3 impaired DAZ's apoptosis-inducing effects on NSCLC cells. Conclusions: DAZ inhibits NSCLC cells by inducing apoptosis by restoring STK4 expression and YAP1 phosphorylation.

**Keywords:** Lung cancer, daidzein, hippo pathway, apoptosis, STK4, YAP1

## Introduction

Lung cancer is not only one of the most frequent cancers but also one of the leading causes of cancer-related deaths in the world. The non-small cell lung cancer (NSCLC) and the small cell lung cancer (SCLC) are the two main pathological types of lung cancer. Approximately, NSCLC accounts for 85%-87% of lung cancers according to previous global data [1]. The prognosis of NSCLC is very poor because its 5-year survival rate is as low as 10%-15% [2]. Though there have been kinds of advanced therapeutic methods against NSCLC nowadays, the outcomes are far from satisfaction [3]. Since last few years, studies concerning natural products such as emodin, matrine [4] and xanthotoxin attracted our attentions and provided now clues for treatment of many diseases including human malignant tumors [5].

Belonged to the isoflavon group, daidzein (DAZ) is a polyphenolic product extracted from many soy-based natural plants such as *Trifolium pratense*, *Leguminosae*, and *Medicago sativa* [6]. The various biological activities including anti-proliferation, anti-fibrosis, anti-inflammation have been reported in previous studies [7-9]. It was found that as a nontoxic compound, DAZ exerted cell death- inducing effect on a variety of human cancer types [10, 11]. Evidences from both *in vivo* and *in vitro* studies indicated that DAZ could inhibit malignant cells by suppressing cell growth and inducing cell death [12, 13]. However, the anti-cancer effect of DAZ on NSCLC is still not reported.

Yes-associated protein 1 (YAP1) is one of the key effectors of Hippo signaling pathway which is conserved in many species. It is believed that the Hippo pathway played its role as a tumor suppressor in many human cancers [14]. The activation of Hippo pathway would promote the phosphorylation of YAP1 by Hippo core complex [15]. However, when Hippo pathway losses its

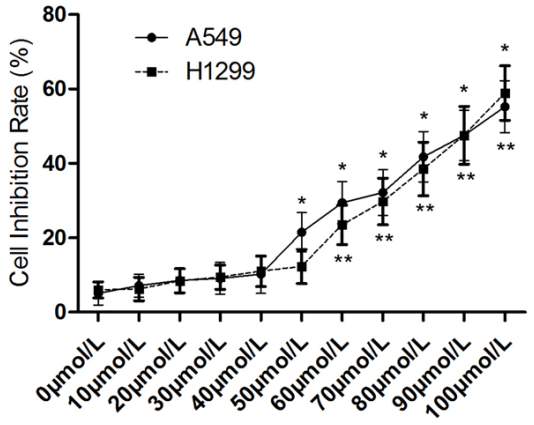


**Figure 1.** DAZ inhibited lung cancer cells in a concentration- dependent manner. Results of MTT assay. The inhibition rate by serially diluted DAZ solution (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μmol/L) against A549 and H1299 cells were demonstrated by full curve and dotted curve respectively. * differences were statistically significant when compared with previous concentration.

activity, the unphosphorylated YAP1 could enter the nucleus and initiate transcriptions of its down-stream genes which are involved in cancer cell proliferation [16, 17]. The Hippo core complex is composed of and the activity is decided by two serine- threonine kinase pairs, namely STK3 and STK4 [18, 19]. Then another kinase pairs, LATS1 and LATS2, are activated and further regulate the phosphorylation of YAP1 [20].

Kinases are ideal molecular targets for cancer treatments [21, 22]. In this study, the anti-cancer effect of DAZ on NSCLC and the involvement of Hippo pathway in NSCLC were investigated. Importantly, for mechanism study, STK3 and STK4 expressions were silenced by RNA interference and then the affection of DAZ incubation on the Hippo core complex was observed. We believe that data collected in this study not only would be helpful in supporting further clinical application of DAZ in NSCLC treatments, but also expand our knowledge of the pharmacological mechanism of DAZ.

## Materials and methods

### *Cell lines*

Human lung cancer A549 and H1299 cells were provided by American Type Culture Collection (ATCC, Rockville, USA). Cells were maintained in RPMI 1640 culture medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), L-glutamine (2.5 mmol/L, Invitrogen, USA) and antibiotics mix (100 U/mL penicillin and 100 μg/mL streptomycin, Sigma, USA) in a cell incubator providing 5% $CO_2$ and 95% fresh air and a humidified environment at 37°C.

### *Cell growth inhibition assessment*

The growth inhibition of A549 and H1299 cells was assessed by MTT assay. Briefly, cells were seeded in a 96-well plate at density of $6\times10^3$/well. Then cells were incubated respectively with DAZ at concentrations of 20, 30, 40, 50, 60, 70 and 80 μmol/L for 24 hours before 20 μL MTT (5 mg/ml) was added to each well. After 4-hour incubation, 100 μL DMSO was added to each well to dissolve the resulted formazan crystals. A plate reader (Bio-Rad, USA) was used to record the absorbance at 490 nm. The cell growth inhibition rate was calculated by comparing the acquired data to that of DMSO-treated control cells.

### *Small interfering RNA transfection*

In this study, the expressions of STK3 and STK4 were knocked down respectively by using small interfering RNAs. Targeting sequence for *stk3* was 5'-ACTACCCTACAGTCATATTACC-3', for *stk4* was 5'-GCCCTCATGTAGTCAAATATT-3'. The siRNAs were designed and synthesized by TaKaRa (Japan). Equal amount of siRNA was transfected into cells at final concentration of 12.5 mmol/L with the assistance of HiPer Fect™ siRNA transfection reagent (Qiagen, USA) per manufacturer's instructions. The transfected cells were cultured 24 hours before subsequent experiments.

### *Cell apoptosis examination*

The cell apoptosis was determined by terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay in the current study. Harvested cells were washed and then fixed in 2% paraformaldehyde (Boster, China) for 30 minutes at room temperature. Then the cells were permeabilized by 0.1% Triton X100 solution (Boster, China) for 30 minutes at room temperature. After washed by PBS, TUNEL assay was carried out by using TUNEL assay kit (Roche, Holland) according to

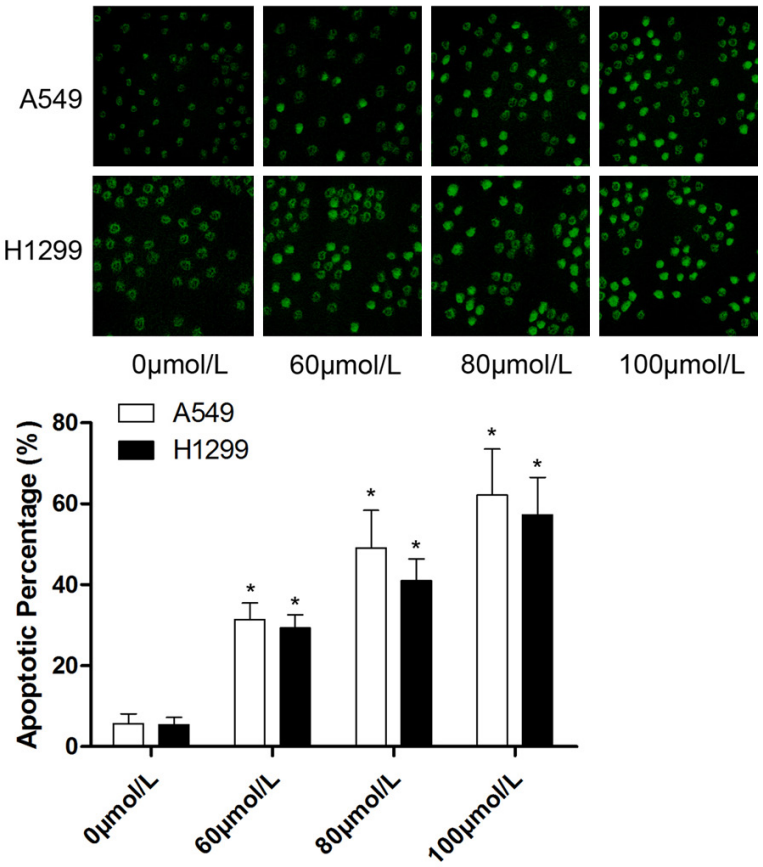


**Figure 2.** DAZ induced apoptosis of lung cancer cells in a concentration-dependent manner. The upper part of this figure demonstrated the captured fluorescent images of TUNEL assay of A549 and H1299 cells incubated with DAZ at concentrations of 0, 60, 80 and 100 μmol/L. Columns on the lower part of this figure demonstrated the apoptotic percentage of A549 (white column) and H1299 (black column) incubated with DAZ at concentrations of 0, 60, 80 and 100 μmol/L. [* differences were statistically significant when compared with previous concentration].

manufacturer's instructions. The TUNEL-positive cells were observed by a fluorescence microscope.

*Real time PCR*

Trizol (Invitrogen, USA) was used to extract the RNA from cultured cells. The extracted RNA was quantified by Nanodrop spectrophotometer. After quantification, the RNA was transcribed to cDNA by using PrimeScript RT Reagent kit (Takara, Japan) according to the instructions provided by the manufacturer. The real-time PCR was carried out by using SYBR Premix Ex TaqTMII (TaKaRa, Japan). The sequence of oligonucleotide primers for STK3 was forward 3'-CGATGTTGGAATCCGACTTGG-3', reverse 5'-GTCTTTGTACTTGTGGTGAGGTT-3'; for STK4 was forward 5'-AGTGCCAAAGGAGTGTCAATAC-3', reverse 5'-GGATTCCTGGCGTTTCAGTTTC-3'; for GAPDH was forward 5'-GAAGGTGAAGGTCGGAGTCA-3', reverse 5'-GGGGTCATTGATGGCAACAATA-3. Data was analyzed and the relative expression levels were calculated by using delta delta Ct method when GAPDH was introduced as the internal reference.

*Western blotting*

Cultured cells were harvested and then lysed by RIPA lysis buffer (Santa Cruz, USA) supplied with PMSF (Santa Cruz, USA). The proteins were extracted from the supernatants which were separated from cell homogenates by centrifugation. After the concentration was detected by a BCA protein assay kit (Thermo, USA), the proteins were subjected to SDS-PAGE and the separated by vertical electrophoresis and transferred to PVDF membranes. Specific antibodies against YAP1 (Abcam, USA), phosphorylated YAP1 (Abcam, USA), STK3 (Cell signaling, USA), STK4 (Cell signaling, USA), and GAPDH (Invitrogen, USA) were used to incubate the membranes at 4°C for 12 hours. After washing, the membranes were incubated with corresponding second antibodies. The membranes were developed with Signal West Pico reagent (Thermo, USA) and visualized on X-ray films.

*Statistics*

Data collected in this study were presented in a (mean ± SD) manner and further analyzed by using SPSS software (16.0, SPSS, USA). One-way analysis of variance (ANOVA) and Student t-tests were used to decide the significance of differences between groups. Differences were considered significant when $P<0.05$.

## Results

The cell growth of A549 and H1299 was significantly inhibited by DAZ incubation in a concentration-dependent manner.

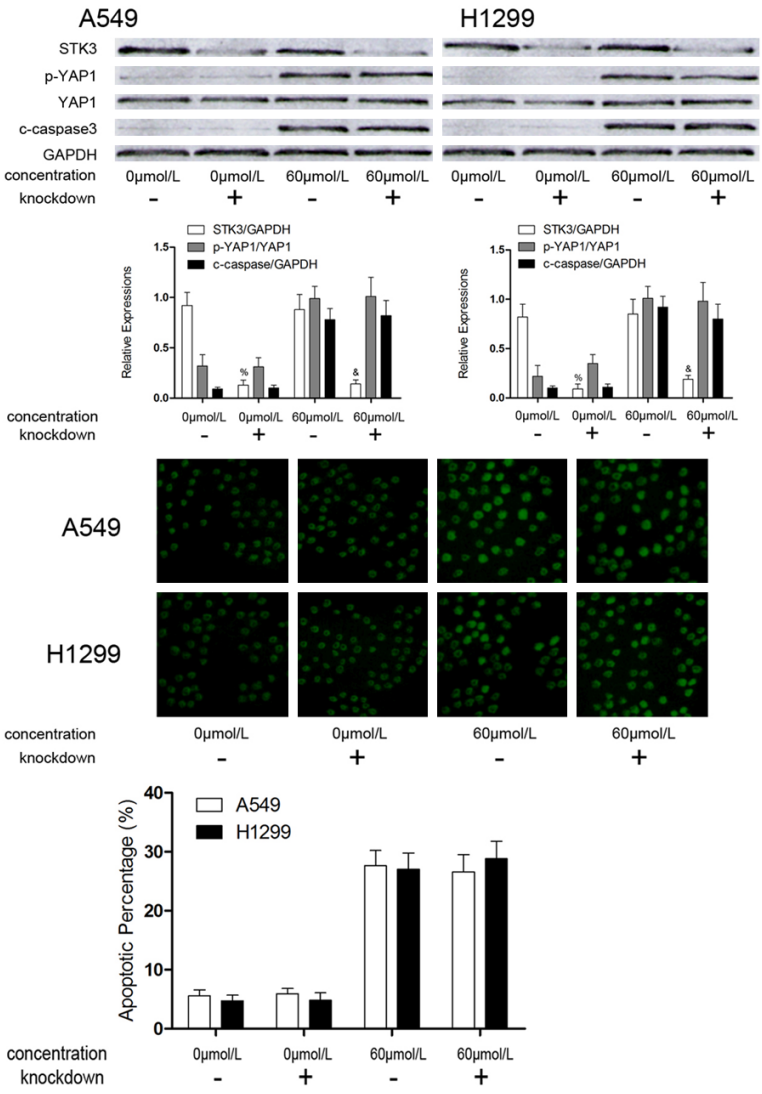


**Figure 3.** Effects of DAZ on activation of STK3/YAP1 and STK4/YAP1 signaling- induced apoptosis in lung cancer cells. The left side of upper part of this figure demonstrated the immunoblots of STK3, STK4, phosphorylated YAP1, YAP1, cleaved caspase3 and GAPDH of A549 and H1299 incubated with DAZ at concentrations of 0, 60, 80 and 100 μmol/L respectively. Columns on the right side of upper part of this figure demonstrated the relative expression levels of STK3, STK4, YAP1 phosphorylation and cleaved caspase3 of A549 and H1299 incubated with DAZ at concentrations of 0, 60, 80 and 100 μmol/L respectively. The lower part of this figure demonstrated the relative mRNA expression levels of STK3 and STK4 of A549 and H1299 incubated with DAZ at concentrations of 0, 60, 80 and 100 μmol/L respectively. [* differences were statistically significant when compared with previous concentration].

As demonstrated in **Figure 1**, the MTT assay revealed that after DAZ incubation, the growth inhibitory rate increased significantly in a concentration-dependent manner in both A549 and H1299 lung cancer cells. The DAZ began to show dramatic inhibitory effect on cell growth on A549 cells at 50 μmol/L and H1299 cells at 60 μmol/L. Thus DAZ concentrations above 60 μmol/L were selected for subsequent experiments.

DAZ induced the apoptosis of both A549 and H1299 lung cancer cells in a concentration-dependent manner.

The results of cell apoptosis which was detected by fluorescent TUNEL assay in **Figure 2**. After DAZ incubation, the percentage of apoptotic cells elevated significantly. Furthermore, the apoptosis-inducing effect of DAZ on both A549 and H1299 cells was in a concentration-dependent manner.

DAZ incubation increased expression of STK4 (not STK3) which further elevated the phosphorylation of YAP1 and cleavage of caspase-3 in both A549 and H1299 lung cancer cells in a concentration-dependent manner.

The related results were demonstrated in **Figure 3**. After DAZ incubation, in both A549 and H1299 cells, the mRNA expressions of STK4 increased significantly but no dramatic changes of STK3 mRNA expression was found. Furthermore, the phosphorylation of YAP1 and protein expressions of cleaved caspase-3 was significantly increased as well as the protein expression of STK. No significant change of protein expression of STK3 was spotted.

*Stk4* knockdown (not *stk3* knockdown) impaired DAZ's inhibitory effect on cancer cell growth and apoptosis-inducing effect on cancer cell apoptosis

In this study, siRNA was used to silent expression of STK3 and STK4 respectively. As shown

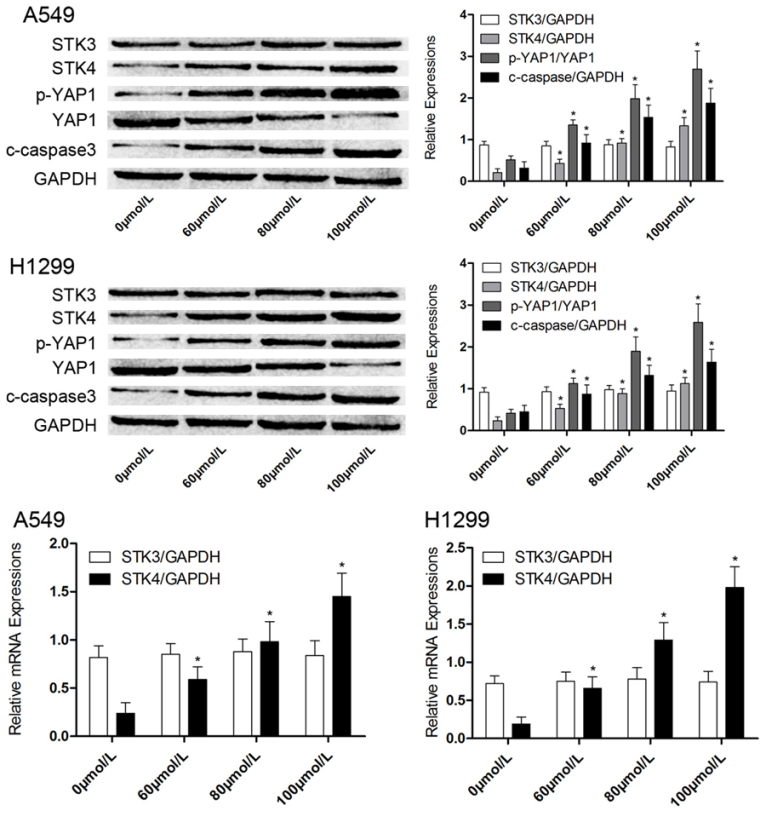


**Figure 4.** Effects of *stk3* knockdown on activation of STK3/YAP1 signaling-induced apoptosis of lung cancer cells. The upper part of this figure demonstrated the immunoblots of STK3, phosphorylated YAP1, YAP1, cleaved caspase3 and GAPDH in A549 and H1299 cells received DAZ incubation at 0/60 μmol/L and/or shRNA against *stk3* respectively. The columns on the upper panel demonstrated the relative expression levels of STK3, YAP1 phosphorylation and cleaved caspase3 in A549 and H1299 cells eceived DAZ incubation at 0/60 μmol/L and/or shRNA against *stk3* respectively. The lower part of this figure demonstrated the captured fluorescent images of TUNEL assay of A549 and H1299 cells received DAZ incubation at 0/60 μmol/L and/or shRNA against *stk3* respectively. Columns on the lower part of this figure presented the apoptotic percentage of A549 (white column) and H1299 (black column) cells received DAZ incubation at 0/60 μmol/L and/or shRNA against *stk3* respectively. [% differences were statistically significant when compared with cells received DAZ incubation of 0 μmol/L without shRNA treatment; & differences were statistically significant when compared with cells received DAZ incubation of 60 μmol/L without shRNA treatment].

in **Figure 4** (for *stk3* knockdown) and **Figure 5** (for *stk4* knockdown), the expressions of STK3 and STK4 were dramatically inhibited in both A549 and H1299 cells. Results from TUNEL assay indicated that *stk4* knockdown but not *stk3* knockdown impaired DAZ's inhibitory effect on lung cancer cell growth by inducing apoptosis.

## Discussion

In this study, two lung cancer cell lines, A549 and H1299, were used to observe the anti-tumor activity of DAZ. We showed that DAZ exerted its anti-tumor activity by inhibiting cancer cell growth. Our data also demonstrated that the inhibitory effect of DAZ on lung cancer cells was mediated by its apoptotic-inducing activity. We made several further investigations of the mechanisms of DAZ's anti-cancer effect. Expression of one of the key composites of the Hippo core complex, STK4, was found elevated after DAZ incubation in A549 and H1299 cells. As a result, the phosphorylation level of YAP1 was increased and further induced activation of caspase cascade, eventually resulting in increased cell death. The gene knockdown of STK4 by RNA interference impaired the apoptotic-inducing and cell growth inhibitory effect of DAZ. We believe that the data we collected would not only be helpful in further explaining the mechanisms of lung cancer, but also provide new clues and basis for DAZ as a potential clinical alternative drug in lung cancer treatment.

In recent years, soy-related products containing phytoestrogens drew much attention because of the various biological activities in promoting human health [23, 24]. The reduced incidences of several human carcinoma such as breast and prostate cancers were found associated with consumption of soy products according to several previous epidemiological studies [25]. DAZ was extracted from much soy-based food such as soybeans and chickpeas. A few investigations reported the capability of DAZ in inducing death of tumor cancer cells by arresting cell cycle and mediating apoptosis [6]. However, the exact mechanisms of DAZ's cell growth inhibitory effects were still unknown. In this study, after DAZ incu-

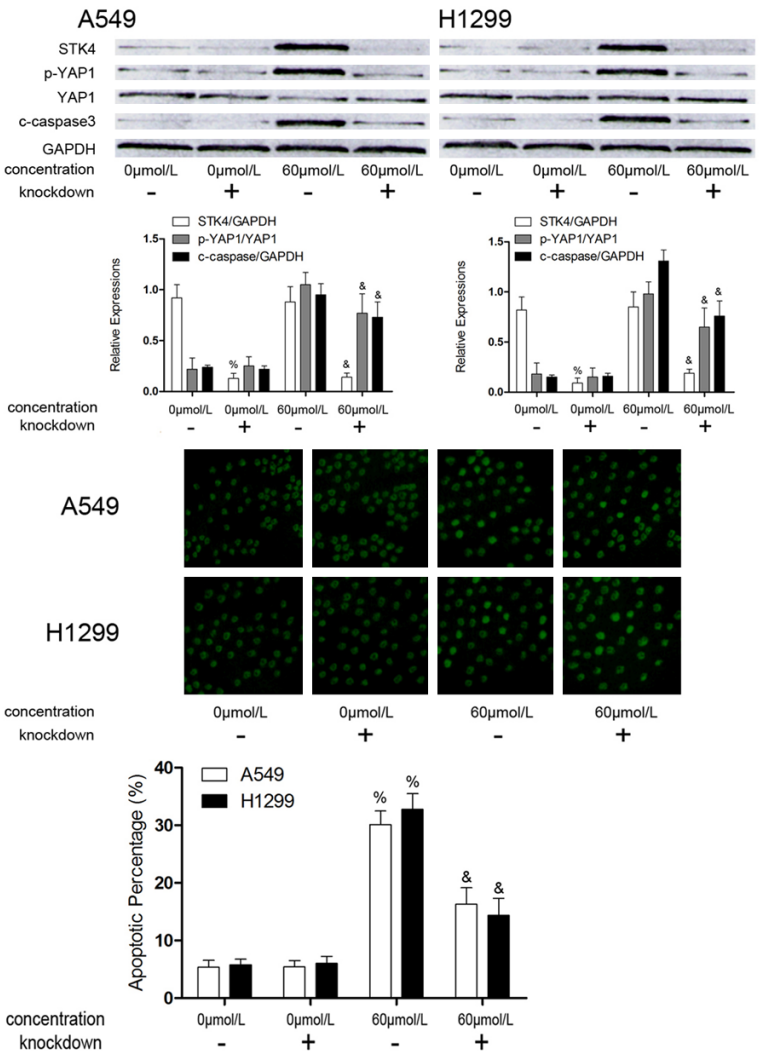


**Figure 5.** Effects of *stk4* knockdown on activation of STK4/YAP1 signaling-induced apoptosis of lung cancer cells. The upper part of this figure demonstrated the immunoblots of STK4, phosphorylated YAP1, YAP1, cleaved caspase3 and GAPDH in A549 and H1299 cells received DAZ incubation at 0/60 μmol/L and/or shRNA against *stk4* respectively. The columns on the upper panel demonstrated the relative expression levels of STK4, YAP1 phosphorylation and cleaved caspase3 in A549 and H1299 cells eceived DAZ incubation at 0/60 μmol/L and/or shRNA against *stk4* respectively. The lower part of this figure demonstrated the captured fluorescent images of TUNEL assay of A549 and H1299 cells received DAZ incubation at 0/60 μmol/L and/or shRNA against *stk4* respectively. Columns on the lower part of this figure presented the apoptotic percentage of A549 (white column) and H1299 (black column) cells received DAZ incubation at 0/60 μmol/L and/or shRNA against *stk4* respectively. [% differences were statistically significant when compared with cells received DAZ incubation of 0 μmol/L without shRNA treatment; & differences were statistically significant when compared with cells received DAZ incubation of 60 μmol/L without shRNA treatment].

bation, the cell growth of lung cancer cells was found inhibited in a DAZ concentration-dependent manner. Furthermore, we also found that DAZ induced the apoptosis of lung cancer cells in a concentration-dependent manner.

The Hippo signaling pathway has gained much attention because some previous studies suggested the disorder in regulation of the components of Hippo pathway would lead to tumorigenesis and maladjustment of organ development [26, 27]. YAP1 is an effecter of Hippo pathway and its role as an oncogene has been reported in many human malignant tumors such as hepatic cancer and breast cancer [28]. Mechanically, the unphosphorylated YAP1 was intended to enter the cell nucleus to initiate the transcriptions of genes involved in proliferation. It was recognized that the phosphorylation of YAP1 was partly dependent on the activity of Hippo core complex which could inactivate and phosphorylate the YAP1 [29]. The components of Hippo core complex were two kinase pairs, namely STK3/STK4 and LATS1/LATS2. The activation of STK3/STK4 could further activate LATS1/LATS2 that could direct the phosphorylation of YAP1 [30].

It was found in this study that the expression levels of both STK3 and STK4 were suppressed in both A549 and H1299 cells. Correspondingly, the phosphorylation level of YAP1 was down-regulated. However, DAZ incubation dramatically restored the expression levels of STK4 without affecting expression levels of STK3. The phosphorylation level of YAP1 was reversed to induce apoptosis which was evidenced by up-regulated cleaved caspase3. These results preliminarily indicated that DAZ induced lung cancer cells apoptosis

by restoring the STK4-induced YAP1 phosphorylation. The RNA interference technique was utilized to testify the conclusion. *stk4* knockdown but not *stk3* knockdown significantly impaired DAZ's cell growth inhibitory and apoptosis inducing effects on both A549 and H1299 cells. Thus, we could further confirm that DAZ inhibited lung cancer cells by targeting STK4, rather than STK3, in Hippo pathway.

## Conclusions

The components of Hippo pathway, namely STK3/4 and phosphorylation of YAP1 were down- regulated in lung cancer cells.

DAZ incubation significantly inhibited growth of lung cancer cells by inducing apoptosis.

DAZ induces lung cancer cell apoptosis by restoring STK4 expression and the phosphorylation of YAP1.

## Disclosure of conflict of interest

None.

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