ISSN 2616-5791 Vol.4, Issue 12: 20-26, DOI: 10.25236/AJMHS.2023.041203

The Effect and Mechanism of Timosaponina-III from Anemarrhena Asphodeloides on Proliferation and Apoptosis of Human Liver Cancer Cell Line MHCC97H

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Abstract: *To explore the effects and mechanism of TimosaponinA-III (Tim-AIII) on the proliferation and apoptosis of human liver cancer cells MHCC97H, an in vitro culture of MHCC97H cells was conducted. The logarithmic growth phase MHCC97H cells were divided into control group, Tim-AIII group, Tim-AIII + pc-DNA group, and Tim-AIII + pc-TGF-β1 group: the control group was MHCC97H cells, the Tim-AIII group was added with 40 nmol/L Tim-AIII, the Tim-AIII + pc-DNA group was transfected with blank control plasmid vector and added with 40 nmol/L Tim-AIII, and the Tim-AIII + pc-TGF-β1 group was transfected with TGF-β1 overexpression plasmid TGF-β1-OE and added with 40 nmol/L Tim-AIII. After culturing for 24 hours, the cloning formation experiment was used to detect the proliferation of each group of cells. Flow cytometry was used to detect the apoptosis rate of each group of cells. Western blot was used to detect the expression levels of TGF-β1 and ERK protein in each group of cells. The results showed that the numbers of clones in the control group, Tim-AIII group, Tim-AIII + pc-DNA group, and Tim-AIII + pc-TGF-β1 group were 221.69 ± 29.71, 32.18 ± 5.82, 31.92 ± 7.27, and 112.28 ± 23.42, respectively; the apoptosis rates were 13.60% ± 1.35%, 29.10% ± 2.71%, 30.00% ± 3.63%, and 21.70% ± 1.99%, respectively. Compared with the control group, the proliferation rate, clone number, and apoptosis rate of the Tim-AIII group and Tim-AIII + pc-DNA group were significantly different ($P < 0.05$); compared with the Tim-AIII group and Tim-AIII + pc-DNA group, the proliferation rate, clone number, and apoptosis rate of the Tim-AIII + pc-TGF-β1 group were significantly different ($P < 0.05$). Compared with the control group, the relative expression levels of TGF-β1 and ERK protein in the Tim-AIII group were significantly decreased ($P < 0.05$), while the relative expression levels of TGF-β1 and ERK protein in the pc-TGF-β1 group were significantly increased ($P < 0.05$); compared with the pc-TGF-β1 group, the relative expression levels of TGF-β1 and ERK protein in the Tim-AIII + pc-TGF-β1 group were significantly decreased ($P < 0.05$); compared with the Tim-AIII group, the relative expression levels of TGF-β1 and ERK protein in the Tim-AIII + pc-TGF-β1 group were significantly increased ($P < 0.05$). These results suggest that Tim-AIII can inhibit the proliferation of human liver cancer cells MHCC97H and promote apoptosis, which may be related to inhibiting the TGF-β1/ERK signaling pathway.*

Keywords: *Liver cancer; TimosaponinA-III; Proliferation; Apoptosis; TGF-β1/ERK*

1. Introduction

Liver cancer is the fourth most common malignant tumor worldwide, with hepatocellular carcinoma being the most frequent form[1]. Due to atypical symptoms and rapid growth, most liver cancer patients are diagnosed at an advanced stage, resulting in a median survival time of less than ten months and a five-year overall survival rate of 10%[2]. Liver cancer is typically caused by alcohol, non-alcoholic fatty liver disease (NAFLD), cirrhosis, and viral infections (including hepatitis B virus (HBV) and hepatitis C virus (HCV))[3]. The current main treatment for liver cancer is surgical resection of the tumor tissue, with multikinase inhibitor sorafenib being the only first-line drug[3]. Therefore, exploring new treatment methods for liver cancer is particularly important. Natural product timosaponin A III (Tim-A III) is used in traditional Chinese medicine to treat joint pain, blood heat, nocturnal fever, and night

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sweats[4]. Tim-A III is a steroid saponin and the main active ingredient of timosaponin. It exhibits various pharmacological activities, such as anti-cancer, anti-neurodegenerative, and anti-inflammatory effects[5, 6]. Tim-A III has been shown to mediate the impact of cancer, particularly in the treatment of cervical cancer and breast cancer, which is considered to have the most potential[7, 8]. However, little is known about the role, target, and potential molecular mechanism of Tim-A III in the treatment of liver cancer. Currently, there are few studies applying Tim-A III to the treatment of liver cancer, but the occurrence and development of liver cancer also involve mechanisms such as DNA synthesis and repair, cell proliferation, and hypoxia. This makes it possible to apply Tim-A III to the treatment of liver cancer. In this study, we investigated the effect of Tim-A III on the proliferation and apoptosis of human liver cancer cells MHCC97H and explored its mechanism.

2. Materials and Methods

2.1 Materials

Tim-A III (HY-N2830) was purchased from MCE Company (USA). Human liver cancer cells MHCC97H were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Annexin V-FITC/PI Kit was purchased from Hangzhou Union Biotechnology Co., Ltd. Antibodies TGF-β1 (ab215715) and ERK (ab32537) were purchased from abcam Company (UK). TGF-β1 overexpression plasmid pc-TGF-β1 and blank control plasmid pc-DNA were purchased from Guangzhou Ruibo Biotechnology Co., Ltd.

2.2 Culture, Grouping, and Plasmid Transfection of Human Liver Cancer Cells MHCC97H

After Resuscitation of liver cancer cells MHCC97H in liquid nitrogen, they were added to DMEM medium (containing 10% fetal bovine serum, 1% penicillin-streptomycin) and cultured at 37℃ with 5% CO2. When the cells adhered to the wall more than 80%, they were passaged for the first time. After three passages, logarithmic growth phase MHCC97H cells were selected for the following experiments. The logarithmic growth phase MHCC97H cells were divided into four groups: control group, Tim-A III group, Tim-A III + pc-DNA group, and Tim-A III + pc-TGF-β1 group. The control group only contained MHCC97H cells, while the Tim-A III group was added with 40 nmol/L Tim-A III. The Tim-A III + pc-DNA group was transfected with the blank control plasmid pc-DNA using Lipofectamine 2000 and added with 40 nmol/L Tim-A III. The Tim-A III + pc-TGF-β1 group was transfected with the Snail overexpression plasmid pc-TGF-β1 using Lipofectamine 2000 and added with 40 nmol/L Tim-A III. The cells were cultured for another 24 hours before the experiment.

2.3 Measurement of Cell Proliferation Activity of Human Liver Cancer Cells MHCC97H

The MTT method was used. Each group of cells was inoculated in 96-well plates with six replicate wells. After culturing for 0, 24, 48, and 72 hours, 0.5% MTT solution was added, cultured at 37℃ for 4 hours, and then the culture medium was removed. DMSO (100 μL/well) was added and the plates were shaken at a constant temperature of 37℃ for 10 minutes. The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay reader, and the cell activity was expressed as OD value.

2.4 Clone Formation Experiment of Human Liver Cancer Cells MHCC97H

The plate cloning method was used. Each group of cells was inoculated in a 6-well plate at a density of 800 cells per well and cultured at 37℃ with 5% CO2 for 14 days. The culture medium was changed every three days, and after culturing, the original culture medium was discarded. The cells were washed three times with PBS, fixed with 4% paraformaldehyde for 30 minutes, stained with 0.1% crystal violet for 15 minutes, washed again, observed under a microscope, and counted using Image J software.

2.5 Measurement of Apoptosis Rate of Human Liver Cancer Cells MHCC97H

Flow cytometry was used. After collection of each group of cells, they were resuspended in Binding Buffer solution (100 μL), stained with Annexin V-FITC solution (5 μL) for 5 minutes in the dark at room temperature, followed by staining with PI solution (10 μL) for 10 minutes in the dark at room temperature before analysis by flow cytometry.

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2.6 Detection of TGF-β1 and ERK Protein Expression

Protein immunoblotting was used. Cells from each group were collected after intervention for 24 hours, protein extracted and quantified, and electrophoresis performed using SDS-PAGE gel electrophoresis, followed by transfer to a PVDF membrane. After blocking for 2 hours at room temperature according to the antibody manual, a diluted primary antibody solution (TGF-β1, ERK, and GAPDH) was added and incubated overnight at 4℃ before washing with TBST three times. Then a goat anti-rabbit IgG secondary antibody was added and incubated for another.

2.7 Statistical methods

The statistical analysis of the results was carried out using GraphPad Prism 10.0 software. t-tests and one-way ANOVA were used to detect differences in indicators between groups, with data presented as mean ± standard deviation. P< 0.05 was considered statistically significant.

3. Results

3.1 Concentration selection of Tim-AIII

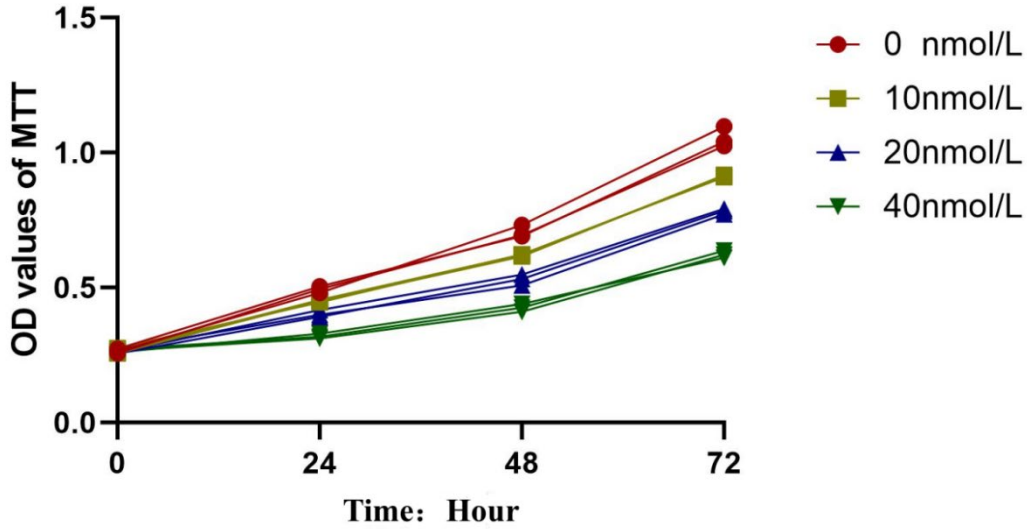

Figure 1: Cell OD values at different time points for each concentration

Comparison of cell OD values at different time points for each concentration (Figure 1). To ensure that Tim-AIII has a significant effect on MHCC97H cells, a dose of 40 nmol/L of Tim-AIII was selected for subsequent experiments.

3.2 Comparison of proliferation abilities of MHCC97H human liver cancer cells in each group

The control group, Tim-AIII group, Tim-AIII + pc-DNA group, and Tim-AIII + pc-TGF-β1 group had 221.69 ± 29.71, 32.18 ± 5.82, 31.92 ± 7.27, and 112.28 ± 23.42 clones, respectively. Compared with the control group, the Tim-AIII group and the Tim-AIII + pc-DNA group had significantly reduced clone numbers ($P < 0.05$). Compared with the Tim-AIII group and the Tim-AIII + pc-DNA group, the Tim-AIII + pc-TGF-β1 group had significantly increased clone numbers ($P < 0.05$) (Figure 2).

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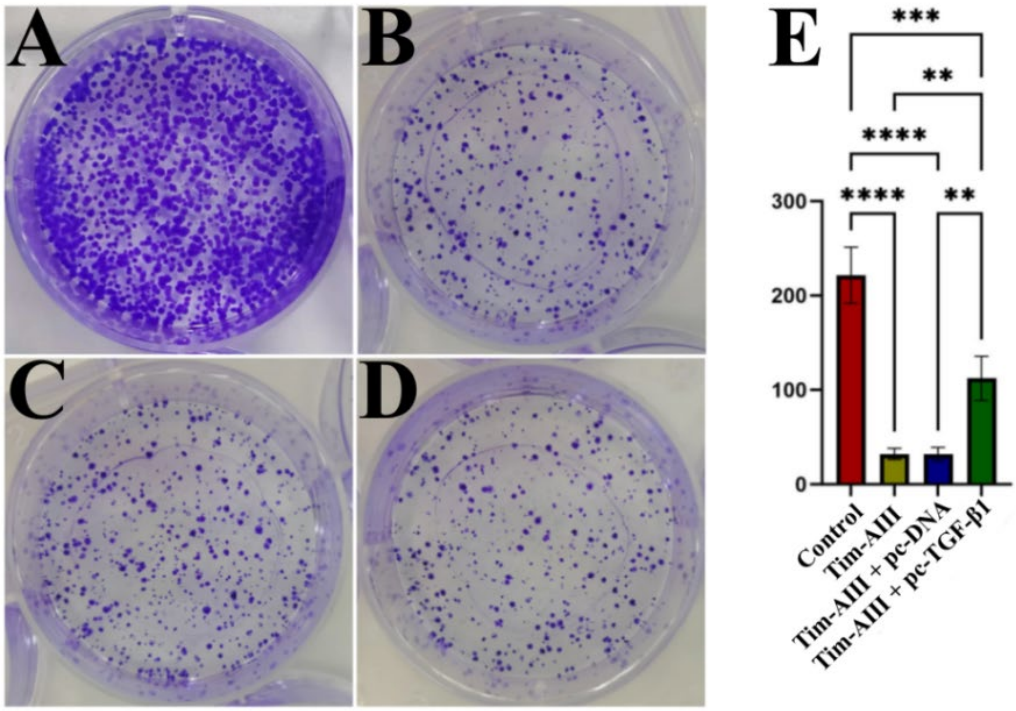

Figure 2: (A) Proliferation of MHCC97H cells in the control group. (B) Proliferation of MHCC97H cells in the Tim-AIII group. (C) Proliferation of MHCC97H cells in the Tim-AIII + pc-DNA group. (D) Proliferation of MHCC97H cells in the Tim-AIII + pc-TGF-β1 group. (E) Comparison of proliferation abilities of MHCC97H cells in each experimental group.

3.3 Comparison of apoptosis rates of MHCC97H human liver cancer cells in each group

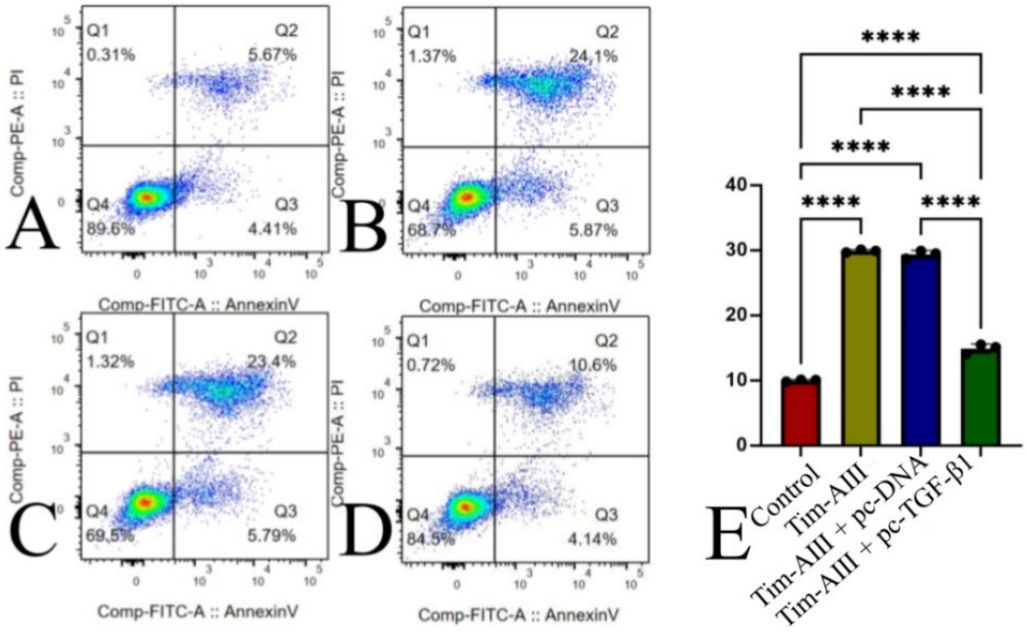

Figure 3: Apoptosis rates of MHCC97H cells in each experimental group. (A) Control group. (B) Tim-AIII group. (C) Tim-AIII + pc-DNA group. (D) Tim-AIII + pc-TGF-β1 group. (E) Comparison of apoptosis rates in each group.

The control group, Tim-AIII group, Tim-AIII + pc-DNA group, and Tim-AIII + pc-TGF-β1 group

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had apoptosis rates of 13.60% ± 1.35%, 29.10% ± 2.71%, 30.00% ± 3.63%, and 21.70% ± 1.99%, respectively. Compared with the control group, the Tim-AIII group and the Tim-AIII + pc-DNA group had significantly increased apoptosis rates ($P < 0.05$). Compared with the Tim-AIII group and the Tim-AIII + pc-DNA group, the Tim-AIII + pc-TGF-β1 group had a significantly reduced apoptosis rate ($P < 0.05$) (Figure 3).

3.4 Comparison of TGF- β1 and ERK protein expression in MHCC97H human liver cancer cells in each group

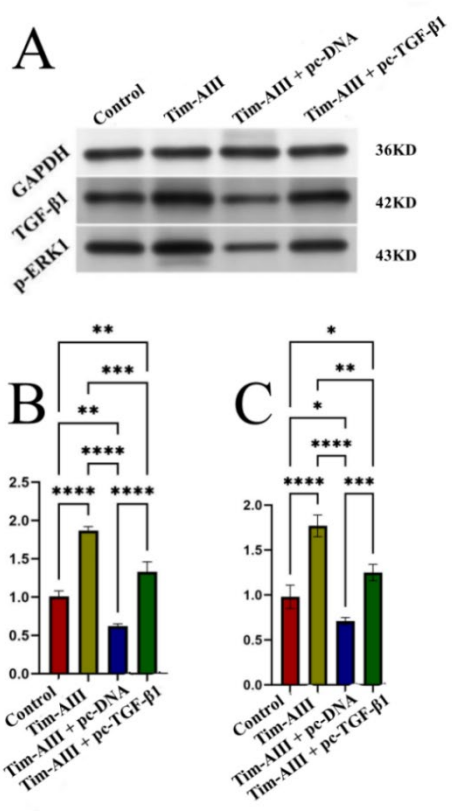

Figure 4: (A) Expression levels of GAPHD, TGFβ1, and Erk proteins. (B) Relative protein expression levels of TGFβ1 in each experimental group. (C) Relative protein expression levels of Erk in each experimental group.

Compared with the control group, the Tim-AIII group had significantly reduced TGF-β1 and ERK protein expression levels ($P < 0.05$), while the pc-TGF-β1 group had significantly increased TGF-β1 and ERK protein expression levels ($P < 0.05$). Compared with the pc-TGF-β1 group, the Tim-AIII + pc-TGF-β1 group had significantly reduced TGF-β1 and ERK protein expression levels ($P < 0.05$). Compared with the Tim-AIII group, the Tim-AIII + pc-TGF-β1 group had significantly increased TGF-β1 and ERK protein expression levels ($P < 0.05$) (Figure 4).

4. Conclusion

Tim-AIII can inhibit the proliferation and promote the apoptosis of human liver cancer cells MHCC97H. One possible mechanism of its action is through inhibiting the TGF-β1/ERK signaling axis.

5. Discussion

The combination of modern science and technology with traditional medicine is receiving increasing attention in the diagnosis and treatment of diseases. Anemarrhena asphodeloides Bunge (Tim-AIII), a traditional medicine used in China and many Asian countries for thousands of years, is a saponin isolated from the root of Anemarrhena asphodeloides[9]. Tim-AIII has a wide range of

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biological activities, including the ability to inhibit the production of superoxide anions by human neutrophils induced by arachidonic acid and to increase the intracellular concentration of Ca2+ in endothelial cells[10][11]. Previous studies have shown that Tim-AIII has potential anti-cancer effects, playing an important role in inhibiting cancer cell proliferation, promoting apoptosis, inducing autophagy, and reversing multidrug resistance[12]. Sy et al. reported that Tim-AIII induced autophagy in human cervical HeLa cells, leading to mitochondrial pathway-dependent apoptosis[7]. Other studies have shown that Tim-AIII can reverse multidrug resistance in leukemia by inhibiting the PI3K/Akt signaling pathway and downregulating the expression and function of MRP1 transcription[13]. Kang et al. confirmed that Tim-AIII can inhibit the proliferation of human colon cancer cells, induce cell cycle arrest, and promote apoptosis[14]. Inhibition of cell proliferation and promotion of apoptosis are prerequisites for maintaining cellular homeostasis, while inhibiting tumor cell proliferation and promoting tumor cell apoptosis are effective methods for cancer treatment. Therefore, studying the role of Tim-AIII in inducing apoptosis and inhibiting tumor proliferation is of great significance for exploring its pathogenic mechanism and treating tumors.

Transforming growth factor β (TGF-β) plays important and complex roles in liver cancer and gastrointestinal tumors. These functions include regulation of multiple processes such as cell proliferation, differentiation, and immune modulation to maintain stem cell homeostasis, promote fibrosis, immune modulation, and inhibit tumors[15]. TGF-β can participate in normal physiological processes in normal cells and can also promote tumor development as a tumor promoting factor[16]. TGF-β1 is the most studied member of the TGF-β superfamily and is closely related to tumor development[17]. Research has shown that miR-494 targets SIRT3 and regulates the SIRT3/TGF-β/SMAD signaling pathway to become a key mediator of EndMT and HCC development[18]. Wu et al. verified that the TGF-β/SMAD signaling pathway is abnormally activated in hepatocellular carcinoma, suggesting that TGF-β/SMAD/lnc-UTGF forms a positive feedback loop that regulates HCC development[19]. Ihling et al. suggest that inhibiting PD-L1+ ic and TGF-β activity as well as their respective immune regulatory pathways may contribute to the anti-tumor effects of HCC[20]. This suggests that TGF-β plays a crucial role in liver cancer development.

In this study, we used Western blot to detect the effect of Tim-AIII on TGF-β1 protein expression and found that Tim-AIII can inhibit TGF-β1 activity. The TGF-β1/ERK pathway is a non-Smad dependent TGF-β signaling pathway. TGF-β1 can activate the MAPK/ERK signaling pathway, triggering a cascade of reactions and producing corresponding biological behaviors[21]. Our experiments also showed that Tim-AIII reduced the relative expression levels of TGF-β1 and ERK protein, indicating that Tim-AIII has an inhibitory effect on the TGF-β1/ERK signaling pathway in human liver cancer cells MHCC97H.

In summary, we analyzed the effect of Tim-AIII on human liver cancer cells MHCC97H and further studied its potential molecular mechanism. We found that Tim-AIII mainly inhibits proliferation to exert its anti-tumor activity and inhibits MHCC97H proliferation through the TGF-β1/ERK signaling pathway. This provides a reference for future research. The characteristic change of apoptosis is the morphological change of cells, which is a programmed cell death process that includes chromatin condensation and nuclear fragmentation while maintaining intact cell membranes and organelles[22]. The mechanism of apoptosis includes signal transduction mechanisms, mitochondrial apoptotic mechanisms, related enzyme mechanisms, gene mechanisms, etc. However, this experiment did not further study the specific mechanism of apoptosis in human liver cancer cells MHCC97H. Future experiments can continue to conduct complementary research. Next, we should continue to explore whether there are other signaling pathways regulated by Tim-AII that regulate liver cancer cell proliferation and apoptosis to provide reference for liver cancer treatment.

Acknowledgement

Funding: This work was supported by Sanya Science and Technology Innovation Fund (2016YW43)

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ISSN 2616-5791 Vol.4, Issue 12: 20-26, DOI: 10.25236/AJMHS.2023.041203

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