

Original Article

c9, t11- conjugated linoleic acid induces HCC cell apoptosis and correlation with PPAR- γ signaling pathway

Guozhong Lu¹, Guoqing Zhang¹, Xing Zheng², Yan Zeng¹, Ziqi Xu¹, Weichi Zeng¹, Kebing Wang¹

¹Department of Pharmacy, 169 Hospital of PLA, 369 Dongfeng Road, Zhuhui District, Hengyang City 421002, Hunan Province, China; ²Department of Pharmacy and Biological Sciences, University of South China, Hengyang City 421002, Hunan Province, China

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Abstract: Objective: *Cis9, trans11* conjugated linoleic acid (c9, t11-CLA) is one of the most important isomers of conjugated linoleic acid, which have a strong anti-tumor effects. Based on previous studies, we further explored the molecular mechanism of inducing cells apoptosis in human hepatocellular carcinoma cell line HepG2 and Hep3B. Methods: Cell Counting Kit 8 (CCK-8) assay was used to investigate the effects of c9, t11-CLA on cell viability and cell proliferation ability; The effects of c9, t11-CLA on cell apoptosis was analyzed by DNA ladder assay, immunofluorescence and flow cytometry, respectively. Apoptotic related gene (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bax, Bak, Bad, Bid and Bim), PPAR family member (PPAR- α , PPAR- β and PPAR- γ), and Cox2 mRNA and protein expression were analyzed by RT-PCR and western blotting. ELISA assay was used to detect the content of Caspase-3. Results: Our data were confirmed that c9, t11-CLA could inhibit the HCC cells proliferation ability and decrease the cells viability. RT-PCR and western blotting assay verified that c9, t11-CLA obviously increased the transcription and protein expression levels of PPAR- γ . The synchronism and correlation between PPAR- γ and apoptotic proteins Bcl-2, Bax and Caspase-3 were found with a dose- and time-dependent manner. PPAR- γ inhibitor GW9662 and activator Rosiglitazone were further verified that there was cooperative relation between them. Conclusion: In our study, we first report that c9, t11-CLA induces apoptosis in HCC cells by activation of PPAR- γ -Bcl-2-Caspase-3 signal pathway. These results indicated that c9, t11-CLA will be useful for clinic therapy of anti-tumor and as a new regulator of PPAR- γ in the future.

Keywords: Conjugated linoleic acid isomer, c9, t11-CLA, peroxisome proliferators activated receptor γ (PPAR- γ), cell apoptosis, Hepatocellular carcinoma cell lines HepG2 and Hep3B.

Introduction

CLA (conjugated linoleic acid) is a group of polyunsaturated fatty acids possess with variously biological activity, mainly presents in beef, cheese and milk fat, which is geometric isomers and positional isomers of linoleic acid (LA) [1, 2]. Recently, CLA has been caused great concern due to its significant anti-tumor activity [3]. Currently, El Roz *et al.* reported that CLA could inhibit the development of tumors by inducing formation of lipid oxidation in mice with early gastric cancer [4]. In addition, Fite *et al.* found that CLA could inhibit formation of lung cancer through lipid metabolism and reduced production of eicosanoids carbon acid [5]. When further explored the molecular mechanism, Huang *et al.* discovered that CLA could inhibit tumor cell proliferation by interfering with normal op-

eration cycle of colon cancer cells [6]. Thus, CLA can suppress various tumors through different molecular pathways.

Recent studies have focused on CLA that how to play regulatory role in the initial stage and/or promotion stage within cancer. Bae *et al.* firstly observed chromatin condensation and DNA cross united by researching the role of CLA on colorectal cancer [7], but its mechanism is still no clear. Moreover, Josyula *et al.* reported that CLA treatment can decrease the expression of Bcl-2 in tumor model of rat thymus and thymic tumor cells, whereas it was no significant effect on the expression of Bcl-XL, Bax, Bak, Bad, p53, p21 and WAF1/CIP [8]. Huot *et al.* also found that CLA can inhibit the cell cycle in estrogen receptor-positive human breast cancer cells MCF-7, whereas it was no response to es-

trogen receptor-negative human breast cancer cells MDA-MB-231, and the down-regulation of c-Myc that closely associated with cell cycle is likely to be the main mechanism [9]. Subsequently, Stachowska *et al.* found that CLA can affect the metabolism of arachidonic acid, and inferring that CLA may be activated legend for peroxisome proliferator-activated receptor family receptors (PRAR) [10].

Although most of studies confirm that CLA has anti-tumor effects, but its molecular mechanism is different in different animal models or different cell types, which may be correlated with species, tissue sources and cell types [11, 12]. Moreover, there is the mixture of CLA used in the experiment, which may include various types of monomer, even mixture that has different composition, so that it is more complicated to discuss these results [12]. Therefore, further study focused on anti-tumor activity of CLA monomers and its molecular mechanism, will establish a foundation to develop the new drugs to fight cancers.

CLA is isomers of linoleic acid includes several biologically active form, of which *cis*9, *trans*11-CLA (c9, t11-CLA) is one of the most strongest anti-cancer active isomer, and c9, t11-CLA is the highest content of CLA monomers in foods [13]. Therefore, based on previous studies on CLA-induced chromatin condensation and DNA cross-linking in tumor cells, we attend to explore whether c9, t11-CLA inhibits proliferation of HCC cells and induce cell apoptosis, and further study its possible molecular mechanisms.

Materials and methods

Materials

c9, t11-CLA powder with 98% purity was purchased from Nanchang Huaxing Biotechnology Company, and formulated as 200 mmol/L of mother liquor using DMSO, then packed storage at -20°C for use. GW9662 (PPAR-γ inhibitor) and Rosilitazone (PPAR-γ agonists, referred as Rosig) was purchased from Jingmei, Ltd. Trizol reagent was come from US Invitrogen Corporation. Retroviral agents Oligo-dT, dNTP mixture were provided by Takara Biological Engineering Company Limited. Reverse transcriptase M-MLV was purchased from Promega Corporation. BCA protein assay kit was purchased from Pierce Company. CCK-8 assay kit was purchased from Japan Dojindo Laboratories. Ra-

bbit monoclonal PPAR-α, PPAR-γ, Bid and Bax were purchased from Abcam Company. Rabbit polyclonal antibody PPAR-β, Bcl-w and Bcl-2 were purchased from Santa Cruze Company. Mouse polyclonal antibody Cox2 and Bak were purchased from the Cell Signaling Technology Corporation. PVDF membrane with 0.2 μm pore was purchased from Millipore Corporation.

Cell culture

Human hepatocellular carcinoma cell lines HepG2 and Hep3B were cultured in DMEM supplemental 10% FBS at 37°C, 5% CO₂, and 0.25% trypsin was used for digestion and passage. The cultured cells in logarithmic growth phase were used in the whole experiments.

CCK-8 assay

CCK-8 assay was performed for detecting the effect of c9, t11-CLA on hepatocellular carcinoma cells proliferation ability. HepG2 and Hep3B cells were seeded in 96-well plates at the concentration of 1×10^4 per well and treatment with different concentration of c9, t11-CLA (0, 5, 10, 20, 50, 100 and 200 μM) or control vehicle at the same time point, or treated HepG2 and Hep3B cells with 50 μM and 100 μM c9, t11-CLA or control vehicle at different time point. After treatment, 10 μl/well CCK-8 solution was add and incubated in 37°C, 5% CO₂ humidified incubator for 2-4 h. The absorbance value at 450 nm was read using a microplatereader (Bio-Rad, CA, USA). Cell viability and/or cell inhibition rate were calculated. The experiment was repeated three times.

Cell apoptosis was observed by fluorescence microscope

Human hepatocellular carcinoma cell lines HepG2 cells were seeded in 12-well plates at 37°C, 5% CO₂ overnight. Then 50 or 100 μM c9, t11-CLA were added into the training system, continually cultured 24 h and 36 h, washed cells with PBS three times, then dyed with fluorescent dye Hoechst33342, anhydrous methanol fixed, eventually cells nuclear morphological changes were observed in inverted fluorescence microscope.

DNA ladder assay

Approximately 5×10^5 cells were collected in sterile 1.5 ml centrifuge tubes, 2000 rpm cen-

trifuged at 4°C for 5 min, the supernatant was discarded, washed once with ice-cold PBS solution, the supernatant was discarded; Add 20 µl lysis buffer [20 mmol/L EDTA, 100 mmol/L Tris, PH8.0, 0.8% (w/v) SDS], mixed with a pipette tip cell pellet; Then add 10 µl RNA enzyme A/T1 mixture (respectively 500 U/ml, 20000 U/ml), mix tip flick. 37°C for 60 minutes; Add 10 µl 20 mg/ml proteinase K, flicking the tip mix, 50°C for overnight; Add 5 µl 6 × DNA loading buffer, DNA samples were added 1.5% agarose gel dry hole. Electrophoresis at the low voltage and imaging.

The proportion of cell apoptosis was assayed by flow cytometry

HepG2 cells were grown to logarithmic growth phase in DMEM medium supplemental 10% FBS and digested by 0.25% trypsin, adjusting the cell density is 5×10^5 /ml, and seeded into a 6 well culture plate. When the cells reached about 70% degree of confluence, HepG2 cells were divided into 3 groups: control group, c9, t11-CLA (50 µM) group and c9, t11-CLA (100 µM) group. Pretreatment HepG2 cells with c9, t11-CLA for 24 h or 36 h. After treatment, cells were digested by 0.25% trypsin and dissociated into single cell after treatment. 5×10^5 cells were harvested and double-stained with fluorescein APC-labeled annexin V and PI (Becton Dickinson). The percentage of apoptotic cells were detected by flow cytometry (Becton Dickinson) after staining. The experiment was repeated three times.

Quantitative real-time PCR assay

Samples total RNAs were isolated from appropriate HepG2 cells using Trizol agent (Invitrogen, USA) according to manufacturer's instruction. NanoDrop-1000 was used to determine the concentration and purity of total RNA. A reverse transcription Kit (TaKaRa) was used to synthesize the first strand of cDNA. Taken 1 µg total RNA synthesized the first strand cDNA according to the method of reverse transcription kit (Promega Corporation USA). RT-PCR assay was used to detect the mRNA expression of apoptotic related genes Bcl-2, Bcl-XL, Bcl-w, Bax, Bak, Bad, Bid, Bim, Cox2, PPAR-α, PPAR-β and PPAR-γ. GAPDH mRNA served as a loading control.

Western blotting

Proteins isolated from appropriate HepG2 cells were mixed with a 5 × Loading buffer and boiled for 10 min at 95-100°C. Subsequently, 20 µg of total proteins were loaded onto a 10% SDS-PAGE for separation. Proteins were transferred to a PVDF membrane, and blocked with 5% milk/PBST, then treated with the corresponding first antibodies. The antibody detection system consisted of Cox2 (1:500 dilution), PPAR-α (1:500 dilution), PPAR-β (1:1000 dilution), PPAR-γ (1:1000 dilution), Bcl-2 (1:500 dilution), Bcl-w (1:300 dilution), Bax (1:500 dilution), Bak (1:500 dilution), Bid (1:500 dilution), GAPDH (1:1000 dilution) and HRP-conjugated anti-rabbit antibody. Analysis of the absorbance values of each band Quantity One software, the experiment was repeated for three times. GAPDH was used as internal loading control, and the representative images were captured and analyzed.

The content of caspase-3 was detected by ELISA

The kit and samples were defrosted at room temperature (RT). Washing liquor, test plasma, standards, and horseradish peroxidase-labeled streptavidin were diluted into required concentration. 50 µL standard substance, reference substance and test plasma were respectively added into each well of ELISA plate and then sealed with closure plate membrane for incubation in shaking table for 2 h. Afterwards the liquid in the plate was abandoned and 300 µL washing liquor was added into each well. The liquid in the plate was abandoned again. The plate was dried by filter paper. The above process was repeated 6 times. 100 µL horseradish peroxidase-labeled streptavidin was added into each well. The plate was sealed with new closure plate membrane for 45 min of incubation in shaking table. Afterwards the liquid in the plate was abandoned and 300 µL washing liquor was added into each well. The liquid in the plate was abandoned again. The plate was dried by filter paper. The above process was repeated 6 times. 100 µL TMB (3, 3', 5, 5'-Tetramethylbenzidine) was added into each well in dark room to incubate for 15 min at RT. The plate frame was knocked gently to mix the liquid thoroughly. The wells turned from colorless to yellow green. In 30 min, OD value was assayed using automatic microplate reader at wavelength of 450 nm. The abscissa represented

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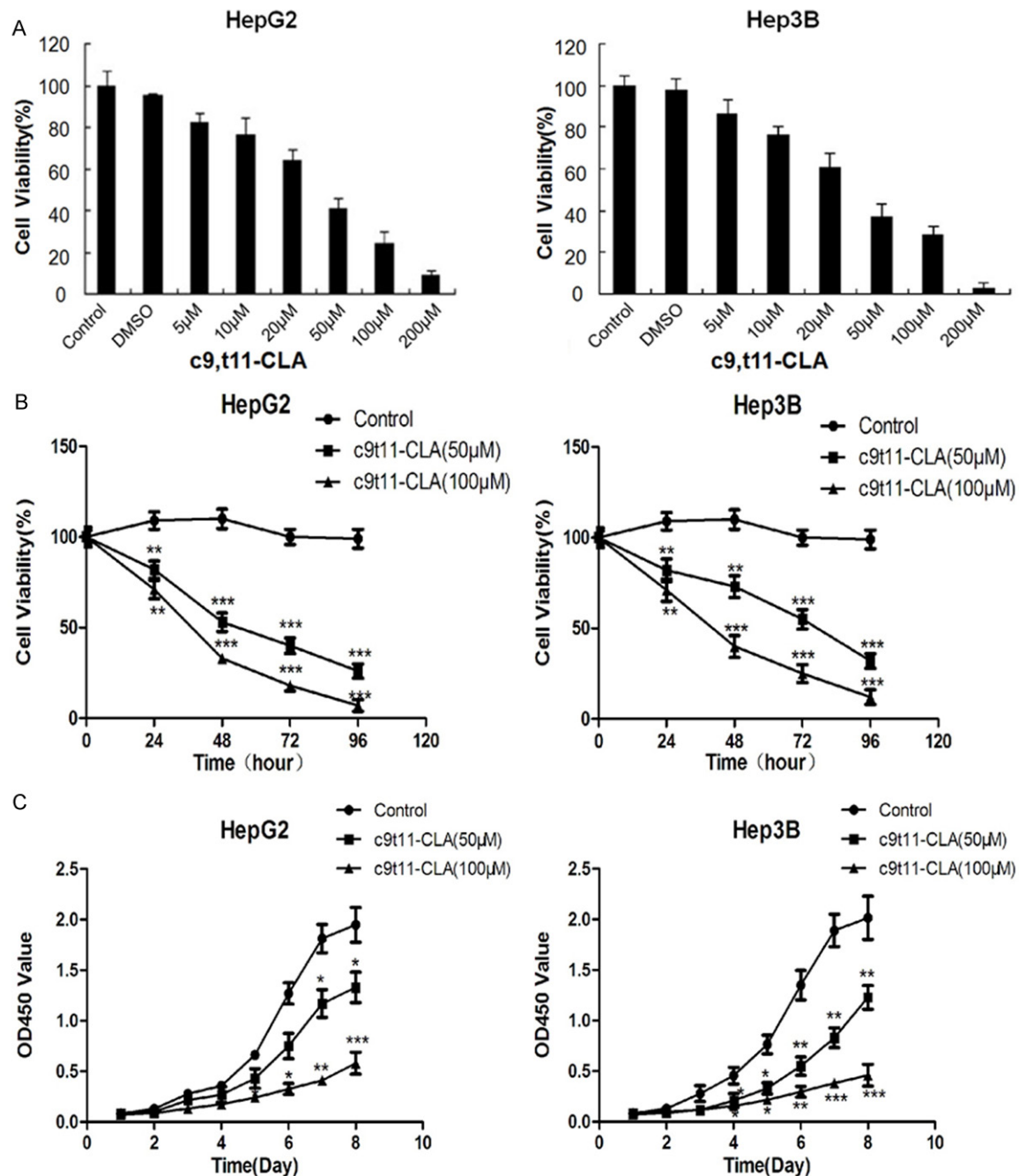


Figure 1. c9, t11-CLA could decrease the cell viability and inhibit the cells proliferation ability in HCC cells line HepG2 and Hep3B. A. CCK-8 assay was used to analyze the effects of different doses of c9, t11-CLA on cell viability of HepG2 and Hep3B at 48 h; B. HepG2 and Hep3B were treated with different doses of c9, t11-CLA (50 μM and 100 μM) for 0 h, 24 h, 48 h, 72 h and 96 h, respectively, and CCK-8 assay was used to analyze the cell viability; C. CCK-8 assay was used to analyze the cell proliferation curve of c9, t11-CLA (50 μM and 100 μM) on HepG2 and Hep3B cells.

the concentration of standard substance. The ordinate represents the corresponding OD value. Standard curve was generated by regression fitting in computer. The concentration of test antibody in test serum was calculated according to the standard curve.

Statistical analysis

SPSS 18.0 statistic software was used for data processing. The results are presented with average \pm standard deviation. Analysis of variance (ANOVA) was used to compare differences

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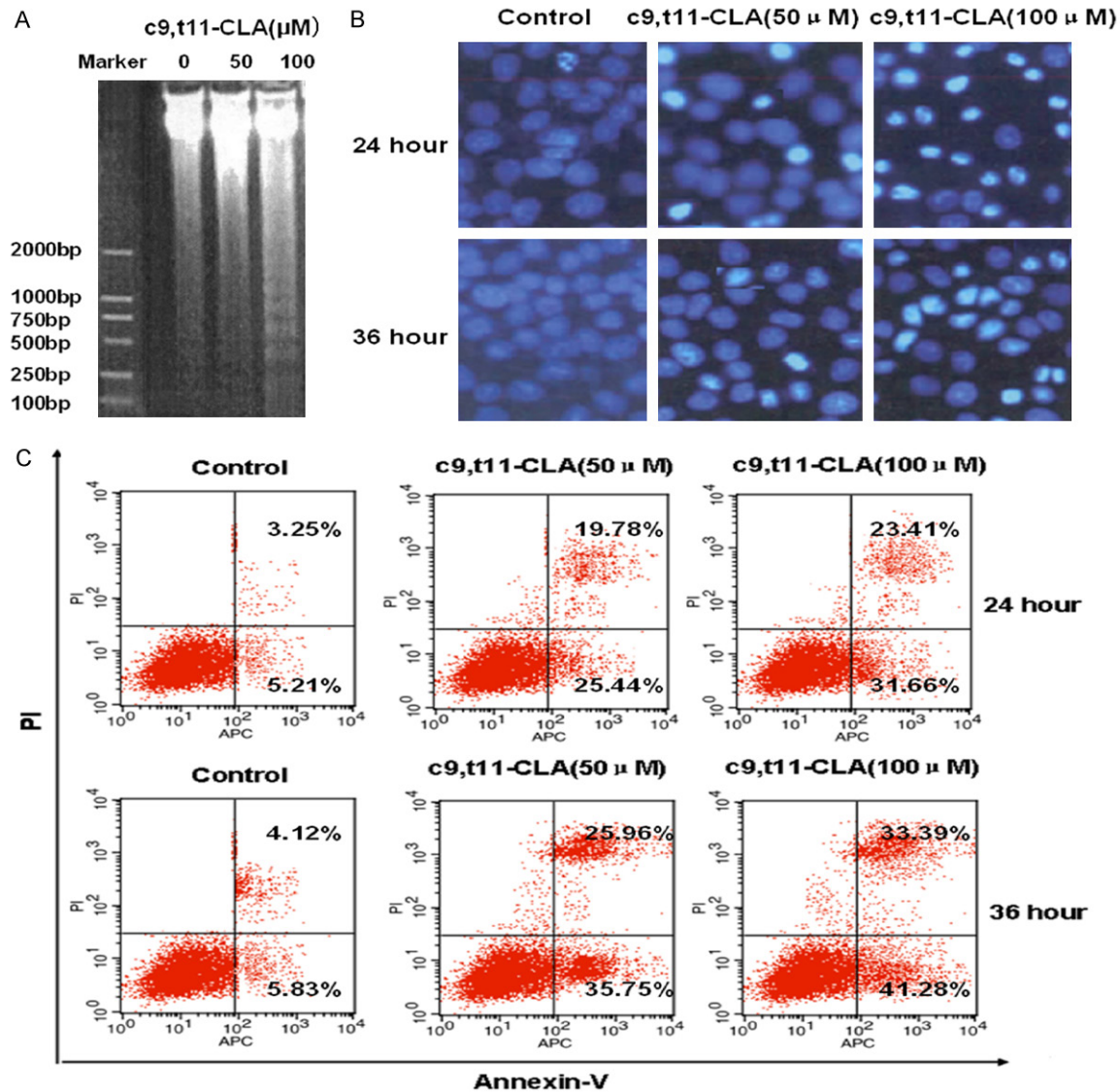


Figure 2. c9, t11-CLA could induce HCC cells apoptosis and showed a dose- and time-dependent manner. (A-C) The effects of c9, t11-CLA (50 μM and 100 μM) on cell apoptosis was analyzed by DNA ladder assay at 24 hours (A), fluorescence microscope (B) and flow cytometry (C) at 24 hours and 36 hours.

between groups. $p < 0.05$ was considered statistically significant.

Results

c9, t11-CLA significantly inhibits the growth of hepatocellular carcinoma cells HepG2 and Hep3B

In order to explore the effect conjugated linoleic acid monomers c9, t11-CLA on the growth of HCC cells, we treated HCC cells HepG2 and Hep3B with different concentrations of c9, t11-CLA (the dose separately were 0 μM, 5 μM, 10

μM, 20 μM, 50 μM, 100 μM and 200 μM) for 48 hours, and detected the effect of c9, t11-CLA on cell viability in HCC cells by CCK-8 assay. Our data suggested that HepG2 cells and Hep3B cell viability were gradually decreased followed by the dose of c9, t11-CLA gradually increased (**Figure 1A**). Especially, when the dose of c9, t11-CLA greater than 50 μM, the survival ratio of HCC cells were significantly decreased, the difference was significant ($p < 0.05$). To further discover whether the cytotoxicity effect of c9, t11-CLA on HCC cells showing time-dependent manner, we treated HCC cells HepG2 and Hep3B with 50 μM and 100 μM of c9, t11-CLA

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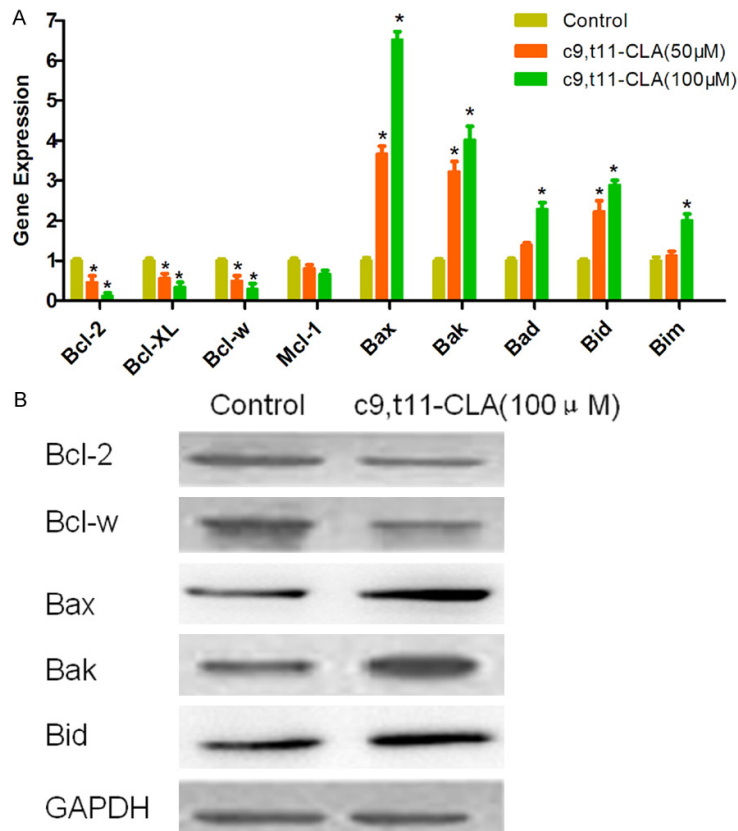


Figure 3. c9, t11-CLA could induce HCC cells apoptosis by up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes. A. RT-PCR assay was used to detect the expression of Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bax, Bak, Bad, Bid and Bim in HepG2 cells treated with c9, t11-CLA (50 μM and 100 μM) for 24 h; B. Western blotting assay was used to detect the expression of Bcl-2, Bcl-w, Bax, Bak and Bid in HepG2 cells treated with c9, t11-CLA (50 μM and 100 μM) for 48 h, GAPDH was used as internal loading control.

for 24 h, 48 h, 72 h and 96 h, respectively, and detected the cell viability by CCK-8 assay. The results were shown in **Figure 1B**, with the continuous extension of treatment time, cell survival rate was extremely significant reduced ($p < 0.05$). **Figure 1C** indicated that 50 μM and 100 μM c9, t11-CLA could significantly inhibited HepG2 and Hep3B cell growth compared with the control group (DMSO). The most significantly inhibitory effect of c9, t11-CLA on proliferation of HepG2 cells has occurred at 8 day, and the inhibition rate was 43.5% (50 μM) and 78.9% (100 μM) separately. However, The most significantly inhibitory effect of c9, t11-CLA on proliferation of Hep3B cells has occurred at 6 day, the variance analysis results by SPSS 18.0 showed that c9, t11-CLA were significantly different compared with the control group ($p < 0.05$).

c9, t11-CLA can promote the apoptosis of HepG2 and Hep3B cells in hepatocellular carcinoma

To further explore the molecular mechanism of c9, t11-CLA effect on HepG2 cells viability, and test whether induce cell apoptosis. We treated HepG2 cells with c9, t11-CLA (50 μM and 100 μM) for 24 and 36 hours, then analyze the effects of c9, t11-CLA on cells apoptosis. The results demonstrated that after treatment HepG2 cells by 50 μM and 100 μM c9, t11-CLA, after extraction of DNA and through electrophoresis detection, DNA exhibits dispersion-like distribution, indicating that the DNA of HepG2 cells breakage has occurred and the cells are gradually apoptosis (**Figure 2A**). Hochest33342 fluorescent dye suggested that specifically binds DNA within the cell, normal cell nuclei (control group) showed diffuse homogeneous fluorescence, fluorescence is weak, the c9, t11-CLA (50 μM and 100 μM) group showed a typical apoptotic nuclei, nuclear volume was significantly smaller, wrinkled, dense granules massive

stain strong fluorescence, even pyknosis or fragmented (**Figure 2B**). To further investigate the effect of c9, t11-CLA on cell apoptosis in Hepatocellular carcinoma cells, we treated HepG2 cells with 50 μM and 100 μM c9, t11-CLA for 24 h and 36 h, respectively and detected the cell apoptosis proportion by flow cytometry. The results showed that the cell apoptosis rate of HepG2 cells were gradually increased, 50 μM c9, t11-CLA induced HepG2 cells apoptotic rates were 45.22% and 61.71% after treated 24 h and 36 h, respectively, compared with the control group (**Figure 2C**, $p < 0.05$). 100 μM c9, t11-CLA induced HepG2 cells apoptotic rates were 55.07% and 74.67% after treated 24 h and 36 h, respectively, compared with the control group were significantly different (**Figure 2C**, $p < 0.05$). The results showed that with the c9, t11-CLA concentration was increased,

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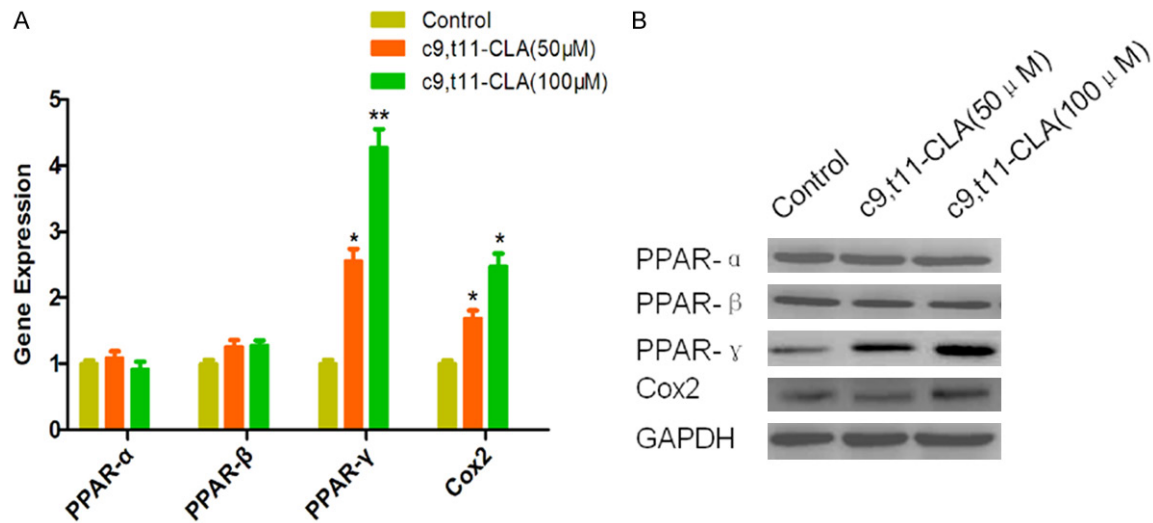


Figure 4. c9, t11-CLA could induce HCC cells apoptosis through activation of PPAR-γ. A. RT-PCR assay was used to detect the expression of PPAR-α, PPAR-β, PPAR-γ, and Cox2 in HepG2 cells treated with c9, t11-CLA (50 μM and 100 μM) for 24 h; B. Western blotting assay was used to detect the expression of PPAR-α, PPAR-β, PPAR-γ, and Cox2 in HepG2 cells treated with c9, t11-CLA (50 μM and 100 μM) for 48 h.

the time of treatment was prolonged, and the apoptosis rates of HCC cells gradually increased efficiency.

c9, t11-CLA can induce cells apoptosis by inhibiting the expression of anti-apoptotic gene and promoting pro-apoptotic gene expression

In order to further investigate the molecular mechanism of c9, t11-CLA on cell apoptosis in hepatocellular carcinoma cells, we detected the expression of apoptotic related gene (anti-apoptotic gene Bcl-2, Bcl-XL and Bcl-w; pro-apoptotic gene Bax, Bak, Bad, Bid and Bim) by RT-PCR assay. The results demonstrated that expression of anti-apoptosis gene Bcl-2, Bcl-XL and Bcl-w were significantly down-regulated in HepG2 cells treated with 50 μM or 100 μM c9, t11-CLA for 24 hours, and the expression of pro-apoptotic gene Bax, Bak, bad, bid and Bim were extremely up-regulated (**Figure 3A**). We further verified the expression of apoptotic related genes by western blotting assay, the results were consistent with the results of RT-PCR assay (**Figure 3B**).

c9, t11-CLA promotes cell apoptosis by activating PPAR-γ expression in hepatocellular carcinoma cells

In order to further explore the possible molecular mechanism of c9, t11-CLA induced HCC

cells apoptosis, we treated HepG2 cells with 50 μM or 100 μM c9, t11-CLA and detected the expression of PPAR-α, PPAR-β, PPAR-γ and Cox2 by RT-PCR and Western blotting assay, which gene are closed related to cell apoptosis. The results indicated that the mRNA expression of PPAR-γ and Cox2 were significantly up-regulated in c9, t11-CLA treated group compared with the control group (**Figure 4A**, $p < 0.05$, $p < 0.05$), only PPAR-γ protein expression was extremely significant increased (**Figure 4B**). However, the mRNA and protein expression of PPAR-α and PPAR-β were no significantly changed among all three groups (**Figure 4A and 4B**, $p > 0.05$). These results implied that c9, t11-CLA only activated the signaling pathways associated with PPAR-γ, but had no effect on PPAR-α and PPAR-β. Furthermore, compared with the control group, the mRNA expression of PPAR-γ was notable up-regulated followed by increased the concentration of c9, t11-CLA, suggested that c9, t11-CLA could promote the transcription of PPAR-γ (**Figure 4A**). In addition, our further analysis found that c9, t11-CLA on PPAR-γ activation was significantly higher than that Cox2 activation (**Figure 4A and 4B**). These data indicated that c9, t11-CLA may be mainly through activation of PPAR-γ related signaling pathways played an important role in inhibition of cell growth and promotion of cell apoptosis.

c9, t11-CLA can significantly increase the transcriptional activity and protein expression of PPAR-γ, and which are synchronous with the changes of the apoptosis related genes

Our data suggested that the effect of c9, t11-CLA on HCC cells apoptosis was achieved by up-regulation of pro-apoptotic gene Bax or Bad and down-regulation of anti-apoptotic gene Bcl-2 (**Figure 3A** and **3B**). We further investigate that c9, t11-CLA activation of PPAR-γ and induced cell apoptosis are synchronization, we detected the mRNA expression of PPAR-γ, Bcl-2, Bax, Bad and Caspase-3 in HepG2 cells treated with c9, t11-CLA for 24 h, 36 h and 48 h. **Figure 5A** showed that the expression of PPAR-γ and pro-apoptotic gene Bax, Bad and Caspase-3 was significantly higher than control group ($p < 0.05$), and these genes expression showed obvious time-dependent manner. The expression of PPAR-γ reached its peak after treated with 48 h, the expression of cell apoptotic gene reached its peak at 36 h, 72 h after began to decrease, the difference has statistical significance (**Figure 5A**, $p < 0.05$). We further investigated whether PPAR-γ are synchronous with the changes of the apoptosis related genes, we treated HepG2 cells with c9, t11-CLA combined PPAR-γ inhibitor GW9662 or PPAR-γ activator Rosig, and observed the expression changes of PPAR-γ and apoptosis related genes. The data showed that expression of PPAR-γ and apoptosis related genes were inhibited by GW9662 and were promoted by Rosig (**Figure 5A**, $p < 0.05$), indicating that expression of PPAR-γ are synchronous with the changes of the apoptosis related genes. Caspase-3 is the most critical apoptotic executive protein, and the content of Caspase-3 increased, implied that cell apoptosis has been activated. So we further detected the activity of Caspase-3 by ELISA. The results showed that activity of Caspase-3 was enhanced with treated c9, t11-CLA, and can be further enhanced by PPAR activator Rosig. However, activity of Caspase-3 was reduced when HepG2 cells treated combined c9, t11-CLA and PPAR-γ inhibitor GW9662 (**Figure 5B**, $P < 0.05$). We further validated our results by flow cytometry. Our results showed that c9, t11-CLA induced cell apoptosis in HCC cells, and the apoptosis effect can be reversed by GW9662 and promoted by Rosig (**Figure 5C**, $p < 0.05$). So we have a sufficient reason to speculate that c9, t11-CLA as a ligand activat-

ed PPAR-γ, and ultimately leads to cells apoptosis through activated of PPAR-γ-Bcl2-Caspase-3 signaling pathway.

Discussion

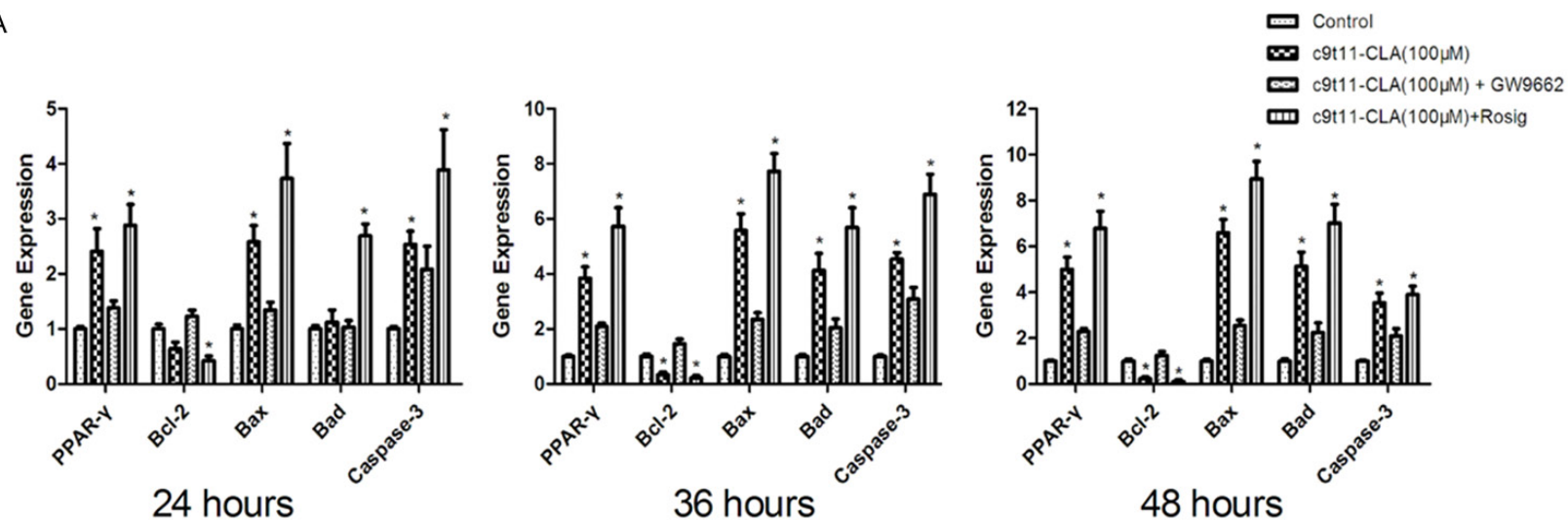
Conjugated linoleic acid (CLA) has anti-cancer effect is shown in the two aspects. Firstly, Numerous studies have demonstrated that CLA can inhibit a variety of cancers that induced by chemical substances in different animal models [14, 15]. Secondly, CLA can inhibit proliferation of tumor cells *in vitro*, and accompanied by cell apoptosis [16, 17]. Previous studies have found that c9, t11-CLA can strongly suppress proliferation of such cells through treating prostate cancer cells PC-3 [18], colorectal cancer cells HT-29 and MIP-101 with c9, t11-CLA [19]. Most of results considered that CLA has obviously anti-cancer effects, but its inhibitory effect is different in different animal models, source of tissues and cell types.

In the current study, proliferation of HCC cell lines HepG2 and Hep3B were all obviously inhibited by c9, t11-CLA, and the inhibitory effects of HepG2 by c9, t11-CLA was similar to Hep3B. In our study, to examine the effect of different concentrations of c9, t11-CLA on HCC cell lines, HepG2 and Hep3B were treated with different concentrations of c9, t11-CLA (0 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM and 200 μM), respectively. The results showed that the killing effect of low concentration of c9, t11-CLA on HCC cells was relatively small. In addition, when the concentration of c9, t11-CLA was equal to or more than 50 μM, c9, t11-CLA showed significant proliferation inhibition and killing effect, and the inhibition effect was closely related to the concentration of c9, t11-CLA. At the same time, the inhibitory effect of also showed a significant time-dependent manner.

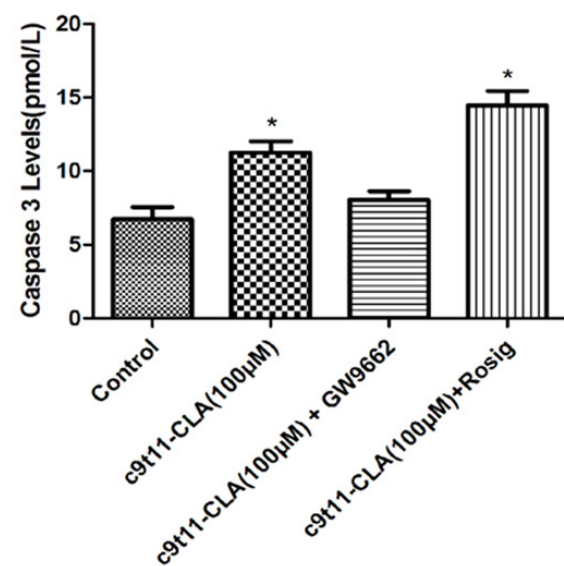
Our study further confirmed that c9, t11-CLA can induce apoptosis of HCC cell lines HepG2. The HCC cells showed typical DNA fragmentation by long-term treatment with c9, t11-CLA. Further detection by Hoechst staining and flow cytometry, the results showed that c9, t11-CLA not only caused cells morphological changes, but also significantly increased apoptosis rate of HCC cells, which was consistent with previous studies. The signal transduction pathway of cell apoptosis mainly concludes death receptor

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A



B



C

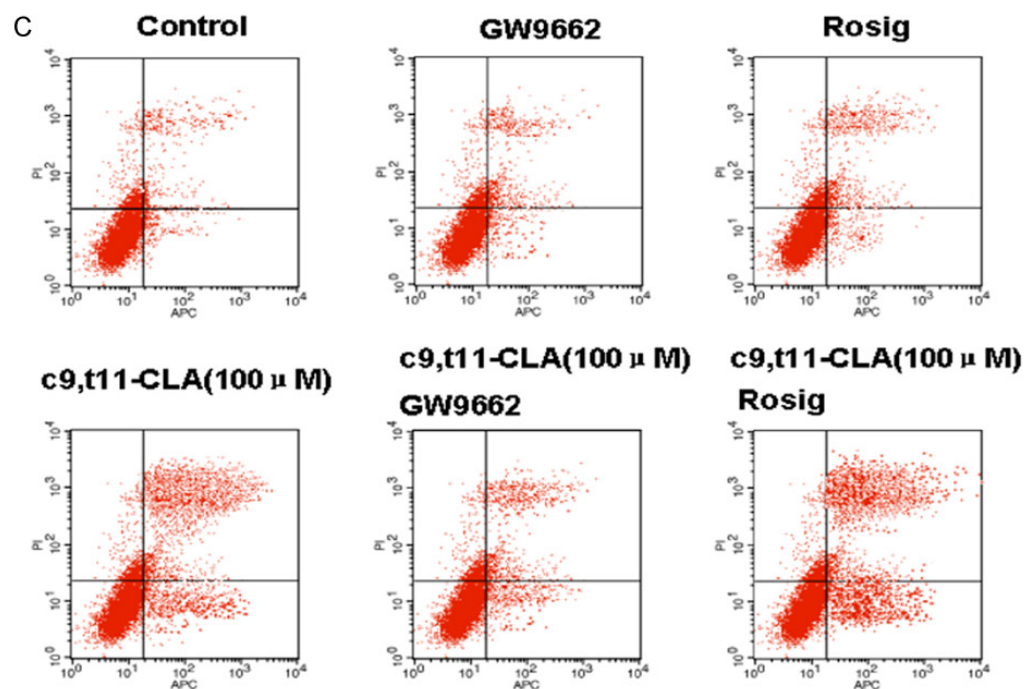


Figure 5. c9, t11-CLA induces cell apoptosis in HCC cells by activation of PPAR- γ -Bcl-2-Caspase-3 signal pathway. A. RT-PCR assay was used to detect the expression of PPAR- γ , Bcl-2, Bax, Bad and Caspase-3 in control group, c9, t11-CLA (100 μ M) group, or c9, t11-CLA (100 μ M) + GW9662 group and c9, t11-CLA (100 μ M) + Rosig group; B. The content of Caspase-3 was detected by ELISA; C. The cell apoptotic rate was discovered by flow cytometry.

pathway and mitochondrial pathway, and the activation of Caspase-3 is common way of the two pathways [20, 21]. Caspase-3 is the most critical apoptotic protein, and the activity of Caspase-3 increased, that is the production of small fragments, implied that cell apoptosis has been activated [22, 23]. Moreover, expression and regulation of Bcl-2 family is one of the key factors that affect cell apoptosis [24], which play an important role in the signal transduction pathway of cell apoptosis [25]. Occurrence of cell apoptosis not only based on the increasing expression of apoptotic protein Bax or the decreasing expression of anti-apoptotic protein Bcl-2, but also depended on strict chemical ratio between Bax and Bcl-2 in the heterologous dimmers [26, 27]. In our study, we confirmed that c9, t11-CLA could promote expression of Bax, and simultaneously inhibit expression of Bcl-2 (increased the ratio of Bax/Bcl-2), which promoted pro-Caspase-3 activation, and increasing expression of small fragments of Caspase-3, and eventually lead to cell apoptosis.

Moreover, we further explored the molecular mechanism of HCC cell apoptosis induced by c9, t11-CLA, and results showed that it was closely correlated to activation of peroxisome proliferators activated receptor γ (PPAR- γ). As a super family member of nuclear receptor transcription factor, PPAR has complexly biological functions [28]. According to the structure and function of PPAR, it can be divided into three different subtypes: PPAR- α , PPAR- β and PPAR- γ [29]. Of which PPAR- γ is in the intersection of different signal transduction, which has many biological effects and closely related to the development of tumors [30]. If activated by ligand, PPAR- γ can not only inhibit the proliferation of tumor cells, but also induce tumor cell apoptosis, thus becoming the new target for cancer therapy [31]. In this study, our results showed that c9, t11-CLA could induce transcriptional activity of PPAR- γ and significantly enhance its protein expression level, and consistent with the change trend of Bax, Bcl-2 and Caspase-3, which showed dose- and time- dependent manner. These results suggested that PPAR- γ was associated with apoptosis of tumor

cells. Accordingly, we further speculated that c9, t11-CLA can be used as a regulator to combine PPAR- γ , then activated expression of PPAR- γ . Moreover, we confirmed that there was collaborative relationship between PPAR- γ and apoptosis related protein Bax, Bcl-2 as well as Caspase-3. These results further implied that c9, t11-CLA could combine the upstream factors in Bcl-2 family through PPAR- γ -Bcl-2-Caspase-3 signaling pathway, and then activate or inhibit the expression of its downstream gene, eventually induced apoptosis of HCC cells.

c9, t11-CLA is a normal metabolite in the body with no toxic and side effects, and it is expected to be a new-typed regulator of PPAR- γ in clinical application. So, how to improve the pre-clinical studies of c9, t11-CLA, and how to reasonably develop c9, t11-CLA to a promisingly effective drugs, which will become a developing direction and be a fighting goal in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Keping Wang, Department of Pharmacy, 169 Hospital of PLA, 369 Dongfeng Road, Zhuhui District, Hengyang City 421002, Hunan Province, China. Tel: +86 13875662288; E-mail: 1339036689@qq.com

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