Experimental study on the mechanism of curcumin inhibiting the proliferation of human hepatocellular carcinoma cells HepG2

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Abstract: Objective: The purpose of this experiment is to study the effects of Curcumin (Cur) on the proliferation, apoptosis and cell cycle of human hepatocellular carcinoma HepG2 cells, and to explore its mechanism of inhibiting the proliferation of HepG2 cells. Methods: The experiment was divided into control group and Cur group. The HepG2 cultured human hepatoma cells were treated with Cur solution of different concentrations. The inhibitory effect of Cur on the proliferation of HepG2 cells was detected by MTT assay. The apoptosis rate and cell cycle distribution of CUR-induced HepG2 cells were detected by flow cytometry. Results: Compared with the control group, the growth of HepG2 cells was inhibited by Cur in a concentration-dependent manner. Apoptosis by flow cytometry. Results: Compared with the control group, the growth of HepG2 cells was inhibited by Cur in a concentration-dependent manner. Flow cytometry showed that the apoptosis rates of the control group and the Cur group were 0.3%, 18.0%, 24.7% and 86.9%, respectively, showing a dose-dependent relationship. Cell cycle detection showed that in the control group and Cur concentration groups, the proportion of S phase was 24.11%, 32.68%, 37.75%, 42.10%, respectively. The apoptosis percentage was most obvious in S phase, and cells were blocked in S phase. Compared with control group, with the increase of Cur concentration. Conclusion: Curcumin may inhibit the proliferation of human hepatoma cells HepG2 by preventing cell division and inducing cell apoptosis.

1. FOREWORD

Hepatocellular carcinoma (HCC), known as the "king of cancers," [1] is a malignant tumor that occurs in the liver and is the third leading cause of cancer-related death [2]. The onset of HCC is occult, the early clinical symptoms are not obvious, and the diagnosis is in the middle and late stage. Although there are many treatment methods for liver cancer, such as: radiotherapy, radiofrequency ablation, liver transplantation, intervention, hepatic artery embolization chemotherapy and so on. Chemotherapy still plays an important role in the comprehensive treatment of liver cancer. However, at present, there is a lack of effective and low-toxicity drugs in the chemotherapy of advanced liver cancer patients. Compared with modern medicine, traditional Chinese medicine is increasingly prominent in the treatment of cancer because of its low toxicity, good curative effect and relatively low price. It has unique advantages in preventing and treating the occurrence and metastasis of liver cancer, prolonging the survival period of liver cancer patients, and improving the quality of life of liver cancer patients [1]. Curcumin (Cur) is a kind of natural plant monomer extracted from the rhizome of curcuma, curcuma, curcuma and calamus. It has the effects of lowering blood lipids, anti-tumor, anti-inflammation, gallbladder and anti-oxidation. Anti-tumor effect is its main effect [3]. Chang et al. Curcumin combined with glycyrrhizic acid acted on HepG2 cells, and it was found that the proliferation of HepG2 cells was blocked in G1 phase and apoptosis increased. Meanwhile, it was confirmed by subcutaneous transplantation tumor model of nude mice that the combination of curcumin and glycyrrhizin could up-regulate the expression of PTEN and inhibit the activation of PI3K/Akt pathway, thus playing an anticancer role [4]. This study aims to investigate whether curcumin can inhibit the proliferation of human hepatocellular carcinoma cells HepG2 by inducing apoptosis and preventing cell division by intervening with Cur in vitro.

2. Materials and methods

2.1. Materials

Human hepatoma HepG2 cells, provided by Translational Medicine Center, the Second Affiliated Hospital of Shaanxi University of Chinese Medicine.

Drug and reagent curcumin (Sigma), purity is 97%. Tetramethyl azazole blue (MTT), dimethyl sulfoxide (DMSO) (Sigma), DMEM medium and trypsin

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(Hyclone); Fetal bovine serum (Hangzhou Sijiqing); Rat anti-human Bcl-2, Bax, Caspase-3 antibody and rabbit anti-rat IgG (Sigma) labeled by HRP.

Main instruments Ultra-clean table (ESCO), inverted microscope (Otte BDS-200), carbon dioxide incubator (BindCD-150), ultra-low temperature refrigerator (Thermo 702), High speed low temperature centrifuge (Gene Company Limited), Enzyme labeling instrument (Bio-Tek ELX808IU), flow cytometer (Beckman Coulter), decolorization shaker (Kylin-Bell BETS-M5).

1.2. Methods

Preparation of curcumin solution 184mg curcumin powder was dissolved in 5mlDMSO to obtain a Cur stock solution with a concentration of 100mmol/L, which was stored at 4℃ for later use. Before the trial take concentrate 1 ul, 2 ul, ul, 3 4 ul, 5 ul, 6 ul, 8 ul concentration of soluble in 10 ml1dmem medium, respectively for a quick end 10 umol/L, 20 umol/L, 30 umol/L, 40 umol/L, umol/L 50, 60 umol/L, 80 umol/L Cur solution.

Detection of cell proliferation by MTT method HepG2 cells were amplified at 37℃ and 5% CO2 saturated humidity. HepG2 cells were taken at logarithmic growth stage and inoculated into 96-well cell culture plates with an adjusted density of 2×104 cells /ml and 100ul/ well. After the cells were attached to the wall, the supernatant was discarded and 7 Cur solutions with different concentrations of 100ul/ well were added. There were 5 compound pores per concentration, and 100ul DMEM was added to the control group. They were incubated for 6,12,24,48 and 72h, and then added 10ul/ well of 5mg/mlMTT solution and continued to culture for 4h. Discard the medium, add 150ul/ well DMSO, shake at a low speed for 10min on the shaking table, and wait for the crystallization to dissolve fully. The optical density (OD) of each hole is measured at the wavelength of 490nm with the enzyme marker. Growth inhibition rate (%) =1-[(drug group A - control group A)/control group A], LC50 value was calculated with half inhibition concentration (LC50) calculation software, and the experiment was repeated three times [5]. The breeding experiments selected the optimal concentration of Cur, 20 umol/L, 40 umol/L, 60 umol/L, follow-up experiments adopt this concentration.

Flow cytometry to detect cell apoptosis HepG2 cells inoculated in 6 orifice plate, can be divided into blank control group and Cur20umol/L, 40 umol/L, 60 umol/L group. Except for the control group, 1ml of Cur solution with corresponding concentration was added to the other groups, which were treated for 48h. The cells of each group were digested by pancreatic enzymes and collected, the supernant was centrifuged, and the overhung cells were washed with cold PBS, and 6×104 cells were counted, according to Annexin V-FITC apoptosis kit. Dyed with Annexin V-FITC and propyl iodide (PI). 5000 cells were counted and the apoptosis rate was detected by flow cytometry, which avoided light for 15min. The experiment was repeated 3 times.

Cell Cycle detection by flow cytometry The cells were treated with the same method for 48h, digested by pancreatic enzymes, washed with sterile PBS once, collected cells were transferred to a centrifuge tube, pre-cooled with PBS, centrifuged at 1000 r/min for 5min, washed cells, discarded supernant, added with PBS at 4℃, and mixed in whirlpools. Fix with 70% ice ethanol and save for later. Before staining, the fixing solution was washed off with PBS, the cells were re-suspended with PBS, the cell density was adjusted to 1×106 cells /mL, and 1mg/mL RNaseA 20ul was added to water bath at 37℃ for 30min, then 50ug/mL PI 400ul was added for staining, and the cells were detected by flow cytometry. Modfit software analyzes the cell cycle.

Statistical Analysis SPSS20.0 Statistical software package was used for statistical analysis. Measurement data were represented by mean ± standard deviation (± s), T-test was used for comparison between groups, and one-way analysis of variance was used for comparison of data above two groups. $p < 0.05$ was considered as significant difference, and $p < 0.01$ was considered as extremely significant difference.

3. Results

Effects of curcumin on proliferation of HepG2 cells MTT method proved that Cur at different concentrations could inhibit the growth of HepG2 cells in a concentration-dependent manner, and the inhibition rate reached 66% at 60umol/L. Compared with blank control group, the difference was statistically significant (P<0.05). After 24,48,72h and 10-80μmol/L concentration screening,48 h, the inhibition rate was in the range of 20.08-65.81%; The optimum concentrations of Cur were 20, 40 and 60μmol/L, and the inhibition rates showed a significant time-concentration dependence (FIG. 1).

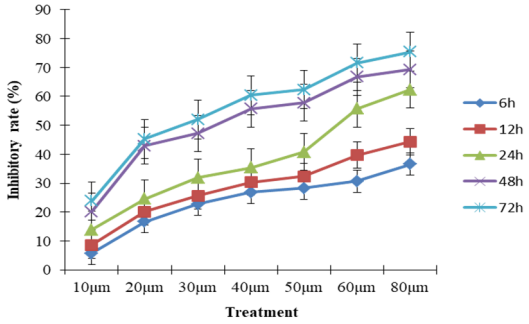


Fig. 1. Effect of Cur on the proliferation inhibition rate of HepG2 cells

Effects of curcumin on apoptosis of HepG2 cells compared with the control group, different concentrations of Cur could induce apoptosis of HepG2 cells, and the apoptosis rate gradually increased with the increase of concentration. The total apoptosis rates of the control group, the 20, 40 and 60μmol/ LCur group were 0.3%, 17.1%, 28.7% and 86.9%, respectively. It is suggested that the effect is concentration-dependent. The differences were significant (P<0.05, P<0.01), as shown in Figures 2A and B

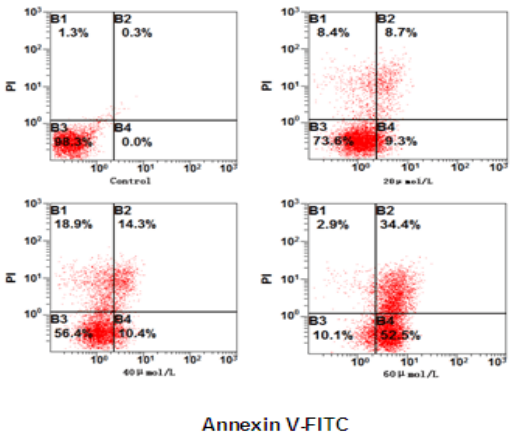


Figure. 2A. 48h apoptosis rate of HepG2 cells treated by each dose group of Cur

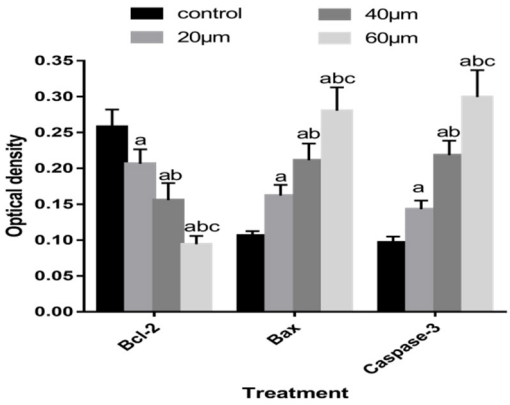
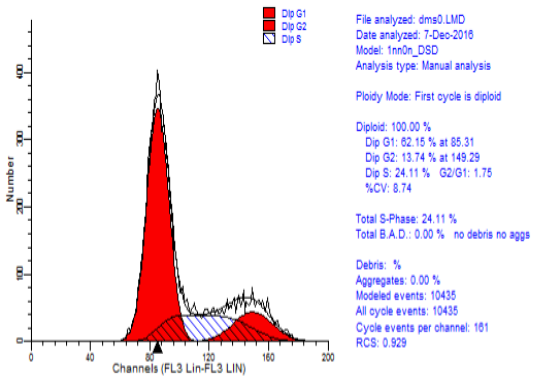


FIG. 2B. Effects of Cur dose groups on apoptosis of HepG2 cells for 48h

Effects of curcumin on HepG2 cell cycle The percentage of HepG2 cells was most obvious in S phase, while the percentage and proportion of HepG2 cells did not change significantly in G1 phase and G2 phase. In the blank group, the 20μmol/L Cur group treated HepG2 cells for 48h, and the percentages of S phase were 24.11% and 32.68%, respectively, which were significantly different from those in the blank group (P<0.05), indicating that Cur could block HepG2 cells in S phase. See Figure 3.



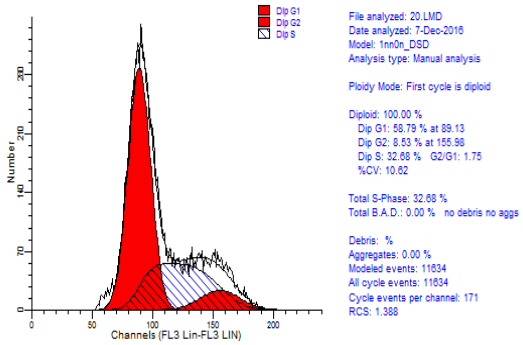


Figure 3. Effect of Cur on HepG2 cell cycle

The effect of curcumin on the expression levels of Bcl-2, Bax and Caspase-3 proteins in HepG2 cells was compared with that in the control group. After treatment for 48h in 20, 40 and 60μmol/L Cur groups, the expression of Bcl-2 protein in HepG2 cells was decreased and the expression of Bax and Caspase-3 proteins was up-regulated. The difference was statistically significant (P<0.05).

4. Discussion

Liver cancer belongs to the Chinese medicine "accumulation" category, due to the body qi machinery is not smooth, Yin and Yang imbalance, qi and blood discord, wet turbidness, phlegm drink, and other pathological products interknot, condensed for a long time, accumulated in the hypochthonic liver cancer as its pathogenesis. In most of the prescriptions, Zedoary is the main medicine, and curcumin is the main active component, and its anti-tumor effect has attracted wide attention. Studies have shown that curcumin can regulate cell cycle, inhibit tumorigenesis and metastasis, and show anticancer activity, and has an inhibitory effect on a variety of tumors [6]. Such as lung cancer, breast cancer, colon cancer, etc., the United States National Cancer Institute has listed it as the third generation of cancer chemoprevention drugs [7]. Curcumin is a diketone compound extracted from the tuber of turmeric, the rhizome of turmeric, the rhizome of zedoary and the rhizome of calamus araceae [8]. These herbs have the functions of activating blood, promoting qi, relieving pain, dispelling dampness and phlegm, breaking blood and eliminating accumulation, which are the basic treatment principles of tumor TCM. Studies have shown that CUR can significantly inhibit the occurrence and development of tumor cells, and has no obvious toxic effect on normal cells [9]. Its mechanism is mainly related to inhibiting the growth and proliferation of tumor cells, inhibiting tumor invasion and metastasis, blocking cell cycle and inducing tumor cell apoptosis [10]. Zeng Yuqun et al treated HCCLM3 cell line with different concentrations of curcumin in vitro, and the cell proliferation ability was significantly reduced, indicating that curcumin has a significant inhibitory effect on

HCCLM3 cell proliferation, and the trend is concentration dependent [11]. In this study, CUR was used to treat HepG2 cells. The results showed that CUR at different concentrations could inhibit the growth of HepG2 cells, and the inhibition rate reached 66% at 60umol/L. And it is concentration-dependent. The results are the same as above.

Cell life activities undergo a complete cycle of G1-S-G2-M. When the DNA integrity of cells is damaged, the cells cannot successfully complete the cycle of G1-S-G2-M [12], resulting in continuous proliferation of cells out of control, and the imbalance between cell proliferation and apoptosis will lead to tumor occurrence [13]. Lu Na et al confirmed that the mechanism of curcumin combined with docetaxel on radiotherapy sensitization of human lung cancer cells is related to tumor cell cycle arrest and induction of apoptosis in a concentration-dependent manner [14]. By flow cytometry, we found that CUR could block HepG2 cells in S phase and unable to carry out cell cycle cycle, which was the same as the results of Lu's study.

Apoptosis is the programmed and active mode of cell death pathway, which is regulated by its own genes in normal physiological or pathological environment [15], among which Bcl-2 and Caspase are particularly important in cell apoptosis. As a key enzyme in the apoptosis cascade, caspase-3 is the core of the apoptosis cascade, and the hydrolytic activity of Caspase-3 can directly induce apoptosis [16]. Bcl-2 and Bax are the most important anti-apoptotic and pro-apoptotic genes in this family, respectively. They can jointly activate the pro-apoptotic factor caspase-3 and other factors in this family bcl-2 are important anti-apoptotic genes, which can prevent the occurrence of apoptosis caused by various injuries, and mainly regulate the increase of Bax protein expression during the apoptosis process. Overexpression of bax can promote the occurrence of apoptosis [17]. Zhang et al. found that Curcumin can inhibit the proliferation of SMMC-7721 by activating JNK signaling pathway and inhibiting ERK and p38 MAPK signaling pathway.Thus, the expression of Bcl-2 and Survivin is down-regulated, and the expression of Bax and caspase-3 is up-regulated, thus inhibiting the proliferation of SMMC-7721 HCC cells and promoting their apoptosis [19]. The apoptosis rate of HepG2 cells was detected by flow cytometry, and the apoptosis rate increased gradually with the increase of CUR concentration. It is suggested that CUR can induce apoptosis of hepatocellular carcinoma cells.

In summary, in this study, curcumin acted on human hepatoma cells HepG2 cultured in vitro with different concentrations, and it was found that curcumin could induce apoptosis and inhibit the proliferation of human hepatoma cells HepG2 by inducing apoptosis, preventing cell division, down-regulating the expression of Bcl-2, up-regulating the expression of Bax and Caspase-3 proteins. As a traditional Chinese medicine monomer, Cur has multi-target, multi-link and multiple functions, and has the characteristics of wide source, low toxicity and high efficiency. Compared with traditional Chinese medicine compound, its efficacy is clear, which is more conducive to the study of its mechanism of action. The mechanism of action of curcumin is complex, and the genesis of tumor is also very complex, involving the changes of different genomes and proteomes, the changes of related proteins of pathways, related signaling pathways, and the changes of oncogenes and their proteins, which still need to be further explored.

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