Inhibitory mechanism of curcumin on tumor cells

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Abstract: Objective: The aim of this study was to observe the effects of Curcumin (Cur) on the proliferation, invasion, migration and apoptosis of human hepatocellular carcinoma HepG2 cells, and to explore its mechanism of tumor inhibition. **Methods:** The experimental design encompassed a control group and a CUR group, with HepG2 cells subjected to varying concentrations of CUR solution in vitro culture. Proliferation was assessed using the MTT assay, apoptosis rates, and cell cycle distribution were examined via flow cytometry, and Bcl-2, Bax, and Caspase-3 protein levels were analyzed using Western blot. Migration and invasion capabilities were evaluated using scratch and Transwell assays. **Results:** A concentration-dependent inhibition of HepG2 cell growth by all CUR concentrations compared to the control group. Flow cytometry demonstrated increased apoptosis rates with rising CUR concentrations, while cell cycle analysis indicated S phase arrest across all groups. Scratch and Transwell assays corroborated a decline in migration and invasion with escalating CUR concentrations. Western blot results illustrated a decrease in Bcl-2 expression and an increase in Bax and Caspase-3 expression compared to the control group. Conclusions: Curcumin emerges as a potent inducer of apoptosis and inhibitor of proliferation in HepG2 cells. Its impact on migration, invasion, and cell division, coupled with the modulation of Bcl-2, Bax, and Caspase-3 proteins, underscores its potential as a therapeutic agent in hepatocellular carcinoma.

1. Introduction

Hepatocellular carcinoma (HCC) stands out as a prevalent malignancy in the digestive system, posing a significant threat to human health globally, particularly in China. The onset of HCC is subtle, with inconspicuous early symptoms leading to diagnoses in the middle to late stages. Current treatments for advanced liver cancer, including surgery, radiotherapy, and transcatheter arterial chemoembolization [1], exhibit limited efficacy. Notably, there is a conspicuous absence of effective and low-toxicity drugs for chemotherapy in advanced liver cancer patients. In contrast to conventional medicine, traditional Chinese medicine is gaining prominence in tumor treatment owing to its low toxicity, cost-effectiveness, and proven efficacy. It demonstrates unique capabilities in preventing and treating liver cancer by impeding occurrence and metastasis, prolonging survival, and enhancing the quality of life for patients [2-3]. Curcumin (Cur), a natural monomer derived from plants like Curcuma sativa, Zedoary turmeric, and Acorus calamus, has garnered attention for its lipid-lowering, anti-tumor, anti-inflammatory, cholelithiasis, and anti-oxidative properties, with a primary focus on its anti-tumor effects [4]. Studies indicate that Cur inhibits tumor growth *in vivo* and *in vitro*, inducing apoptosis in various tumor cells [5]. In a study by Chang et al., the combined action of Curcumin and glycyrrhizic acid in HepG2 cells resulted in suppressed proliferation, G1 phase cell cycle arrest, and heightened apoptosis. Confirming these anti-cancer effects, a nude mice subcutaneous transplantation tumor model revealed increased PTEN expression and the inhibition of the PI3K/Akt pathway activation [6]. This study aims to delve into the potential of Curcumin to impede HepG2 cell proliferation by inducing apoptosis, suppressing cancer cell metastasis, and halting cell division.

2. Materials and methods

2.1. Cell line

Human liver cancer HepG2 cells, provided by Translation of Medicine Center, the Second Affiliated Hospital of Shaanxi University of Chinese Medicine.

2.2. Experimental drug

Curcumin (Sigma, batch number: 110823-201405) with a purity of 97%.

2.3. Experimental reagents

Methyl thiazol tetrazolium (Sigma, batch number: M5655-500MG), dimethyl sulfoxide (DMSO) (Sigma, batch number: D265-100ML), DMEM culture medium and trypsin (Hyclone, batch number: SH30022.01B); Transwell chamber (Minipore, batch number: 3422);

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Fetal bovine serum (Hangzhou Sijiqing, batch number: 11011-8611); Rat people the Bcl - 2, Bax, antibody of Caspase 3 and 2 (Abcam British company, batch number: ab182858 ab32503, ab184787, ab6721).

2.4. Main instrument

The altraclean workbench (ESCO and models: AC2-4 s1), inverted microscope (Japan being future-proof models: IX53) and carbon dioxide incubator (German Heraeµs, models: BB5060µV), ultra-low temperature refrigerator (Thermo, model: 702), high speed cryogenic centrifuge (Sigma, Germany, model: SIGMA 2-16P), microplate reader (Bio-Tek, USA, model: ELX808IU), flow cytometer (Beckman Coulter, model: CytoFLEX), decolorization shaker (Killin-Bell, model: BETS-M5).

3. Methods

3.1 Preparation of curcumin solution

184 mg curcumin powder was dissolved in 5ml DMSO to obtain a Cur stock solution with a concentration of 100 mmol/L, which was stored at 4℃. Take concentrate 1ul,2 ul, 3ul, 4 ul, 5 ul, 6ul, 8 ul of original liquid respectively dissolved in 10 ml1DMEM medium, a quick final concentration of 10 umol/L, 20 umol/L, 30 umol/L, 40 umol/L,50 Umol/L, 60 umol/L, 80 umol/L of Cur solution.

3.2. MTT method to detect cell proliferation

Adjust the HepG2 cells in the logarithmic growth phase to a density of 2×104 cells /ml and seeded in a 96-well cell culture plate at 100ul/ well. When the cells were adherent, the supernatant was discarded, then add 7 different concentrations of Cur solution at 100ul/ well. Each concentration had five replicates.Add 100ul DMEM to the control group for culture. Each well was respectively incubated for 6,12,24,48 and 72 hours. Then add MTT solution (10ul/ well, 5mg/ml) and the cells cultured for another 4 hours.Add DMSO (150ul/ well) and discarded the culture medium . After the crystals were fully dissolved by 10 minutes shaker oscillating at a low speed, measured the the optical density (OD) value of each well at 490nm wavelength by microplate reader. The growth inhibition rate (%) =1-[(drug group-A control group)/control group A], the LC50 value was calculated by the calculation software, and the experiment was repeated for 3 times [7]. The breeding experiments selected the optimal concentration of Cur at 20 umol/L, 40 umol/L, 60 umol/L, follow-up experiments adopt this concentration.

3.3. Technology of flow cytometry detection of cell apoptosis

Inoculated HepG2 cells in 6 orifice plate, which divided into blank control group and 20umol/L, 40 umol/L, 60 umol/L group of the Cur. In addition to the control group added 1mlDMEM, the other groups were added with the corresponding concentration of Cur solution 1ml at treatment for 48h, trypsin digestion and collection of each group of cells, centrifugation abandoned the supernatant, cold PBS washing resuspension cells, counting 6×104 /ml cells, according to Annexin V-FITC apoptosis kit instructions,the cells were stained with Annexin V-FITC and propidium iodide (PI). Counted 5000 cells and detected the apoptosis rate by flow cytometry in the dark for 15min.Repeated for 3 times.

3.4. Flow cytometry technology to detect the cell cycle Treatment of the cells

for 48 h with the pancreatic enzyme digestion, washed with sterile PBS, collecting cells into the centrifugal tube, add precooling PBS. Centrifugal 5 min at the speed of 1000r/min before washing cells and abandon supernatant. Add PBS at 4℃, spiral way grinder. The cells were fixed with 70% ice-cold ethanol and stored for later use. Used PBS wash liquid before dyeing, PBS re-suspension cells, adjusted cell density to 1 x 106 / mL, add 1 mg/mL RNaseA 20 ul and 37℃ water bath for 30 min, then plus 50 ug/mL PI 400 ul for dyeing, ice bath for 30 min, detected by flow cytometry instrument. Modfit software was used to analyze the cell cycle.

3.5. Scratch test of cell migration

The logarithmic phase of HepG2 cells, digested by pancreatic enzyme, made into single cell suspension, adjusted the density to 1 x 106 / ml, vaccination orifice on 1 ml each holes of 6-well plate, in addition add 1ml DMEM cultures of 24h incubation to cell wall, abandon the supernatant, After being washed twice with PBS, add 20,40, and 60umol/L Cur solution respectively. The equal volume of DMEM culture medium was added to the control group. In each group, there were 3 repeated Wells. The cells were cultured at 0h,6h,12h,24h, and the scratch width of the cells was observed and photographed under an inverted microscope. The cell migration ratewas calculated (cell migration rate = cell migration distance/cell scratch width).

3.6. Transwell invasion experiment method

Digested the celss same as the method of the 2.5. Adjusted the cell concentration to 5 x 104 / ml by used different concentration of serum-free medium. Joint the cell suspension of 150µl in upper chamber of Transwell little room. Diluted the Matrigel matrix by serum-free at rate of 1: 8, 50µl of dilution glue was added to the upper chamber. In the lower chamber, 600µl of DMED culture medium containing 10% fetal bovine serum was added.In the control group, only cell suspension was added without drug solution. The cells were incubated at 37℃ for 12 hours. The cells in the upper chamber were wiped off with a cotton swab, cleaned with PBS, fixed with 4% paraformaldehyde for 10min, and stained with crystal violet after cleaning with PBS. Five fields were randomly selected under the microscope for observation and

photographed, and the total number of cells in the five fields was recorded for statistical analysis.

3.7. The expression of protein of Bcl-2, Bax and Caspase3 in HepG2 cells

As same in method 2.4, the cells in each group were digested with trypsin and collected, the supernatant was discarded by centrifugation, and the cells were re-suspended after washing with cold PBS. BCA method was used to determine the total protein concentration of samples. 25ug of total protein samples from each group were separated by 12% SDS polyacrylamide gel electrophoresis. The separated proteins were transferred to PVDF membrane and blocked with 5% skim milk powder. Separately in rat resistance to the Bcl - 2 antibodies (1:3000), rat Bax antibodies (1:2000), rat Caspase3 (1:2000) incubation for the night at 4℃. The second anti-body was RHP marking rabbit anti rat IgG (1:1000), incubated for 1h at room temperature, DAB chromogenic, Image J software analysis results above, with objective stripe and internal beta actin said the ratio of the average grey value of protein levels, semi-quantitative analysis.

3.8. Statistical analysis SPSS20.0

Statistical software package for statistical analysis. The measurement data were expressed as mean ± standard deviation $\bar{x}$ (± s), T test was used for comparison between groups, and one-way analysis of variance was used for comparison between more than two groups. $p < 0.05$ was considered as significant difference, and $p < 0.01$ was considered as extremely significant difference.

4. Results

4.1. Curcumin's Impact on HepG2 Cell Proliferation

Utilizing the MTT method, we observed a concentration-dependent inhibition of HepG2 cell growth by various concentrations of Curcumin. The inhibition rate reached 66% at 60 µmol/L, and this difference was statistically significant compared to the blank control group (P<0.05). Over a 24-hour, 48-hour, and 72-hour period, with concentrations ranging from 10 to 80 µmol/L, the inhibition rate spanned from 20.08% to 65.81% at 48 hours. Notably, the optimal concentrations of Curcumin were identified as 20, 40, and 60 µmol/L, showcasing a substantial time-concentration dependence (see FIG. 1). This underscores Curcumin's potential in impeding the proliferation of HepG2 cells.

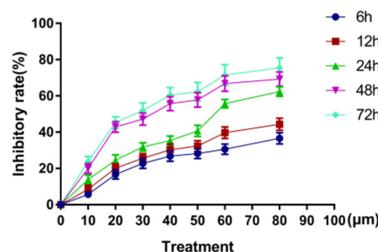


Figure 1. Effect of Cur on the proliferation inhibition rate of HepG2 cells

4.2. Curcumin's Influence on HepG2 Cell Apoptosis

Compared to the control group, introducing varying concentrations of Curcumin exhibited an inducible effect on HepG2 cell apoptosis. Notably, as the concentration increased, the apoptosis rate gradually rose. Specifically, the total apoptosis rates for the control group and the 20, 40, and 60 µmol/L Curcumin groups were 0.3%, 19.5%, 24.7%, and 86.9%, respectively. These findings underscored the concentration-dependent nature of Curcumin's impact on apoptosis, with statistically significant differences (P<0.05, P<0.01), as illustrated in Figures 2A and B. The data emphasizes the potential of Curcumin to induce apoptosis in HepG2 cells.

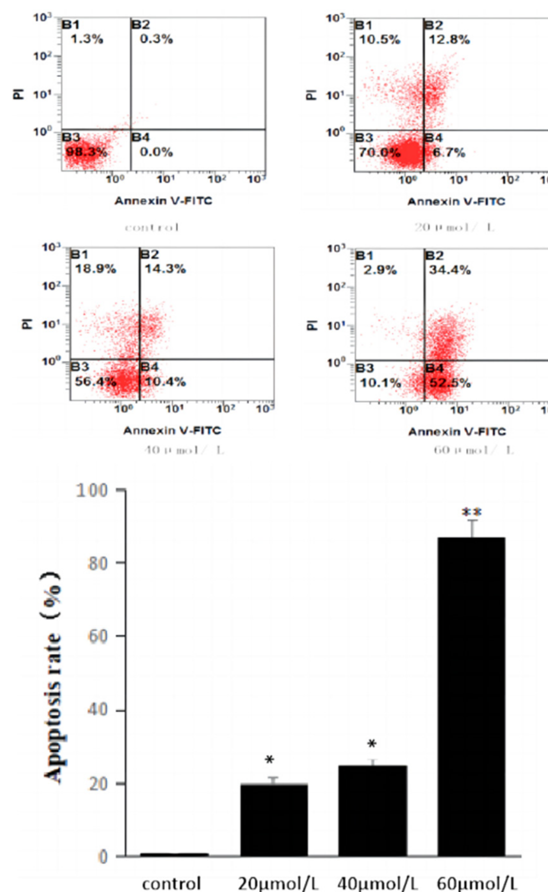


Figure 2. Effect of Cur on apoptosis of HepG2 cells Figure 2B Apoptosis rate (%)
*P<0.05,**P<0.01 compared with control group

4.3. Curcumin's Impact on HepG2 Cell Cycle

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 control group, the proportion of S-phase in Cur concentration groups was 24.11%, 32.68%, 37.75% and 42.10%, respectively. The difference between Cur concentration group and blank group was significant (P<0.05), indicating that Cur could block HepG2 cells in S phase. See Figure 3.

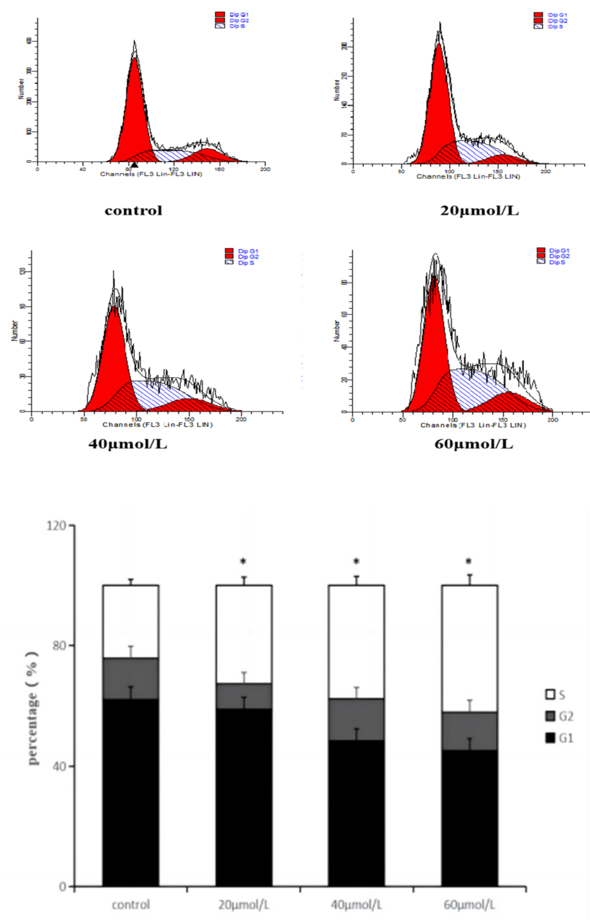


Figure 3. Effect of A Cur on HepG2 cell cycle
*P<0.05, compared with the control group

4.4. Curcumin's Impact on HepG2 Cell Migration and Invasion

Curcumin emerges as a potent agent in diminishing the migration and invasion capabilities of HepG2 cells. In the wound healing experiment, HepG2 cells treated with 20, 40, and 60 μmol/L Curcumin showed notable migration rate reductions after 24 hours—18%, 13%, and 7%, respectively. This contrasted with the blank control group, which exhibited a migration rate of 23%. The decrease in cell migration ability was statistically significant (P < 0.01), underscoring Curcumin's efficacy in impeding HepG2 cell migration. Refer to Figures 4a and 4b for a graphical representation of these impactful outcomes.

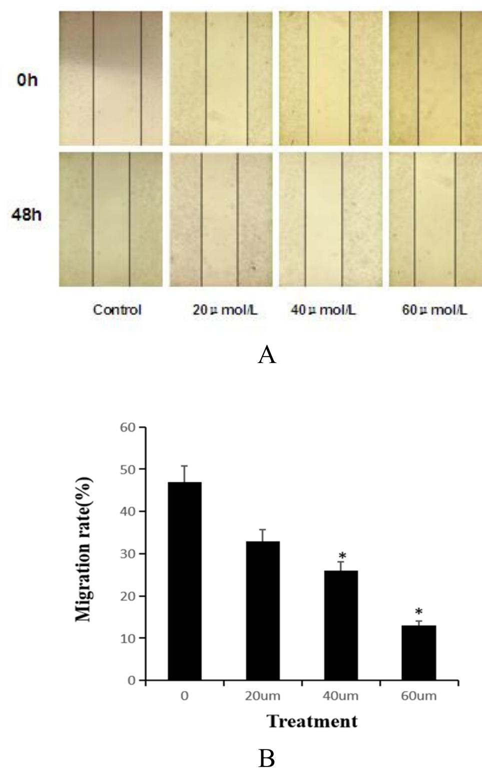


Figure 4. A, B Effect of Cur on HepG2 cell migration
*P<0.01, compared with control group

4.5. Transwell invasion experiment Effect

In the Transwell invasion experiment conducted 12 hours after Curcumin treatment on HepG2 cells, the impact on invasion and metastasis was evaluated across varying concentrations of Curcumin. The findings demonstrated a significant inhibition of cell invasion ability in each concentration of the Curcumin group compared to the blank control group (P<0.05), as depicted in Figure 5. These results underscore the effectiveness of Curcumin in curtailing the invasive potential of HepG2 cells. Refer to the figure for a visual representation of these noteworthy inhibitory effects.

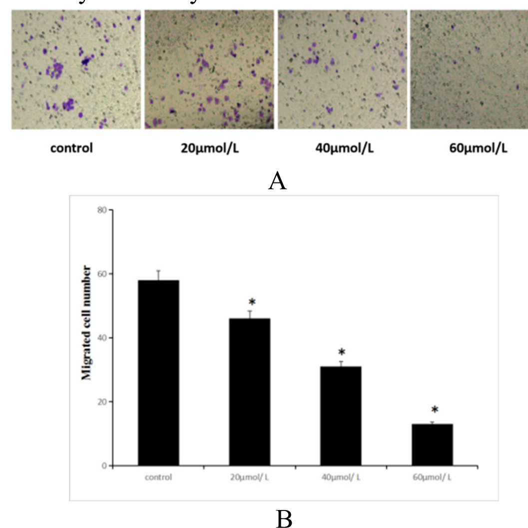
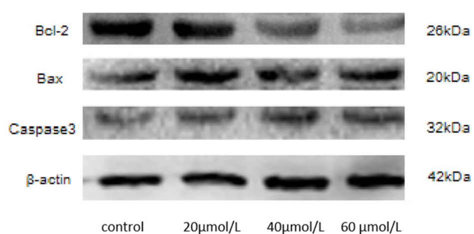


Figure 5. A, Effect of BCur on HepG2 cell invasion
*P<0.05, compared with control group

4.6 Protein Expression in HepG2 Cells:

Impact of Curcumin Treatment. Upon 48 hours of Curcumin treatment, the protein expression profile in HepG2 cells underwent significant alterations. Compared to the control group, the expression of Bcl-2 protein decreased, while the expression of Bax and Caspase-3 proteins increased markedly. These changes were statistically significant (P<0.05, P<0.01), as illustrated in Figures 6A, 6B, 6C, and 6D. These findings further emphasize the regulatory influence of Curcumin on key proteins associated with apoptosis in HepG2 cells, suggesting its potential as a modulator of apoptotic pathways. Refer to the figure for a visual representation of these notable protein expression changes.



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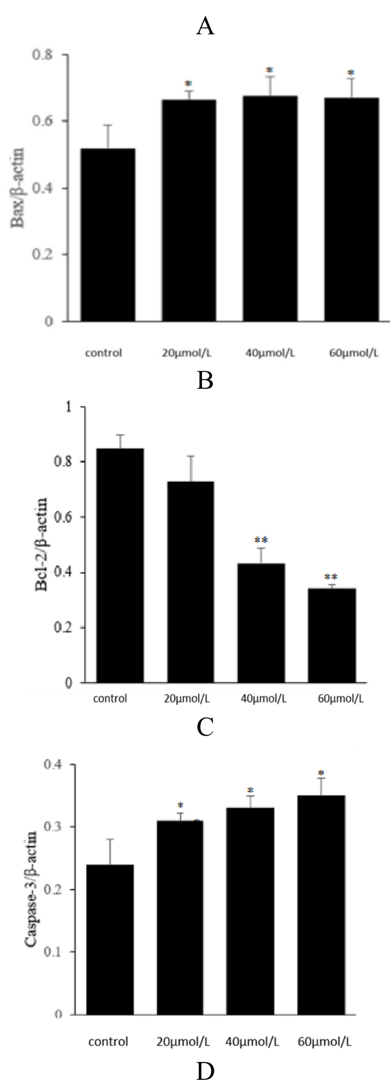

D

Figure 6. Expression of Bcl-2, Bax, and Caspase3 proteins in Hep G2 cells detected by Western Blot
*P<0.05,**P<0.01 compared with control group

5. Discussion

Liver cancer, classified under the traditional Chinese medicine concept of "accumulation," arises from imbalances in the body's qi, disruptions in Yin and Yang, disharmony of qi and blood, as well as the accumulation of pathological products such as dampness, turbidity, and phlegm. This prolonged accumulation in the hypogastrium contributes to the development of liver cancer. Curcuma, the primary active ingredient in traditional prescriptions, has garnered attention for its remarkable anti-tumor effects. Research has highlighted Curcumin's ability to regulate the cell cycle, inhibit tumorigenesis, and demonstrate anti-tumor activity across various cancer types, such as lung, breast, and colon cancer [8, 9]. The United States National Cancer Institute recognizes Curcumin as a third-generation cancer chemopreventive drug [10]. Extracted from the tuber root of Curcuma curcuma, a rhizome of Curcuma turmeric, rhizome of Zedoary turmeric, and rhizome of Acaraceae calamus [11], Curcumin is a diketone compound. These traditional Chinese medicines share the fundamental principles of tumor treatment in traditional Chinese medicine, focusing on activating blood circulation, promoting qi circulation, relieving pain, breaking blood stasis, and eliminating accumulation. Studies indicate that Curcumin significantly inhibits the occurrence and development of tumor cells while demonstrating minimal toxic effects on normal cells. Its primary mechanisms involve inhibiting the growth and proliferation of tumor cells, impeding invasion and metastasis, arresting the cell cycle, and inducing apoptosis in tumor cells [12, 13]. A study by Zeng Yuqun et al. using different concentrations of Curcumin on the HCC cell line LM3 further supports these findings, revealing a concentration-dependent reduction in cell proliferation ability [14]. In alignment with previous research, our study on HepG2 cells treated with Curcumin demonstrated a concentration-dependent inhibition of cell growth, reaching a significant inhibition rate of 66% at 60 μmol/L. These results affirm Curcumin's potential as a therapeutic agent in inhibiting the proliferation of HepG2 cells.

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], which leads to the continuous proliferation of cells out of control, and the imbalance between cell proliferation and apoptosis will lead to the occurrence of tumors. 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 [13]. Flow cytometry showed that Cur could block HepG2 cells in S phase and could not carry out cell cycle cycle, indicating that Cur alone could better inhibit the growth of cancer cells.

Apoptosis, a programmed and active mode of cell death, is regulated by specific genes in typical physiological and pathological environments. Notably, Bcl-2 and Caspase play pivotal roles in apoptosis, with

Caspase-3 as a critical enzyme in the apoptosis cascade. The hydrolytic activity of Caspase-3 directly induces cell apoptosis.Bcl-2 and Bax are crucial genes in apoptosis regulation, with Bcl-2 acting as an anti-apoptotic factor and Bax promoting apoptosis. The balance between these factors is essential in controlling cell fate. Zhang et al.'s research revealed that Curcumin inhibits the proliferation of SMMC-7721 cells by modulating the JNK signaling pathway, inhibiting the ERK and p38 MAPK signaling pathways, and regulating the expression of Bcl-2, Survivin, Bax, and Caspase-3. This modulation resulted in increased apoptosis of SMMC-7721 cells. In this study, the apoptosis rate of HepG2 cells was detected by flow cytometry, and it was found that CUR could induce apoptosis of HepG2 cells in a concentration dependent manner. In order to further verify the strong pro-apoptotic effect of CUR, apoptotic proteins Bcl-2, Bax and Caspase-3 were detected by Western blot, and it was found that CUR could significantly express Bcl-2 and up-regulate the expression of pro-apoptotic factor Bax in a concentration dependent manner, suggesting that CUR could induce apoptosis of liver cancer cells. This is consistent with the above research results. Tumor cell invasion and metastasis are critical characteristics of malignancies, and their inhibition is pivotal in evaluating the efficacy of anti-cancer drugs. Previous research has reported that Curcumin inhibits migration and invasion of A549 cells in a concentration-dependent manner. Our study echoes these results, demonstrating that Curcumin treatment for 48 hours significantly reduces HepG2 cell migration and invasion capabilities, as observed in scratch and Transwell invasion tests. These findings underscore Curcumin's efficacy in effectively impeding the migration and invasion of tumor cells, reinforcing its potential as a therapeutic agent in combating cancer progression.

In conclusion, this study investigated the impact of varying concentrations of Curcumin on in vitro cultured human liver cancer cells (HepG2). The findings reveal that Curcumin induces cell apoptosis, inhibits cancer cell metastasis, and modulates the expression of Bcl-2, Bax, and Caspase-3 proteins, thereby promoting apoptosis and restraining the proliferation of HepG2 cells. As a monomer from traditional Chinese medicine, Curcumin exhibits diverse targets, acts on multiple pathways, and elicits various effects. It stands out for its wide availability, low toxicity, and high efficiency. Compared to traditional Chinese medicine compounds, the apparent drug effect of Curcumin facilitates the study of its mechanism of action. The mechanism of Curcumin is intricate, and the causes of cancer involve a complex interplay of genome and protein changes, alterations in pathways, and modifications in cancer-related genes and proteins. Further progress is necessary to comprehensively understand the detailed mechanisms underlying the effects of Curcumin in the context of cancer.

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References

1. Zhou Yanzhao Wang Zhengzheng, qing-jun li, etc. [1] Wang ZH, LI Q J, et al. Immunotherapy of hepatocellular carcinoma [J]. Chin J Hepatobiliary Surgery Tzu chi, 2021, 26 (6) : 472-476.
2. Zhao Tong, Yin Zifei. Research progress on the effect of traditional Chinese medicine on liver cancer stem cells [J]. Liaoning Journal of Traditional Chinese Medicine, 2021,47 (7) : 209-211. (in Chinese)
3. Lin Tong, SHA Yong-qiang, Chen Ze-xiong. [3] Lin Tong, XIA Yongqi, Chen Zxiong. Research progress of autophagy induced by active ingredients of traditional Chinese medicine in hepatocellular carcinoma in vitro [J]. China Medicine Guide, 2021, 15 (34) :49-56.
4. Cui Qi, Dong Yan 'e, Huo Yunlong, et al. [4] Cui Q, Dong Y E, Huo Y L, et al. Effects of Curcumin on proliferation, invasion and metastasis of hepatocellular carcinoma HepG2 cells and its related mechanism [J]. Advances in Anatomical Science, 2021, 25(4):364-367.
5. Zhang Meilan, Shen Zheshi, Zhang Yingzhe, et al. [5] ZHANG M L, Shen Z S, Zhang Y Z, et al. The effect of endoplasmic reticulum stress on human liver cancer BEL-7404 cell apoptosis induced by Curcumin [J]. Jilin Medical Science,2021,42(1):5-7.
6. Chang M, Wu M, Li H. Curcumin combined with glycyrrhetinic acid inhibits the development of hepatocellular carcinoma cells by down-regulating the PTEN/PI3K/AKT signalling pathway.Am J Transl Res 2017; 9: 5567-5575.
7. LIU Yueming, Zhu Jun, Zheng Sipeng, et al. [7] LIU Y M, ZHU J, ZHENG S P, et al. In vitro expansion of NK cells from peripheral blood of healthy adults in Xinjiang [J]. Journal of Xinjiang Medical University, 2013, 35(6):763-767.
8. Chikara S, Nagaprashantha LD, Singhal J, Horne D, AwasthiS, Singhal SS. Oxidative stress and dietary phytochemicals:Role in cancer chemoprevention and treatment. Cancer Lett 2018; 413: 122-134.
9. Li H, Wang Y, Li T Y, et al. The research progress of Curcumin in the treatment of primary liver cancer [J]. Journal of Jilin University of Medicine, Li H, Wang Y, et al.2018,39(2):135-137.
10. Zhang Jun, Tong Zhong, Li Yingxia, et al. [10] ZHANG J, Tong Z, LI Y X, et al. Effect of Curcumin on tumor cell proliferation [J]. Anhui Medicine, 2014(8):1545-1548.

11. Zhu J J, An Y W, Hu G, et al. Simultaneou determination of Multiple sesquiterpenes in Curcuma wenyujin herbal Medicines and related products with one single reference standard [J]. Molecules, 2013, 18(2): 2110-2121.
12. Tuorkey MJ. Curcumin a potent cancer preventive agent:mechanisms of cancer cell killing [J]. Interventional Medicine &Applied Science, 2014, 6(4):139-146.
13. Zeng Y Q, LI B, Dou C W.Zang Y Q, Li B, DOU C W. Curcumin inhibits proliferation, migration and invasion of hepatocellular carcinoma by regulating TUFT1/AKT signaling pathway [J]. New traditional Chinese medicine, 2021,52 (11) : 6-9.
14. Zhao Jian Cui Limin, Dong Liang, etc.ZHAO J A, CUI L M, DONG L L, et al. Comparison and mechanism of Curcumin and curcumin-linked amino group against liver cancer [J]. China Pharmacy, 2020,31(22):2741-2749.