

# Study on the mechanism of AMPK signaling pathway and its effect on apoptosis of human hepatocellular carcinoma SMMC-7721 cells by curcumin

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**Abstract.** – **OBJECTIVE:** Liver cancer is a common malignant tumor in the digestive system. Curcumin is a kind of phenolic pigment, which is extracted from herbage and has a plenty of physiological roles in anti-inflammation, anti-oxidation and anti-tumor. In our study, human hepatoma SMMC-7721 cell lines were selected and treated with curcumin to detect its effects on the apoptosis and AMPK signaling pathway.

**MATERIALS AND METHODS:** Human liver cancer cell strain SMMC-7721 was cultured and treated with different curcumin concentrations for different times followed by measuring the changes of cell proliferation activity and cycle by MTT and flow cytometry, respectively. Protein expression of Bcl-2, Bax and Caspase-3 were tested by Western blot, and the activation level of AMPK was also detected.

**RESULTS:** Different concentrations of curcumin could inhibit the proliferation of tumor cells in a dose-dependent manner. After 48 h inhibition by curcumin with a concentration of 40 mmol/L, the inhibitory effect was more obvious with statistically significant ( $p < 0.05$ ). The number of human liver cancer SMMC-7721 cells increased in G1 stage and decreased in S stage after treated with different concentrations of curcumin. During the G1 stage to the S stage, inhibition occurred and the effect of curcumin intervention group with 40 mmol/L was more evident than that of 10 mmol/L group, 20 mmol/L group and the control group with statistically significant ( $p < 0.05$ ). SMMC-7721 cell stains had been intervening by curcumin with concentrations of 10 mmol/L, 20 mmol/L and 40 mmol/L for 12 h, 24 h and 48 h, as the drug concentration increased, the reaction time prolonged, the protein expressions of Bcl-2 and Survivin were significantly decreased and Bax protein expression was significantly increased ( $p < 0.05$ ).

**CONCLUSIONS:** Curcumin decreased the proliferation activity of tumor cells, increased the cell quantities in G1 stage and decreased the cell numbers in S stage in human liver cancer

SMMC-7721 cells. The Bcl-2 and Survivin proteins were downregulated and Bax protein was upregulated; furthermore, the AMPK signaling pathway was activated.

Key Words:

Curcumin, SMMC-7721, Apoptosis, AMPK.

## Introduction

Curcumin is a phenolic pigment extracted from the rhizome of *Curcuma longa*, whose pharmacological action is broad and has the effects on anti-tumor, anti-inflammation, anti-oxidation and anti-virus<sup>1</sup>. Previous studies<sup>2</sup> showed that curcumin exerts its physiological role based on protecting normal cell of the body from the damage of adverse factors. Hepatocellular carcinoma (HCC) is a common malignant tumor in clinic, with incidence rate and mortality rate ranked as fifth and fourth, respectively. For most of the patients with liver cancer, which were found at the advanced stage and lost the best time for surgery, liver cancer has poor sensitivity to radiotherapy and cytotoxic drugs, and the treatment effect is poor caused by the advanced stage<sup>3</sup>. At present, clinical doctors and researchers hope to find a treatment method with a better treatment and less toxic effects, to improve the prognosis of patients with HCC and reduce the possibility of the various complications. Researches<sup>4</sup> have pointed out that the development of malignant tumor may perhaps have a relationship with the imbalance between tumor cell proliferation and death. In the basic experimental study of curcumin, the curcumin intervention on human lung adenocarcinoma cell line A549/DDP can inhibit cell proliferation, which is accomplished by blocking the cell cycle and inducing apoptosis<sup>5</sup>.

In this study, by selecting human liver SMMS-7721 cell line and the intervention of different time with different concentrations of curcumin, apoptosis of SMMC-7721 cells was detected and the proteins expression of Bcl-2, Bax, Survivin and Caspase-3 were detected, whose relationship with MAPK pathway were also analyzed.

## Materials and Methods

### **Experimental Cell**

HCC SMMC-7721 cells were kindly offered by North China Pharmaceutical Group New Drug Research and Development (Co., Ltd. Shijiazhuang, Hebei, China).

### **Experimental Reagents**

Curcumin was purchased from Shanghai Chinese medicine Reagent Co., Ltd. (lot number: 20050913, Shanghai, China); AMPK antibody was purchased from Cell Signaling Technology Co., Ltd. (Danvers, MA, USA); antibodies against Bcl-2, Bax, Survivin were purchased from Bioworld Technology, Inc. (Nanjing, China); secondary antibody rabbit anti-mouse was purchased from Santa Cruz Biotechnology, Lnc. (Santa Cruz, CA, USA); Dulbecco's Modified Eagle Medium (DMEM) media, Penicillin/Streptomycin and fetal bovine serum (FBS) were purchased from Gibco Co., Ltd (Grand Island, NY, USA).

### **Test Facilities**

Incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA); carbon dioxide incubator and the refrigerator with -80°C were purchased from Sanyo Electric Co., Ltd. (Moriguchi City, Osaka, Japan); inverted microscope was purchased from Nikon Co., Ltd. (Minatoku, Tokyo, Japan); Polymerase Chain Reaction (PCR) with Biometra T1 type was purchased from Biometra Co., Ltd. (Göttingen, Germany); nucleic acid concentration detector was purchased from Eppendorf Co., Ltd. (Hamburg, Germany); bench type low-temperature high-speed centrifuge was purchased from Beckman Co., Ltd (Brea, CA, USA).

### **Experimental Methods**

#### **Cell Culture**

Human HCC SMMC-7721 cells were routinely cultured in RPMI1640 medium, placing in an incubator with 37°C and 5% CO<sub>2</sub>.

### **Human HCC SMMC-7721 cells Were Intervened by Curcumin**

Human HCC SMMC-7721 cells in logarithmic growth phase were selected and were inoculated into 96-well plate for conventional culturing. 2% fetal calf serum (FCS) was used to culture for 24 h overnight. After replacement of 10% fetal bovine serum (FBS) DMEM medium, 10 mmol/L curcumin were added as the experimental group. Human HCC SMMC-7721 cells without being intervened were regarded as the control group.

### **Detection cell Proliferation Activity of Human HCC SMMC-7721 Cells Being Intervened with Curcumin by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT)**

Curcumin was added into the human HCC SMMC-7721 cells with a final concentration of 10 mmol/L, 20 mmol/L and 40 mmol/L, respectively. Cells activity were detected at the time points of 12 h, 24 h and 48 h. 20 μL MTT with concentration of 5 mg/mL were added into to the above solution and terminated incubation after incubation for 4 h under the same conditions; each well was added with 150 μL dimethyl sulfide, and the low-velocity oscillation 10 min to dissolve crystallization. The absorbance of each well was measured at 570 nm.

### **The Effect of Curcumin on the Cell Cycle of Human HCC SMMC-7721 Cells as Detected by Flow Cytometry**

Annexin double labeling staining was used to detect the apoptosis rate of SMMC-7721 cells induced by curcumin. After the cells were treated with synchronization, curcumin at a concentration of 10 mmol/L, 20 mmol/L, 40 mmol/L were added into the experimental group, while the control group was without drug intervention. Cells were collected after 48 h digestion and were adjusted the concentration to 10<sup>6</sup> mmol/L; 490 μL buffer were added and cells were oscillatory mixed, 5 μL Annexin and V-FITC 5LPI dye were added at room temperature, staining 10 min in the condition of avoiding light. The two parameters analysis were carried out by flow cytometry.

### **Detection of Bcl-2, Bax and Survivin Protein Expression in Human HCC Cell Line SMMC-7721 by Western Blotting**

Human HCC SMMC-7721 cells in logarithmic growth stage were cultured in DMEM medium with 10% fetal bovine serum (FBS)

and then curcumin was added to a final concentration of 10 mmol/L, 20 mmol/L, 40 mmol/L. 40 µg/well proteins were taken to carry out electrophoresis separation with 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which were closed 1 h at room temperature. The closed liquid was discarded and immediately joined the primary anti-solution to incubate for 30 min with filtration membrane (1:200 dilution, β-action, 1:500), 4°C for the night. The primary anti-solution was discarded, after washing with tris buffered saline-tween (TBS-T) buffer secondary anti-solution was added (1:2000 dilution) to incubate for 1 hour. Secondary anti-solution was discarded, after washing with TBS-T buffer, 2 ml developer A and B were added for chemical development. Quantity One analysis software was carried out to analyze the optical density of the developing bands.

#### ***The Effect of Curcumin on The Activation of AMPK Cells in Human Hepatocellular Carcinoma SMMC-7721 Cells was Detected by Western Blotting***

Curcumin with a concentration of 40 mmol/L was added to hepatocellular carcinoma SMMC-7721 cells to being intervened for 12 h, 24 h and 48 h, 40 µg/well proteins were taken to carry out electrophoresis separation with 8% SDS-PAGE, which were closed 1 h at room temperature. The closed liquid was discarded and immediately joined the primary anti-solution to incubate for 30 min with filtration membrane (1:200 dilution, β-action, 1:500), 4°C for the night. The primary anti-solution were discarded, after washing with TBS-T buffer secondary anti-solution of anti-rabbit IgG-HRP diluent were added (1:2000 dilution) to incubate for 1 h. Secondary anti-solution was discarded, after washing

with TBS-T buffer, 2 ml developer A and B were added for chemical development. Quantity One analysis software was carried out to analyze the optical density of the developing bands.

#### ***Statistical Analysis***

The data were processed by SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA),  $\chi^2$ -test was used for count data, ANOVA (analysis of variance) with Tukey's post-hoc test was used for measurement data and all results were presented as mean ± standard deviation (SD).  $p < 0.05$  was considered as statistically significant.

## **Results**

#### ***The Effect of Curcumin on the Proliferation of Human Hepatocellular Carcinoma SMMC-7721 Cells***

Human HCC SMMC-7721 cells were intervened by curcumin with concentration of 10 mmol/L, 20 mmol/L and 40 mmol/L for 12 h, 24 h and 48 h and the results showed that different concentrations of curcumin could inhibit the proliferation of cells at different time periods in a dose-dependent manner. The inhibitory effect was more obvious after being treated with 40 mmol/L curcumin with statistically significant difference ( $p < 0.05$ ) (Table I).

#### ***The effect of Curcumin on the Cell Cycle of Human Hepatocellular Carcinoma SMMC-7721 cells was Detected by Flow Cytometry***

Detection of SMMC-7721 cell cycle in human HCC showed that the cell number was increased in G1 phase and reduced in S phase and inhibited from G1 to S phase which were intervened by curcumin with different concentrations. Moreover, the effect of curcum-

**Table I.** The effect of curcumin on the proliferation of human HCC SMMC-7721 cells.

Time	Experimental Group			Control Group
	10 mmol/L	20 mmol/L	40 mmol/L	
12 h	0.985±0.072 <sup>1</sup>	0.723±0.046 <sup>12</sup>	0.232±0.024 <sup>123</sup>	0.091±0.023
24 h	0.811±0.045 <sup>14</sup>	0.512±0.034 <sup>124</sup>	0.176±0.016 <sup>1234</sup>	0.072±0.012
48 h	0.321±0.031 <sup>145</sup>	0.256±0.026 <sup>1245</sup>	0.121±0.011 <sup>12345</sup>	0.034±0.016

<sup>1</sup>Compared to control group, the difference was statistically significant,  $p < 0.05$ .

<sup>2</sup>Compared to 10 mmol/L of experimental group, the difference was statistically significant,  $p < 0.05$ .

<sup>3</sup>Compared to 20 mmol/L of experimental group, the difference was statistically significant,  $p < 0.05$ .

<sup>4</sup>Compared to administration of 12 h, the difference was statistically significant,  $p < 0.05$ .

<sup>5</sup>Compared to administration of 24 h, the difference was statistically significant,  $p < 0.05$ .

**Table II.** The effect of curcumin on the cell cycle of human hepatocellular carcinoma SMMC-7721 cell.

Group	Cell Cycle				
	G1	S	G2/M	AP	PI
10 mmol/L	73.1±0.5 <sup>1</sup>	11.8±1.1 <sup>1</sup>	5.4±0.8 <sup>1</sup>	15.3±1.5 <sup>1</sup>	8.2±1.4 <sup>1</sup>
20 mmol/L	82.4±1.2 <sup>12</sup>	7.6±0.8 <sup>12</sup>	3.1±0.7 <sup>12</sup>	8.2±0.7 <sup>12</sup>	15.7±1.6 <sup>12</sup>
40 mmol/L	92.2±1.7 <sup>123</sup>	3.2±0.5 <sup>123</sup>	0.3±0.3 <sup>123</sup>	1.1±0.2 <sup>123</sup>	19.8±1.8 <sup>123</sup>
Control group	71.1±0.2	18.7±1.3	7.6±1.1	0	24.5±2.5

<sup>1</sup>Compared to control group, the difference was statistically significant,  $p < 0.05$ .

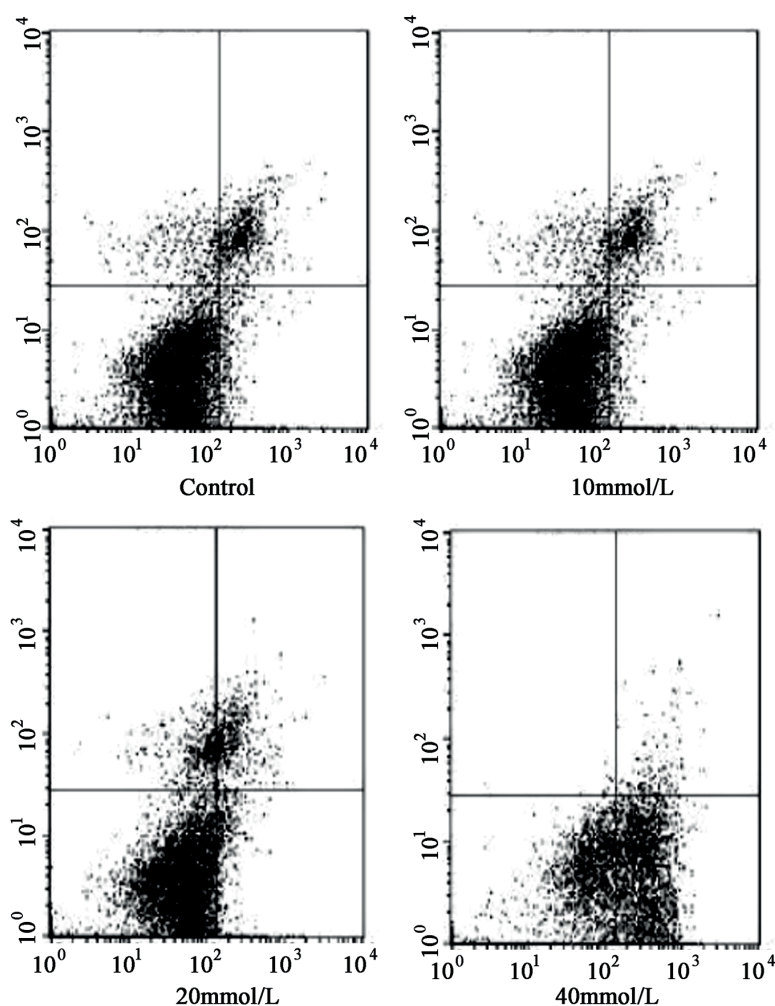
<sup>2</sup>Compared to 10 mmol/L of experimental group, the difference was statistically significant,  $p < 0.05$ .

<sup>3</sup>Compared to 20 mmol/L of experimental group, the difference was statistically significant,  $p < 0.05$ .

in intervention group with 40 mmol/L was more obvious than that of 10 mmol/L, 20 mmol/L and control group with statistical significance ( $p < 0.05$ ). Without drug intervention, SMMC-7721 cells grew well with normal cell cycle (Table II, Figure 1).

**Detection of Bcl-2, Bax and Survivin Protein Expression in Human Hepatocellular Carcinoma Cell Line SMMC-7721 by Western Blotting**

The expression levels of Bcl-2, Bax and Survivin protein in human hepatocellular carcinoma



**Figure 1.** The effect of curcumin on the cell cycle of human hepatocellular carcinoma SMMC-7721 cell.

**Table III.** The expression of Bcl-2, Bax and Survivin protein in human hepatocellular carcinoma cell line SMMC-7721 intervened by curcumin.

Index	Experimental Group			Control Group
	10 mmol/L	20 mmol/L	40 mmol/L	
Bcl-2				
12 h	0.51±0.21 <sup>1</sup>	0.36±0.18 <sup>12</sup>	0.16±0.11 <sup>123</sup>	0.74±0.37
24 h	0.44±0.15 <sup>14</sup>	0.24±0.13 <sup>124</sup>	0.10±0.03 <sup>1234</sup>	0.72±0.38
48 h	0.23±0.13 <sup>15</sup>	0.17±0.09 <sup>125</sup>	0.14±0.01 <sup>12345</sup>	0.69±0.41
Bax				
12 h	0.43±0.21 <sup>1</sup>	0.37±0.18 <sup>12</sup>	0.19±0.11 <sup>123</sup>	0.10±0.09
24 h	0.54±0.25 <sup>14</sup>	0.42±0.21 <sup>1245</sup>	0.22±0.12 <sup>1234</sup>	0.10±0.08
48 h	0.76±0.39 <sup>145</sup>	0.57±0.24 <sup>1245</sup>	0.33±0.17 <sup>12345</sup>	0.11±0.07
Survivin				
12 h	0.85±0.34 <sup>1</sup>	0.56±0.23 <sup>12</sup>	0.37±0.09 <sup>123</sup>	0.12±0.06
24 h	0.71±0.25 <sup>14</sup>	0.43±0.17 <sup>124</sup>	0.26±0.08 <sup>1234</sup>	0.10±0.09
48 h	0.53±0.21 <sup>145</sup>	0.29±0.14 <sup>1245</sup>	0.18±0.07 <sup>12345</sup>	0.12±0.07

<sup>1</sup>Compared to control, the difference was statistically significant,  $p < 0.05$ .

<sup>2</sup>Compared to 10 mmol/L of experimental group, the difference was statistically significant,  $p < 0.05$ .

<sup>3</sup>Compared to 20 mmol/L of experimental group, the difference was statistically significant,  $p < 0.05$ .

<sup>4</sup>Compared to administration of 12 h, the difference was statistically significant,  $p < 0.05$ .

<sup>5</sup>Compared to administration of 24 h, the difference was statistically significant,  $p < 0.05$ .

SMMC-7721 cells were detected by Western blot and the results showed that with the increasing of drug concentration and the extension of action time, the expressions Bcl-2 and Survivin protein were significantly decreased while Bax was significantly increased with statistically significant difference among groups ( $p < 0.05$ ) (Table III).

#### **The Effect of Curcumin on the Expression of AMPK in Human Hepatocellular Carcinoma SMMC-7721 Cells**

Human hepatocellular carcinoma SMMC-7721 cells were intervened by curcumin at a concentration of 40 mmol/L and the results showed AKMP was not activated until 12 h and reached the highest level at the 48 h with a statistically significant difference ( $p < 0.05$ ) (Table IV, Figure 2).

## **Discussion**

Hepatocellular carcinoma (HCC) is a kind of malignant tumor with a high incidence. Although clinical treatments have made great progress in recent years, due to the side effects of chemotherapy and drug resistance and other factors, the overall prognosis of HCC is poor. Therefore, it is important to find the high efficiency and low-toxicity of anticancer drugs for the treatment of liver cancer<sup>6</sup>. Curcumin is a kind of plant polyphenols extracted from turmeric, which have the effects of anti-inflammation, anti-oxidation anticoagulation and lowering blood pressure, anti-atherosclerosis, anti-tumor<sup>7,8</sup>. A previous work<sup>9</sup> found that curcumin has a certain preventive effect on colon cancer, breast cancer, lung cancer and prostate

**Table IV.** The effect of curcumin on the activation of AMPK protein in human hepatocellular carcinoma cell line SMMC-7721.

Group	Action Time			Control Group
	12h	24h	48h	
10 mmol/L	0.084±0.0011	0.212±0.00214	0.257±0.011145	0.107±0.001
20 mmol/L	0.115±0.00212	0.413±0.087124	0.634±0.0211245	0.109±0.003
40 mmol/L	0.223±0.005123	0.702±0.0111234	0.987±0.03612345	0.114±0.002

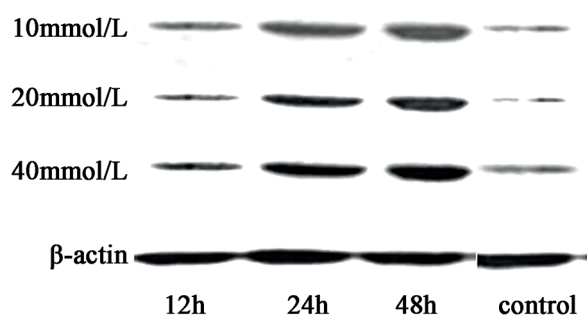
<sup>1</sup>Compared to control, the difference was statistically significant,  $p < 0.05$ .

<sup>2</sup>Compared to 10 mmol/L of experimental group, the difference was statistically significant,  $p < 0.05$ .

<sup>3</sup>Compared to 20 mmol/L of experimental group, the difference was statistically significant,  $p < 0.05$ .

<sup>4</sup>Compared to administration of 12 h, the difference was statistically significant,  $p < 0.05$ .

<sup>5</sup>Compared to administration of 24 h, the difference was statistically significant,  $p < 0.05$ .



**Figure 2.** The expression level of AMPK protein in human hepatocellular carcinoma cell line SMMC-7721.

cancer. At present, most scholars<sup>10</sup> believe that the inhibition of cell apoptosis is one of the important reasons for the occurrence of tumor and the induction of apoptosis may be the basic strategy for the treatment of malignant tumors.

In our study, human hepatocellular carcinoma SMMC-7721 cells were cultured, and were intervened by curcumin with concentrations of 10 mmol/L, 20 mmol/L and 40 mmol/L for 12 h, 24 h and 48 h. MTT assay showed that different concentrations of curcumin could inhibit cell proliferation at different time in a dose-dependent manner. After being treated by 40 mmol/L curcumin for 48 h, the inhibition of proliferation activity is more obvious. In maintaining the normal morphology and function of the body's tissues and organs, cell proliferation and apoptosis play an important role<sup>11</sup>. Previous investigations<sup>12</sup> have found that curcumin can inhibit the growth of hepatocellular carcinoma cells in a dose-dependent manner. Under the electron microscope, the ultra-structural changes of the cancer apoptotic cells were significantly increased after treated with curcumin<sup>13</sup>. Basic research shows that curcumin has a significant inhibitory effect on mice ascites hepatoma cells, and the cell proliferation was inhibited in a dose-dependent manner<sup>14</sup>. All of those findings are consistent with the results of the present study. The most significant feature of tumor cells is split and reproductive endlessly and disorderly. In the occurrence and development of malignant tumors, cell cycle disorder plays an important role in the process of cell cycle, leading to increased cell proliferation and decreased apoptosis<sup>15</sup>. If the control function of G1/S and G2/M are blocked, it will make cell proliferation out of control and lead to the occurrence of malignant tumors<sup>16,17</sup>. The detection of SMMC-7721 cell cycle in human hepatocellular carcinoma showed that human hepatocellular

carcinoma SMMC-7721 cells increased in G1 cycle and decreased in S cycle, and the inhibition happened from G1 phase to S phase after treated with curcumin at different concentrations for 48 h, and the 40 mmol/L curcumin. The intervention group was more obvious than that of 10 mmol/L, 20 mmol/L, and control group. All those results show that curcumin can inhibit the human hepatocellular carcinoma SMMC-7721 cells conversion from G1 to S phase of the cell cycle, leading to inhibition of tumor growth and proliferation.

Previous reports<sup>18,19</sup> have confirmed and showed that the use of curcumin in the intervention of human hepatocellular carcinoma cell SMMC-7721 can inhibit cell proliferation and lead to cell apoptosis with increased expression of Bax, Caspase-3 and decreased Bcl-2, Survivin. Also, curcumin can inhibit ERK and p38 MAPK signaling pathway by activating the JNK signaling pathway in human hepatocellular carcinoma cell line SMMC-7721, which can affect the expression of Bax, Bcl-2, Survivin protein<sup>20</sup>. In our study, the expression levels of Bcl-2, Bax and Survivin protein in human hepatocellular carcinoma SMMC-7721 cells were detected by Western blot and the results showed that after human hepatocellular carcinoma cell line SMMC-7721 were intervened by curcumin at concentrations of 10 mmol/L, 20 mmol/L and 40 mmol/L for 12 h, 24 h and 48 h, with the increase of drug concentration, the time of action was prolonged, the expressions of Survivin and Bcl-2 decreased, and the expression of Bax increased. After being intervened by 40 mmol/L curcumin for 48 h, Bcl-2 and Survivin protein expression were the lowest, and the expression of Bax protein was the highest. At the same time, after being intervened for 12 h, AMPK started to be activated and reached the highest level at 48 h. It shows that curcumin can reduce the expression of Bcl-2, Survivin protein in SMMC-7721 cells, increase the expression of Bax protein as well as activate AMPK signaling pathway.

## Conclusions

Curcumin can decrease the cell proliferation activity, increase the cell quantities in G1 cycle, reduced cell numbers in S cycle in human hepatocellular carcinoma cell line SMMC-7721. The expression of Bcl-2 and Survivin proteins in SMMC-7721 cell line was decreased and the Bax expression increased with activated AMPK sig-

naling pathway. However, the specific mechanism of curcumin's action remains unclear and needs further investigation.

### Conflict of interest

The authors declare no conflicts of interest.

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