Basic Study

Molecular mechanisms of apoptosis in hepatocellular carcinoma cells induced by ethanol extracts of Solanum lyratum Thumb through the mitochondrial pathway

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Abstract

AIM

To explore the induction effects and mechanism of Solanum lyratum Thumb (ST) on human hepatocellular carcinoma cells induced by ethanol extracts of Solanum lyratum Thumb through the mitochondrial pathway.
carcinoma SMMC-7721 cells through the mitochondrial pathway.

**METHODS**

The experiments were conducted on three groups: an experimental group (with ST ethanol extracts’ concentration being 2.5, 5 and 10 mg/L), a negative control group (with only nutrient solution, 0 mg/L ST ethanol extracts), and a positive control group (2.5 mg/L DDP). The inhibition rate of cell proliferation was checked by using the methyl thiazolyl tetrazolium method, and cell apoptosis was tested by TUNEL method. Furthermore, RT-PCR was used to examine mRNA expression of Fas, FasL, caspase-8, caspase-3, p53 and Bcl-2 in the apoptosis mechanism was related to the expression and mechanism of ST on tumor cells are unclear. The occurrence of tumors is closely related to the abnormality of cell differentiation and is a disordered cell apoptosis. Cell apoptosis is strictly controlled by multiple genes and factors. With the development of the technologies used in molecular biology and proteomics, cell apoptosis is gradually being understood, and some new regulatory genes have been found, with the result that the pathway of cell apoptosis is now better recognized. The mitochondrial pathway is currently recognized as one of the important methods of signal transmission in the process of cell apoptosis. Genes including Fas, FasL, caspase-8, caspase-3, p53 and Bcl-2 are involved in regulation of this pathway. Furthermore, the coordinated network regulation system formed by these genes promotes or inhibits cell apoptosis.

**RESULTS**

Compared with the negative control group, the inhibition and apoptosis rates of the experimental group with different concentrations of ST extracts on human hepatocellular carcinoma SMMC-7721 cells significantly increased ($P < 0.05$). Besides, the mRNA expression of FasL and Bcl-2 significantly decreased ($P < 0.05$) while the mRNA expression of Fas, caspase-8, caspase-3 and p53 increased significantly. When compared with the positive control group, the experimental groups with 5 mg/L ST ethanol extracts showed effects similar to the positive control group.

**CONCLUSION**

ST ethanol extracts induced the apoptosis of hepatocellular carcinoma SMMC-7721 cells through up-regulated Fas, caspase-8, caspase-3 and p53, and down-regulated FasL and Bcl-2 in the mitochondrial pathway.

**Key words**: Solanum lyratum Thumb; Hepatocellular carcinoma cell; Cell apoptosis; Mitochondrial pathway; Molecular mechanism

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**Core tip**: Chinese herbal medicine has a very good effect on the tumor. *Solanum lyratum* Thumb (ST) belonging to Solanacea, is generally used to treat tumors, including liver, gastric, esophageal and bladder cancers, with exact curative effects, and it is a commonly used anticancer drug. However, the effects on tumor cells are unclear. The occurrence of tumors is closely related to the abnormality of cell differentiation and is a disordered cell apoptosis. Cell apoptosis is strictly controlled by multiple genes and factors. With the development of the technologies used in molecular biology and proteomics, cell apoptosis is gradually being understood, and some new regulatory genes have been found, with the result that the pathway of cell apoptosis is now better recognized. The mitochondrial pathway is currently recognized as one of the important methods of signal transmission in the process of cell apoptosis. Genes including Fas, FasL, caspase-8, caspase-3, p53 and Bcl-2 are involved in regulation of this pathway. Furthermore, the coordinated network regulation system formed by these genes promotes or inhibits cell apoptosis.

To date, there is no report on whether ST extracts can induce the apoptosis of hepatocellular carcinoma cells through the mitochondrial pathway or by what mechanism such apoptosis occurs. This research aimed to fill this gap in the current knowledge.

**MATERIALS AND METHODS**

**Materials**

**Tumor cells**: Human hepatocarcinoma SMMC-7721 cells were purchased from the Shanghai Institute of Cell Biology of Chinese Academy of Science, China.

**Main reagents**: ST was purchased from the biological medicine chain in Baise, Guangxi Province, China. RPMI 1640 cultural medium and fetal bovine serum were purchased from Gibco Company, United States. The detection kit for in situ cell apoptosis was sourced from Beijing Zhongshan Jinqiao Biotech Company, China. Methyl thiazolyl tetrazolium (MTT) was produced by Shanghai Pufei Biotech Co., Ltd, China. The polymerase chain reaction (PCR) primer was bought from Sangon Biotech Shanghai Co., Ltd, China. In addition, the Trizol Reagent Kit and the 2 × SYBRGreen qPCR Mix were purchased from Shanghai Invitrogen Company, China and Beijing Zhuangmeng Co., Ltd, China respectively. The RevertAid First Strand cDNA Synthesis Kit and DNase I were obtained from Fermentas, United States.
Ethanol extracts of ST: After being smashed, ST of 50 g was immersed for 3 h at 40 °C in 75% ethanol and filtered. The immersion and filtration were conducted three times. Afterwards, the filter liquors were mixed and dried by using a rotary evaporator, thus obtaining ST extractum.

Setting ST of different concentrations: The ST extractum was dissolved using dimethylsulphoxide (DMSO) to prepare the drug solution with a concentration of 10 mg/mL, which was then diluted by RPMI 1640 cell culture solution to obtain ST extract solutions with concentrations of 0, 2.5, 5 and 10 mg/L.

Medicine intervention for tumor cells: Hepatoma carcinoma SMMC-7721 cells were cultured using the culture solution for 24 h and then the culture solution was discarded. Then, the prepared drug solution containing ST extracts was added to the cells. Moreover, a negative control group and a positive control group were set by adding the drug solution without ST extracts. The preparation of the drug solution was done by adding the drug solution (DMSO) to the cells. Furthermore, a table-top, high-speed freezing centrifuge (1-15PK), a microcentrifuge (Uni Force 6K) and a RT-PCR instrument (IQ5) were also used in this study.

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Main instruments: The main instruments used included a carbon dioxide incubator (MCO-18AIC), a biosafety cabinet (BHC-1300 II A/B33), an automatic microplate spectrophotometer (Multiskan MK3), an inverted microscope (Cioc), and a BX51 microscope (Olympus). Furthermore, a table-top, high-speed freezing centrifuge (1-15PK), a microcentrifuge (Uni Force 6K) and a RT-PCR instrument (IQ5) were also used in this study.

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MTT method: Hepatocellular carcinoma SMMC-7721 cell suspension of 200 μL was put into a culture plate with 96 holes at density of 1 × 10⁴ mL⁻¹ to be cultured for 24 h. Afterwards, the culture solution was removed and discarded, and 200 μL of the prepared ST solutions with different concentrations were added to the culture plate. Four holes were established for each concentra-

Table 1 Reverse transcription polymerase chain reaction primers

| Gene name | Primer sequence | Product length, bp |
|-----------|----------------|-------------------|
| Fas       | Forward: 5′-GAATGCAAGGGACTGATAGC-3′ | 414 |
|           | Reverse: 5′-TGTTCTGTGGCAACTTCGTC-3′ | 239 |
| FasL      | Forward: 5′-GAATGCAAGGGACTGATAGC-3′ | 270 |
|           | Reverse: 5′-CTTCTGTGGCAACTTCGTC-3′ | 427 |
| Caspase-3 | Forward: 5′-GCCTCCGTATCTGCTGCGT-3′ | 180 |
|           | Reverse: 5′-CTTCTGCTGATCTGCTGCGT-3′ | 231 |
| p53       | Forward: 5′-ACACTGGCACCAGCCCTT-3′ | 621 |
|           | Reverse: 5′-GGGCGCGACTTCGTCATACT-3′ | 239 |
| Bcl-2     | Forward: 5′-GCCTCCGTATCTGCTGCGT-3′ | 270 |
|           | Reverse: 5′-CTTCTGCTGATCTGCTGCGT-3′ | 231 |
| β-actin   | Forward: 5′-ACACTGGCACCAGCCCTT-3′ | 621 |
|           | Reverse: 5′-GGGCGCGACTTCGTCATACT-3′ | 239 |
The comparison between ST groups and the negative control group shows that $P < 0.05$, while that of ST groups and the positive control group indicates that $P > 0.05$. ST: Solanum lyratum Thumb.

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(10 μmol/L), 1 μL downstream primer R (10 μmol/L), 7 μL ddH2O and 1 μL template (cDNA). The heat cycle was conducted by pre-denaturation for 2 min at 95 ℃, denaturation for 10 s at 95 ℃, and annealing for 40 s at 60 ℃, for a total of 40 cycles; and (5) relative quantitative analysis method: The $2^{ΔΔCT}$ method was used for relative quantitative analysis of data. The calculation formulae were expressed as: $ΔCT = CT$ target gene - CT reference gene, and $ΔΔCT = ΔCT$ target gene - the average value of target gene $ΔCT$ in the control groups. $2^{ΔΔCT}$ represents the relative expression of target genes.

Statistical analysis
Inhibition and apoptosis rate were analyzed by using SPSS17 software and data were represented by mean ± standard variance (± s). A homogeneity test of variance and one-way ANOVA were used for comparison among groups. As for further pairwise comparison, if the variance was homogeneous, the Student-Newman-Keuls method was used, while Games-Howell was used when the variance was inhomogeneous. Moreover, $χ^2$ was used for the comparison of the rates. If $P < 0.05$, the difference was deemed to have been statistically significant.

RESULTS

**Inhibition rate of hepatocellular carcinoma cells**

The inhibition rate of hepatocellular carcinoma SMMC-7721 cells in the experimental group of each ST concentration was significantly higher than that of the negative control group ($P < 0.05$), and the higher the ST concentration, the higher the inhibition rate. Furthermore, the inhibition rate of the group with 5 mg/L ST was equal to that of the positive control group (Table 2).

**Apoptosis rate of hepatocellular carcinoma cells**

The apoptosis rate of hepatocellular carcinoma SMMC-7721 cells in each ST group with different concentrations was significantly higher than that of the positive control group ($P < 0.05$). The higher the ST concentration, the higher the cell apoptosis rate. Moreover, the apoptosis rate of the group with 5 mg/L ST was identical to that of the positive control group (Table 3).

**Influences of ST on the mRNA expressions of genes relating to hepatocellular carcinoma cells**

After intervention of ST extracts in each concentration on the hepatocellular carcinoma SMMC-7721 cells, the mRNA expressions of FasL and Bcl-2 decreased markedly ($P < 0.05$) and the reductions were negatively correlated with the ST concentration. However, the mRNA expressions of Fas, caspase-8, caspase-3 and p53 increased and the increase showed positive correlation with the ST concentration. When the concentration was greater than, or equal to, 5 mg/L, the mRNA expressions increased ($P < 0.05$). Moreover, the mRNA expressions of these genes in 5 mg/L ST group were equivalent to those of the positive control group, as demonstrated in Figure 1 and Tables 4 and 5.

DISCUSSION

ST has functions including cooling and dehumidifying, detoxifying, detumescence and anticancer effects, and it can cure cold and fever, icteric hepatitis, gallstone disease, cholecystitis, nephritis and uterine erosion. Clinically, it has certain effects on the treatment of various types of cancers, especially for lung, liver, stomach and cervical cancers[8-10]. The experimental results show that the inhibition and apoptosis rates of hepatocellular carcinoma SMMC-7721 cells in ST groups with different concentrations were significantly higher than those of the negative control group and showed positive correlation with the ST concentration,

| Group                          | Concentration, mg/L | n  | OD value        | Inhibition rate, % |
|--------------------------------|---------------------|----|----------------|-------------------|
| Three ST groups with different | 2.5                 | 12 | 1.516 ± 0.205  | 18.22 ± 2.32      |
| concentrations                 | 5                   | 12 | 1.242 ± 0.236  | 36.56 ± 2.51      |
|                                | 10                  | 12 | 0.815 ± 0.276  | 55.98 ± 3.12      |
| Negative control group         | 0                   | 12 | 1.842 ± 0.183  | -                 |
| Positive control group         | 2.5                 | 12 | 1.135 ± 0.172  | 38.60 ± 1.78      |

**Table 2** Comparison of the inhibition rates of proliferation of hepatocellular carcinoma SMMC-7721 cells in the groups with different Solanum lyratum Thumb concentrations, $x ± s$.

| Group                          | Concentration, C/mg•L | Apoptosis rate of SMMC-7721 cells, % |
|--------------------------------|-----------------------|-------------------------------------|
| Three ST groups with different | 2.5                   | 10.75 ± 2.51                         |
| concentrations                 | 5                     | 17.31 ± 2.33                         |
|                                | 10                    | 30.21 ± 3.62                         |
| Negative control group         | 0                     | 3.75 ± 1.23                          |
| Positive control group         | 2.5                   | 17.36 ± 1.62                         |

**Table 3** Comparison of apoptosis rates of hepatocellular carcinoma SMMC-7721 cells in different groups, $x ± s$.
confirming that ST can inhibit the growth of hepatocellular carcinoma SMMC-7721 cells. However, the molecular mechanism of this anti-hepatoma behavior is not clear and requires further clarification.

At present, cell apoptosis has been studied and found to be a normal metabolic procedure; however, if the apoptosis process is disordered, a lot of diseases, tumors for instance, can appear. Cell apoptosis is a multi-factor and multi-pathway process regulated by a network, involving a series of proteins, such as

![Figure 1](image-url)
caspase family proteins, Bcl-2 family proteins, p53 protein and survivin. Therefore, the exact mechanism of apoptosis is unclear, while the intrinsic and extrinsic pathways of apoptosis have been clarified[11]. As shown in Figure 2, the extrinsic pathway is triggered by death receptors such as Fas and tumor necrosis factor receptor (TNF-R) on the surface of cells[12,13]. While the intrinsic or mitochondrial pathway is induced by many stress conditions, chemical treatment reagents, and medicines.

Under physiological or pathological conditions, no matter whether the apoptosis is induced by DNA damage or other receptors relating to organelles, various receptors affect the intrinsic pathway of apoptosis[14,15]. In the intrinsic pathway of cell apoptosis, the mitochondria control cell activities and are important in regulating cell apoptosis. There are two main changes in mitochondria in the early stage of apoptosis. One is the decrease of mitochondrial internal transmembrane potential, and the other is the increase of mitochondrial external transmembrane permeability.

For the change in interaction of these two aspects, if the difference in the internal and external potentials of the mitochondrial membrane decreases, the mitochondrial transmembrane potential decreases and then the mitochondrial membrane permeability increases, resulting in the release of caspase activated cytochrome C and the activation of caspase protein. The activated caspase affects other protein substrates in the cells, leading to cascade reaction of apoptosis and finally cell apoptosis[16-18].

Caspase, as a cysteine-aspartic specific protease, can excise fragments containing aspartic acids. So far, at least 14 sub-types of caspase with similar molecular structure and high homology have been found. According to their functions, the sub-types can be divided into two categories, namely, initiator and effector caspases. Moreover, caspase plays an essential role in apoptosis. The initiator caspase acts on the inactive effector caspase, thereby activating the effector caspase.

Caspase-8, belonging to the initiator caspase sub-type, is the initiator of the cascade reaction of cell apoptosis. It can self-activate and transmit apoptotic signals in the participation of other proteins and activate downstream effector caspase, thus forming a cascade amplification system to induce cell apoptosis. Caspase-3 (being subjected to the effector caspase) is the most important final excision enzyme and is also an important part of the killing mechanism of cytotoxic T lymphocyte (CTL) cells and can be activated by a variety of factors. As to the killing effects on CTL cells, caspase-3 can be activated by the Fas/FasL pathway and the B pathway of granzymes. When caspase-8, upstream of cells, is activated, the activated caspase-3 excises poly (ADP-ribose) polymerase (PARP) into two fragments, separating two zinc fingers binding with DNA in PARP from the C-terminal catalytic region to influence its normal function. As a result, the activity of Ca/Mg-dependent endonuclease is adversely affected by PARP increases, so that the DNA between nucleosomes is cracked, leading to cell apoptosis[19-21].

p53, as a tumor suppressor gene, slows down or monitors cell division and the integrity of the genome.
when checking DNA damage loci in the G1 phase. If there is damage, the p53 protein prevents DNA replication and provides sufficient time to repair the damaged DNA\cite{22}.

Bcl-2, as a kind of negative regulatory gene of cell apoptosis, can control the membrane potential by changing the redox state of mitochondrial thiols. Furthermore, it can also regulate the permeability of the mitochondrial membrane for some apoptosis protein precursors and locate apoptosis protein precursor Apaf-1 on the mitochondrial membrane to stop apoptosis\cite{23}.

Fas and FasL are membrane surface molecules, which regulate the apoptosis induced by toxicity in T-cell development\cite{24}. FasL, with its high-level expression on cell surfaces, identifies Fas on the target cell surface after CTL cells recognize target cells. Then, by triggering the apoptosis process in the interior of target cells using Fas, the programmed cell death of target cells occurs\cite{24,25}.

It can be found from this experiment that, after the intervention of ST extracts with different concentrations on hepatocellular carcinoma SMMC-7721 cells, the inhibition and apoptosis rates of the cells increased significantly ($P < 0.05$). This indicated that ST inhibited hepatocellular carcinoma cells. The mRNA expressions of FasL and Bcl-2 were markedly reduced ($P < 0.05$), which showed that the negative feedback regulation protected CTL cells to certain extent. In addition, the mRNA expressions of Fas, caspase-8, caspase-3 and p53 increased. Compared with the positive control group, the effects of 5 mg/L ST extracts were equivalent to those of the positive control group, which suggested that these genes were positive regulatory genes for apoptosis of hepatocellular carcinoma cells induced by ST through the mitochondrial pathway.

Therefore, ST regulated hepatocellular carcinoma SMMC-7721 cells by the combination of Fas/FasL on the surface of the cells with that on the surface of T cells. The mitochondrial pathway was then stimulated to transmit apoptosis signals, which activated the upstream caspase-8 and further the downstream caspase-3. In this way, a cascade amplification system was formed to induce apoptosis. In addition, the apoptosis process was affected by the synergistic action of tumor suppressor gene p53 and negative feedback gene Bcl-2 for apoptosis, jointly forming the regulation network of ST inducing apoptosis of hepatocellular carcinoma SMMC-7721 cells, as shown in Figure 3.

Although this experiment verified that ST can induce the apoptosis of hepatocellular carcinoma SMMC-7721 cells, the apoptosis mechanism was related to the expression of Fas, FasL, caspase-8, caspase-3, p53 and Bcl-2 in the mitochondrial pathway. This result provides powerful evidence of the improved apoptosis effects of ST on hepatocellular carcinoma cells; however, due to only a few genes being involved in apoptosis, the regulatory effects of other genes were unable to be determined here. Moreover, the apoptosis may be related to other proteins in the caspase family, so the molecular mechanism of the apoptosis induced by ST is not completely explained and needs to be further verified by other studies.

### Comments

#### Background

Solanum lyratum Thumb (ST) belonging to Solanaceae is generally used to treat tumors, and it is a commonly used anticancer drug. However, the effects and mechanism of ST on tumor cells are unclear.

#### Researchfrontiers

Precision treatment of Chinese herbal medicine is the focus of the current research and hot spots of investigations. This study verified the mechanism of Chinese herbal medicine by using molecular biological technology, providing powerful evidence of the improved apoptosis effects of Solanum lyratum Thumb on hepatocellular carcinoma cells.

#### Innovations and breakthroughs

The experiment verified that ST ethanol extracts up-regulated Fas and down-regulated FasL in the mitochondrial pathway, inducing the up-regulation for the expression of caspase-8 and caspase-3. In addition, ST ethanol extracts induced the apoptosis of hepatocellular carcinoma SMMC-7721 cells through feedback regulation by up-regulating the p53 gene that inhibits cancers and down-regulating the Bcl-2 gene.

#### Applications

These findings provide powerful evidence of the improved apoptosis effects of ST on hepatocellular carcinoma cells.

#### Peer-review

This study is well designed and the results are interesting. The authors try to explore the induction effects and mechanism of ST on human hepatocellular carcinoma SMMC-7721 cells through the mitochondrial pathway.

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