Inhibiting the Expression of Cyclin D_1 Protein to Reduce the HBV-DNA Level in CHB patients

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Abstract

HBV virus is now affecting over 400 million people worldwide. Chronic Hepatitis B (CHB) is one of the significant clinical symptoms of HBV infection. Previous studies have reported that antiviral therapy is a practical method for patients. However, the current treatment has reached a bottleneck due to economic burden and drug resistance. As a practical component in ligustrum lucidum, ursolic acid has been found to have an antiviral effect. This study focuses on one particular mechanism of its effect. It tests its function in reducing the expression of Cyclin D1 protein to provide a possible reason for its ability to inhibit cell proliferation.

Keywords: TCM, HBV, CHB, ursolic acid, Cyclin D_1 protein, ERK signaling pathway

1. Introduction

HBV always refers to the Hepatitis B virus, which is now affecting over 400 million subjects worldwide [1]. In China, approximately 60%~70% of people have a history of HBV infection, among which about 9% are developed further into Chronic Hepatitis B (CHB) [2]. After the infection, if it is allowed to progress, the patient will potentially develop chronic viral hepatitis B, severe hepatitis, cirrhosis, or even liver cancer [3]. Research has already proved that viral load quantitation has a very vital correlation with the lymphocyte immune process in patients after HBV invasion. According to the REVEAL study from Taiwan, China, viral suppression is closely related to the disease progression in the treatment of CHB: the lower the viral load, the lower the risk of its further development into cirrhotic Hepatocellular Carcinoma (HCC) [4]. This study of the natural history of HBV infection revealed that HBV DNA levels were associated with the development of cirrhosis and HCC. Besides, the study conducted by Liu's team also confirmed that HBV-DNA levels are a good indicator for assessing the degree of liver fibrosis and cirrhosis in patients with HBV-related liver disease, especially in HBeAg-positive patients [5]. Therefore, to reduce the incidence of HBV infection-related mortality, and the morbidity of liver fibrosis, cirrhosis, and liver cancer, antiviral therapy can be considered a good solution for CHB patients. Currently, the main therapeutic drugs in clinical use are *Interferon (IFN), Lamivudine (LAM), Entecavir (ETV), etc*. However, due to the long duration of chronic hepatitis B, high relapse rate, and poor prognosis, the long-term drug therapy will aggravate the economic burden on patients, slowly increase drug toxic side effects and drug resistance, resulting in virus mutation, as well as increasing the difficulty of clinical treatment [6].

Traditional Chinese Medicine (TCM) has been used for CHB treatment for centuries. And in the scientific community, the TCM method emphasizes overall coordination and dialectical treatment, which on the one hand adjusts the functions of organs and tissues, improves the immunity of the body, and increases body resistance; on the other hand, it exerts cytotoxic effects to kill cancer cells. Clinically, CHB was named as stagnancy of liver-qi and blood or hot liver. A study showed that TCMs had a similar beneficial effect when compared with IFN or LAM for CHB on the antiviral activity as evidenced by the loss of serum HBeAg and HBV-DNA [7]. Meanwhile, as an empirical Chinese medicine formula for CHB, the Bushen formula has been shown to have the effect of reducing serum ALT and HBV DNA levels[1]. However, since the complex composition of herbal medicines in the Bushen formula, the exact mechanism by which the drugs exert their pharmacological effects has not yet been well defined, which has become a research bottleneck in the current study of compounding and splitting formula protocols for the treatment of CHB with Chinese medicine.

As one herb in the Bushen formula, Ligustrum lucidum plays an important role in anti-tumor and liver protection [8]. Its main component, ursolic acid, has been proven to have anti-tumorigenic and liver-protecting effects and inhibit the growth of many malignant tumor cells, and its derivatives also have inhibitory activity against viruses [9]. In recent years, with the in-depth research on the anti-tumor properties of ursolic acid, it has been found that ursolic acid has the characteristics of low anti-tumor toxic side effects [10]. According to Zhu's review, the main

anti-tumor mechanisms of ursolic acid that have been clinically confirmed so far are：

“Induction of apoptosis, inhibition of tumor cell proliferation, blocking cell cycle, tumor drug sensitization, and reversal of drug resistance, induction of autophagy in tumor cells, inhibition of tumor cell growth through regulation of oncogenes and oncogenes, cytotoxic effects, inhibition of tumor metastasis and invasion, etc [10].”

However, because of its multiple mechanisms of action and a broad spectrum of tumor inhibition, some scholars have suggested that the mechanism of action of ursolic acid may be different for different cell line types [11]. So, it is important to study the concrete mechanism of action of ursolic acid and clarify the actual sites of its recognition. In the currently available literature, only Li’s team has published that they have isolated ursolic acid from the n-butanol and chloroform fractions of the heartwood of Streblus asper and confirmed its anti-HBV activity by primary bioassays [12]. But the specific mechanism of action and the effect of ursolic acid on the proliferation of HBV-infected cells have not yet been reported in both literature and clinical trials. According to Lei’s experiment, ursolic acid can stop the cell cycle of hepatocellular carcinoma cells in the G1 phase by inhibiting the activation of Cyclin D1, and preventing the cell cycle from progressing through G0/G1 to the S phase, thus achieving a complete arrest of hepatocellular carcinoma cell growth and inhibiting their proliferation [11].

Therefore, I came up with my research question: Does ursolic acid potentially reduce the HBV DNA level in CHB patients via interfering with the interaction between Cyclin D_1 protein and the ERK signaling pathway? And I predict that ursolic acid can inhibit the expression of Cyclin D_1 protein so as to stop the cell cycle in the G_1 phase and block the cell cycle from G_0/G_1 to the S phase. By stopping the growth of the cells that were infected by HBV and inhibiting their proliferation, the HBV DNA level in CHB patients could then be reduced.

2. Methods

2.1 *Measuring the HBV-DNA level Animal Model*

Note. In the field of HBV research, the HBV virus usually cannot infect ordinary mice, and the main methods commonly used are HBV transgenic mice and transfection models established by the high-pressure hydrodynamic method [13]. But, because transgenic animals are born with the virus, their immune tolerance patterns may differ significantly from those caused by infection, and therefore are not suitable as an animal model for this experiment. So, when designing the experiment for this section, we choose the high-pressure hydrodynamic method to establish the hepatitis B mice model.

It has been demonstrated that the pAAV-HBV1.2 plasmid can be used to establish a mouse model of hepatitis B [13]. The plasmid point mutation kit is purchased from Takara Bio Inc., the point mutation primers are synthesized by BioSune Biotechnology (Shanghai) Co., Ltd, and SPF-grade 5- to 6-week-old male C57BL/6 mice are provided by the Laboratory Animal Department of Shanghai Public Health Clinical Center [13].

As is shown in *Table.1*, the mice will be divided into five groups of 6 based on the negative/positive controls and the different concentration gradients of ursolic acid. According to the experiment conducted by Wang’s team, the semi-lethal dose LD_{50} is 22.68mg/kg [14]. So, this study will design three experimental groups including: Low-dose group (1/20 LD_{50} , 1.13mg/kg), Medium-dose group (1/10 LD_{50} , 2.27mg/kg), High-dose group (1/5 LD_{50}, 4.54mg/kg). Based on the data from Qiao’s experiment, we choose lamivudine (LAM) to conduct our positive control group (10mg/kg) [15] 它具有抗逆转录病毒 , 抗疟疾和抗炎特性。该研究主要目的是观察白桦脂酸对 Payw1.3 质粒急性感染小鼠模型的 HBV DNA 复制的抑制作用。实验的 Payw1.3 质粒急性感染小鼠模型 (n=15. And the negative control group (blank group) will be injected with the same dose of saline at the same time until the end of the experiment. Peripheral blood will then be extracted from mice in each group at 24, 48, and 72 hours of incubation, respectively.

Table 1. Animal model

		Controls
Negative	Group 1	Saline
Positive	Group 2	Lamivudine (LAM), 10mg/kg
Experimental	Group 3 (Low dose)	1/20 LD_{50} , 1.13mg/kg
	Group 4 (Medium dose)	1/10 LD_{50} , 2.27mg/kg
	Group 5 (High dose)	1/5 LD_{50} , 4.54mg/kg

RT-PCR

Serum HBV DNA load in peripheral blood is measured with real-time PCR. The handling procedures are performed in strict accordance with the instructions in the reagent Kit (Shenzhen PG Biotech Co., Ltd.). The RNA is treated with RNase-free DNase I (Takara Bio

Inc.) and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega). Real-time PCR is performed on a Mastercycler ep relplex 4 real-time PCR system (Eppendorf) with an SYBR Green qPCR Master Mix (Fermentas), according to the manufacturer's protocol. The serum ALT levels are assayed by DXC 800 Fully-auto Bio-Chemistry Analyzer.

2.2 *Measuring the apoptosis and proliferation rate of the cells and the expression of Cyclin D1 protein*

Note. In this section, we decide to switch our experimental sample from the mice model to in vitro cell culture model of human peripheral blood mononuclear cells (PBMC) since the study had shown that in the acute hepatitis B mice model, the HBV DNA could be cleared by its immune system in six weeks [13]. So, when it comes to the in vitro cell culture experiment, in which we suppose the sample would be cultivated for a long time, the cells from the mice model are not suitable. Besides, in the first section, our goal is simply to test the effect of ursolic acid on reducing the HBV-DNA level. However, in this section, we narrow down our focus to the mechanism of ursolic acid in human bodies (the CHB patients), so it would be less convincing if we still choose the cells from the mice sample.

3. Materials

Peripheral blood mononuclear cells are separated from patients and healthy volunteers [1], MTT (Sigma-Aldrich®, USA), RPMI-1640 medium, ursolic acid (Bioengineering Development Center of Yichun College, Jiangxi Province), High quality fetal bovine serum (Gibco Corporation, USA), DMSO (Sigma-Aldrich®, USA), β-acting (Bioss Biotechnology Co., Beijing), Cyclin D_1 Monoclonal Antibody (ZSGB-Bio Co., Beijing), extracellular signal-regulated kinase pERK1/2 Monoclonal Antibody (ZSGB-Bio Co., Beijing).

In vitro Cell Culture

Peripheral blood mononuclear cells will be inoculated with RPMI-1640 medium and fetal bovine serum, placed in a constant temperature incubator with 5% CO_2 saturation humidity and 37°C. Logarithmic growth phase cells will be selected for the experiment.

MTT Colorimetric Assay

24-well plates will be used to incubate the cells and the density should be adjusted before the treatment. The cells will be divided into six groups of 4: (1) blank control group, nutrient solution; (2) five experimental groups, based on different concentration gradients of ursolic acid treatment (10, 20, 30, 40, and 50μg/mL). The incubation will continue at 37 °C with a 5% CO_2 humidified environment, and then different concentrations of drugs were added. MTT will then be added to each well to test the absorbance (A) value at 490 nm by an enzyme calibrator at 24, 48, and 72 hours of incubation, respectively.

Proliferation inhibition rate (%) =

$$(1 - \frac{experimental\ group}{blank\ control\ group}) \times 100\%$$

3.1 *Flow Cytometry (FCM)*

The density of the cells in the logarithmic growth phase is adjusted before the treatment. Ursolic acid is added to each group at different concentration gradients (10, 20, 30, 40, and 50μg/mL) while a blank control group (nutrient solution) is set up. The cells are incubated under the condition of 37 °C with 5% CO_2. Annexin V is labeled with fluorescein (FITC) as a fluorescent probe. Apoptosis is detected by flow cytometry at the 24, 48, and 72 hours of incubation, respectively. The apoptosis rates of experimental groups are compared with the blank control group to evaluate the result.

3.2 *Western Blot*

The density of the cells in the logarithmic growth phase is adjusted before the treatment. Ursolic acid is added to each group at different concentration gradients (10, 20, 30, 40, and 50μg/mL) while a blank control group (nutrient solution) is set up. The cells are collected and lysed after cultivating at 37 °C in a 5% CO_2 humidified environment for 72h. Protein will be separated by using SDS-PAGE gel electrophoresis. The primary antibody (pERK1/2, Cyclin D_1) and internal reference antibody (β-acting), secondary antibody (HRP) will be used for the treatment. The protein expression level will then be determined with the cumulative A-value by the scanning and recording results of Gel-analyze software. The expression rates of Cyclin D_1 protein in the experimental groups are compared with the result of the blank control group.

3.3 *Statistical Analysis*

All the numerical data collected from the experiments including RT-PCR, Flow Cytometry (FCM), and Western blot will be analyzed using the student's T-Test on GraphPad Prism9 at $P<0.05$.

4. Results

Possible results on HBV-DNV level, cell proliferation, and apoptosis based on the treatment of ursolic acid (the testing procedure is shown in graph 1).

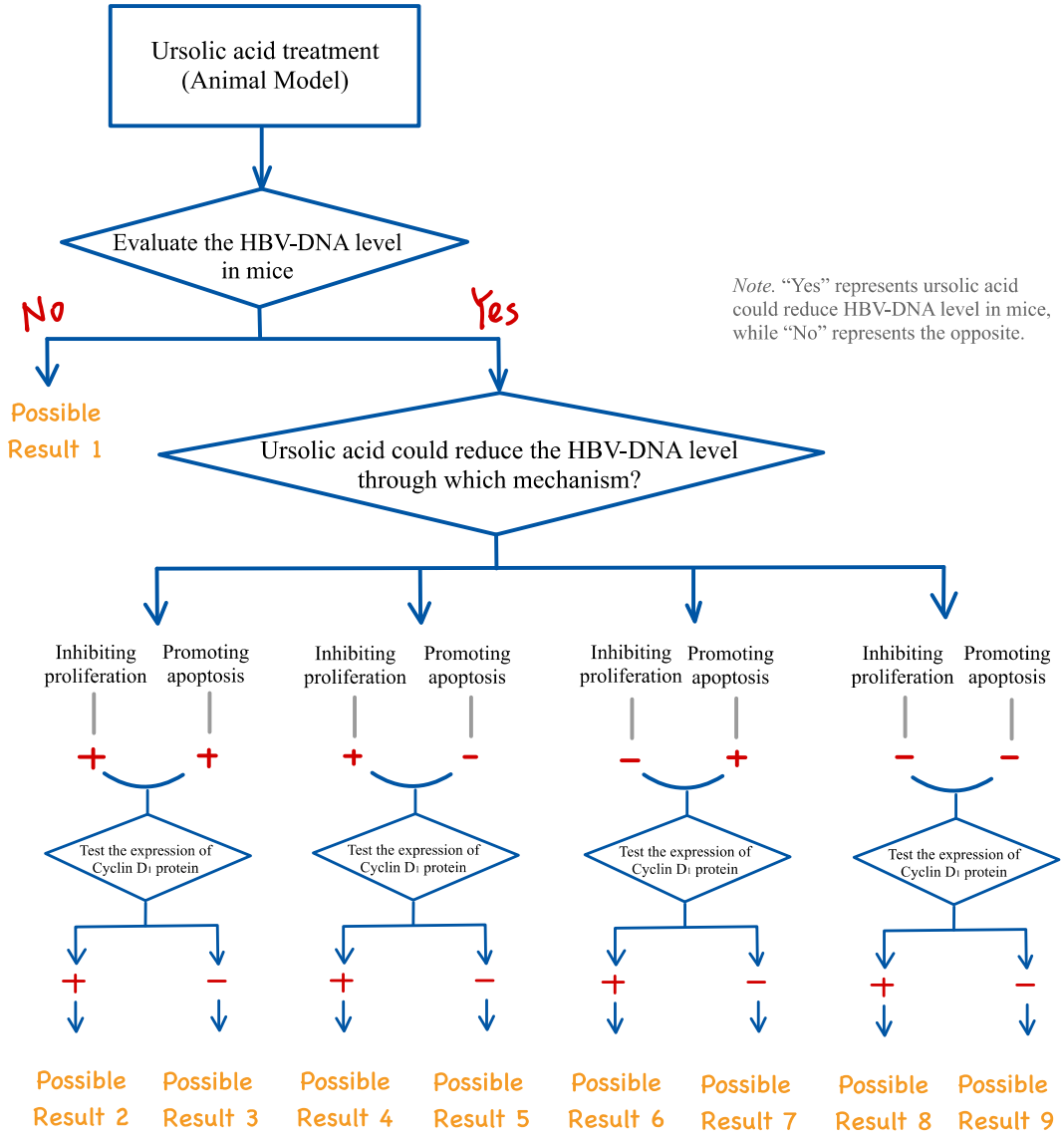

Figure 1. Testing procedure

4.1 *Possible Result 1: The animal model of mice does not show a clear result of reduction of the HBV-DNA level based on the ursolic acid treatment.*

Compared to the positive group, the experimental groups of different concentration gradients of ursolic acid do not show a reduction of the HBV-DNA level in mice. The HBV-DNA levels, however, are similar to the negative control group.

4.2 *Possible Result 2: Applying ursolic acid treatment reduces the HBV-DNA level in mice, inhibits the cell proliferation, and promotes the apoptosis. The expression of Cyclin D1 protein is decreased.*

Compared to the positive group, ursolic acid could reduce the HBV-DNA level in mice in experimental groups, and its effect is elevated with the increase of the concentration. Meanwhile, based on the results of previous experiments, ursolic acid is predicted to exert its inhibitory effect when acting on peripheral blood mononuclear cells for 24h, and the apoptosis rate reached its maximum at 72h [11]. The Western Blot results show that ursolic acid inhibits the

expression of intracellular Cyclin D_1 protein in a dose- and time-dependent manner when acting on the cells.

4.3 *Possible Result 3: Applying ursolic acid treatment reduces the HBV-DNA level in mice, inhibits the cell proliferation, and promotes the apoptosis. But based on the results, Cyclin D1 could be expressed normally.*

Compared to the positive group, ursolic acid could reduce the HBV-DNA level in mice in experimental groups, and its effect is elevated with the increase of the concentration. Meanwhile, ursolic acid could inhibit cell proliferation and promote apoptosis. However, the Western Blot results show that the Cyclin D_1 proteins in peripheral blood mononuclear cells are expressed normally.

4.4 *Possible Results 4: Applying ursolic acid treatment reduces the HBV-DNA level in mice, inhibits the cell proliferation, but does not promote the apoptosis. The expression of Cyclin D1 protein is decreased.*

Compared to the positive group, ursolic acid could reduce the HBV-DNA level in mice in experimental groups, and its effect is elevated with the increase of the concentration. Also, based on the results of previous experiments, ursolic acid is predicted to exert its inhibitory effect when acting on peripheral blood mononuclear cells for 24 hours [11]. However, the Flow Cytometry results show that ursolic acid does not promote apoptosis very significantly. In addition, Western Blot results show that ursolic acid inhibits the expression of intracellular Cyclin D_1 protein in a dose- and time-dependent manner when acting on human peripheral blood monocytes.

4.5 *Possible Result 5: Applying ursolic acid treatment reduces the HBV-DNA level in mice, inhibits the cell proliferation, but does not promote the apoptosis. Based on the results, Cyclin D1 could be expressed normally.*

Compared to the positive group, ursolic acid could reduce the HBV-DNA level in mice in experimental groups, and its effect is elevated with the increase of the concentration. Also, based on the results of previous experiments, ursolic acid is predicted to exert its inhibitory effect when acting on peripheral blood mononuclear cells for 24 hours [11]. However, the Flow Cytometry results show that ursolic acid does not promote apoptosis very significantly. In addition, Western Blot results show the Cyclin D_1 proteins in the cells are expressed normally.

4.6 *Possible Result 6: Applying ursolic acid treatment reduces the HBV-DNA level in mice, promotes the apoptosis, but does not inhibit the cell proliferation. The expression of Cyclin D1 protein is decreased.*

Compared to the positive group, ursolic acid could reduce the HBV-DNA level in mice in experimental groups, and its effect is elevated with the increase of the concentration. Also, the Flow Cytometry results show that ursolic acid could promote apoptosis distinctively. However, ursolic acid treatment does not inhibit the proliferation of peripheral blood mononuclear cells. In addition, Western Blot results show that ursolic acid inhibits the expression of intracellular Cyclin D_1 protein in a dose- and time-dependent manner when acting on human peripheral blood monocytes.

4.7 *Possible Result 7: Applying ursolic acid treatment reduces the HBV-DNA level in mice, promotes the apoptosis, but does not inhibit the cell proliferation. Based on the results, Cyclin D1 could be expressed normally.*

Compared to the positive group, ursolic acid could reduce the HBV-DNA level in mice in experimental groups, and its effect is elevated with the increase of the concentration. Also, the Flow Cytometry results show that ursolic acid could promote apoptosis distinctively. However, ursolic acid treatment does not inhibit the proliferation of peripheral blood mononuclear cells. In addition, Western Blot results show the Cyclin D_1 proteins in the cells are expressed normally.

4.8 *Possible Result 8: Applying ursolic acid treatment reduces the HBV-DNA level in mice but does not inhibit the cell proliferation as well as promote the apoptosis. Based on the results, Cyclin D1 could be expressed normally.*

Compared to the positive group, ursolic acid could reduce the HBV-DNA level in mice in experimental groups, and its effect is elevated with the increase of the concentration. However, the Flow Cytometry results show that ursolic acid does not promote apoptosis very significantly and the ursolic acid treatment does not inhibit the proliferation of peripheral blood mononuclear cells. In addition, Western Blot results show that ursolic acid inhibits the expression of intracellular Cyclin D_1 protein in a dose- and time-dependent manner when acting on human peripheral blood monocytes.

4.9 *Possible Result 9: Applying ursolic acid treatment reduces the HBV-DNA level in mice but does not inhibit the cell proliferation as well as promote the apoptosis. The expression of Cyclin D1 protein is decreased.*

Compared to the positive group, ursolic acid could reduce

the HBV-DNA level in mice in experimental groups, and its effect is elevated with the increase of the concentration. However, the Flow Cytometry results show that ursolic acid does not promote apoptosis very significantly and the ursolic acid treatment does not inhibit the proliferation of peripheral blood mononuclear cells. In addition, Western Blot results show the Cyclin D_1 proteins in the cells are expressed normally.

5. Discussion

Due to the specific pathological features of Chronic Hepatitis B (CHB), many patients with mildly elevated ALT levels (between 1-2 ULN) are excluded from antiviral therapy in the early stages [1]. However, the harm caused by CHB should not be underestimated. As is said previously, current treatments for CHB patients can cause a serious economic burden and affect their life quality. Therefore, it is necessary to provide appropriate therapy for the patients to effectively prevent the progression of the disease.

Based on the previous studies, this experiment is conducted to investigate the ligustrum lucidum, a single TCM material in the Bushen formula [1]. Current research has found that its main component, ursolic acid, has an obvious antiviral effect. Our study predicts the mechanism by which ursolic acid exerts its pharmacological effects and tests this hypothesis through constructing animal models and conducting in vitro cell culture, which can provide a theoretical basis for clinical use, especially in the compounding and splitting of TCM therapy.

Result 1 demonstrates that ursolic acid could not inhibit HBV-DNA levels in mice, which is contradictory to our hypothesis. Excluding the errors caused by improper operation during the experiment, based on previous studies, our study speculates that the mice's immune system could clear the HBV virus. And since HBV cannot infect ordinary mice, the transfection model established by the high-pressure hydrodynamic method in this experiment is partly an acute hepatitis B infection model, and virus clearance usually occurs within 6 weeks [13]. Besides, result 7, 8, and 9 are also contradictory to our hypothesis. However, result 7 reveals that ursolic acid could reduce the HBV-DNA level but not through the way of inhibiting the expression of Cyclin D_1 protein. On the other hand, both results 8 and 9 show that ursolic acid could not inhibit the proliferation as well as promote the apoptosis. As is said before, the mechanisms by which ursolic acid exerts its clinical effects are broad and general, so, there still exist many other possible conditions for those with similar research directions to follow.

Possible results 2 and 4 reveal the fact that ursolic acid can reduce HBV-DNA levels in CHB patients by inhibiting cell proliferation and promoting apoptosis, which fully supports our hypothesis. Result 2 shows that ursolic acid could also promote the apoptosis, while result 4 proves that inhibiting the proliferation is the only way for ursolic acid to reduce the HBV-DNA level. Both of them can provide a valuable theoretical basis for subsequent clinical use.

Unlike the results above, the possible results 3, 5, and 6 partially support our hypothesis. Results 3 and 5 show that ursolic acid does not inhibit cell proliferation by suppressing the expression of Cyclin D_1 protein. As is previously described, it is possible that one of the remaining multiple pharmacological effects of ursolic acid could be its actual mechanism of action. On the other hand, result 6 reveals that the ursolic acid could reduce the HBV-DNA level by promoting the apoptosis, but it is confusing that the expression of Cyclin D_1 protein was also reduced. Since the previous study has found that Cyclin D_1 protein, which could activate the procedure of the cell cycle, is one of the most important constituents of the Cyclins [11]. Therefore, we suggest that the result should be retested under the same condition to evaluate its fidelity.

6. Conclusion

Generally, this study investigated the specific mechanism of action of ursolic acid in reducing HBV-DNA levels in CHB patients. Our results will show whether ursolic acid could affect the ERK pathway by inhibiting the expression of Cyclin D_1 protein, stop the cell cycle of HBV-infected cells, and provide theoretical experience for subsequent clinical use, especially for the compounding and splitting of TCM therapy. Due to the multiple mechanisms of action of ursolic acid and the different pharmacological effects exerted in different cells, the mechanism of action of ursolic acid on apoptosis can be a direction for future research. In addition, ursolic acid's inability to inhibit Cyclin D_1 protein expression, which is contrary to the hypothesis of this study, will also provide a feasible direction for future experiments. Restricted by the development of science and technology, people have only recently started to predict and investigate the specific sites of action of ursolic acid through network pharmacology. Therefore, the remaining mechanisms of ursolic acid's pharmacological action still need to be further studied in order to make the clinical treatment of CHB patients more effective and economical.

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