

## Original Article

# Effect of chlorogenic acid on the proliferation and apoptosis of human hepatocellular carcinoma HepG2 cells by miR-181b/SIRT1/HO-1 signaling pathway

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**Abstract:** Objective: To investigate the effect of chlorogenic acid (CGA) on the proliferation and apoptosis of human hepatocellular carcinoma HepG2 cells and to explore the possible mechanisms. Methods: Cell line HepG2 was cultured *in vitro* and treated with CGA at 10 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL. Results: The growth inhibition rates of HepG2 cells that were treated with the four concentrations of CGA were significantly raised after 48 h and 72 h (all  $P < 0.05$ ); the apoptosis rates of HepG2 cells that were treated with the four concentrations of CGA were significantly higher than those in the blank group (all  $P < 0.05$ ). Compared with the blank group and those treated with CGA at 10 µg/mL, miR-181b, SIRT1, HO-1, Bcl-2, and PCNA expressions in HepG2 cells that were treated with CGA at 25 µg/mL, 50 µg/mL and 100 µg/mL significantly decreased (all  $P < 0.05$ ), and Bax expression significantly increased ( $P < 0.05$ ). Conclusion: CGA can inhibit the proliferation of HepG2 cells and promote their apoptosis, which may be achieved through the regulation of the miR-181b/SIRT1/HO-1 signaling pathway.

**Keywords:** Chlorogenic acid, SIRT1/HO-1 pathway, HepG2, proliferation, apoptosis

## Introduction

Hepatic carcinoma is a common digestive system malignancy found in human beings. Hepatocellular carcinoma (HCC) is the most common histopathological type of hepatic carcinoma, accounting for about 80% of primary liver cancer, with a fatality rate of 93% [1]. Viral hepatitis is the main pathogenic factor of primary liver cancer, and liver damage induced by long-term drinking, aflatoxin and toxic compounds are high-risk pathogenic factors of hepatic carcinoma [2]. The incidence of hepatic carcinoma is raised with the increase of people who acquire non-alcoholic fatty liver disease [3]. Recently, some progress has been made in the diagnosis and treatment of hepatic carcinoma with the improvement of medical technology. The treatment methods for hepatic carcinoma are mainly liver transplantation and transcatheter arterial chemoembolization in clinical practice, but patients are prone to suffer from lesion recurrence and metastasis after treatment. Local treatment is limited by the size and loca-

tion of lesions, which causes little improvement of patient's overall survival. Currently, hepatic carcinoma is a highly lethal disease [4, 5]. Therefore, finding a way to decrease the incidence of chronic liver disease developing into hepatic carcinoma by controlling cell canceration is of great significance in clinical practice.

In recent years, epidemiological investigation has found that coffee consumption is closely related to the incidence of hepatic carcinoma. In the study by Bravi et al., there was a negative correlation between drinking coffee and the risk of suffering from HCC and chronic liver disease [6]. Godos et al. through prospective systematic evaluation and meta-analysis found that there was a negative correlation between coffee consumption and the risk of hepatic carcinoma, and more than one cup of coffee daily was associated with a 15% reduction of risk of hepatic carcinoma [7]. Chlorogenic acid (CGA), mainly derived from coffee, is a kind of natural polyphenolic compound and the an important secondary metabolite found in plants, with anti-

**Table 1.** Primer sequence of miR-181b and  $\beta$ -actin gene

Gene	Forward	Reverse primer
miR-181b	5'-AACAUUCAUUGCUGUCGGUGGG-3'	5'-UUGUACUACACAAAAGUACUG-3'
$\beta$ -actin	5'-TGAGAGGGAATCGTGCCTG-3'	5'-TTGCTGATCCACATCTGCTGG-3'

tumor, anti-inflammation, anti-oxidation, anti-virus, anti-bacteria and other biological effects [8-10].

Previous studies have showed that CGA plays an anti-tumor role in the treatment of various malignant tumors [11, 12]. However, there are few studies on the effect and mechanism of CGA on the biological function of human hepatocellular carcinoma cell HepG2. In this study, we hypothesized that CGA could inhibit the proliferation of HepG2 cells and promote their apoptosis. Therefore, we performed a quasi-*in vitro* study and investigated the mechanism of action of CGA, expecting to provide pharmacological experimental evidence for CGA in the prevention and treatment of hepatic carcinoma in clinical practice.

## Materials and methods

### Main laboratory apparatus and reagents

The conducted research is not related to either human or animal use. Human hepatocellular carcinoma HepG2 cells were purchased from ATCC (Rockville, MD, USA). DMEM and fetal bovine serum were from Thermo Scientific (San Jose, CA, USA). Total RNA extraction kit EasyPure miRNA Kit and PCR + reverse transcription kit TransScript Green miRNA Two-Step qRT-PCR SuperMix were from TransGen Biotech Co., Ltd. (Beijing, China), and microRNA PCR premix kit was from JRDUN Biotechnology Co., Ltd. (Shanghai, China). Ultraviolet and visible spectrophotometer was purchased from BioRad (Hercules, USA). CGA was from Chengdu Mansite Bio-Technology Co., Ltd. (Chengdu, China). Methyl thiazolyl tetrazolium (MTT) kit, rabbit anti-human SIRT1 polyclonal antibody, rabbit anti-human HO-1 monoclonal antibody, mouse anti-human PCNA monoclonal antibody, rabbit anti-human Bax monoclonal antibody, mouse anti-human Bcl-2 monoclonal antibody, mouse anti-human  $\beta$ -actin monoclonal antibody, and horseradish peroxidase-labeled goat anti-mouse secondary antibody were all from Beyotime Biotechnology Co., Ltd. (Shanghai, China). FACSCanto flow cytometry was from Becton

Dickinson (Franklin Lakes, NJ, USA). Elx-800 microplate reader was from BioTek (Winooski, VT, USA) and 7500 fluorescent quantitative PCR was from Applied Biosystems (Foster City, CA, USA). Primers of miR-181b and  $\beta$ -actin were designed and synthesized by the Shanghai Haling Biological Technology Co., Ltd., China (**Table 1**).

### Cell culture

HepG2 cells were seeded into a high-glucose DMEM containing 10% fetal bovine serum, 100  $\mu$ g/mL streptomycin and 100 U/mL penicillin. The cells were incubated in an incubator with 5% CO<sub>2</sub> and a relative saturated humidity of 90% at 37°C. The culture solution was changed every other day. Cell passage was performed once at an interval of 2-3 d. HepG2 cells that grew along the median line were collected for subsequent experiments.

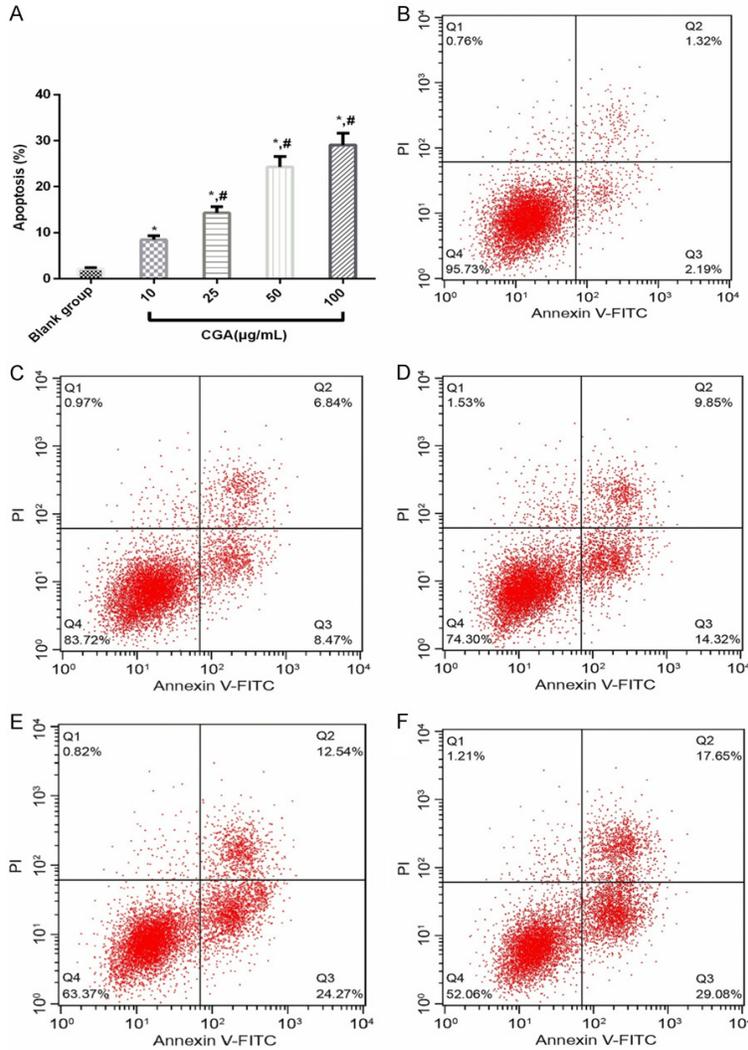
### Cell proliferation detection by MTT assay

Cell density was adjusted to  $2 \times 10^5$  cells/mL. Each well of the 96-well plate was seeded with 200  $\mu$ L cell suspension. The plate was incubated in an incubator with 5% CO<sub>2</sub> at 37°C for 24 h, and then given different concentrations of CGA (10  $\mu$ g/mL, 25  $\mu$ g/mL, 50  $\mu$ g/mL and 100  $\mu$ g/mL). Blank control wells were given the same volume of medium. Three replicate wells were set for each drug. After 24 h, 48 h, and 72 h incubation, each well was given 10  $\mu$ L 5 mg/mL MTT solutions and incubated in an incubator with 5% CO<sub>2</sub> at 37°C for 4 h. The supernatant was discarded. Each well was mixed with 100  $\mu$ L DMSO solutions. The optical density (OD) at 570 nm was determined by using an Elx-800 microplate reader. Each well was repeatedly measured three times. Cell growth inhibition rate =  $(1 - \text{OD value in the experimental group} / \text{OD value in blank group}) \times 100\%$ .

### Cell apoptosis detection by flow cytometry

HepG2 cells in the logarithmic phase were digested using trypsin. Cell density in each well was adjusted to  $1 \times 10^5$  cells/mL. Cells were

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**Figure 1.** HepG2 cell apoptosis. A. Effect of different concentrations of CGA on HepG2 cell apoptosis; B. HepG2 cell apoptosis in the blank group; C. HepG2 cell apoptosis treated with 10 μg/mL CGA; D. HepG2 cell apoptosis treated with 25 μg/mL CGA; E. HepG2 cell apoptosis treated with 50 μg/mL CGA; F. HepG2 cell apoptosis treated with 100 μg/mL CGA. CGA: chlorogenic acid; PI: propidium iodide. Compared with the blank group, \*P<0.05; compared within the group, #P<0.05.

incubated in an incubator with 5% CO<sub>2</sub> at 37°C for 24 h. Wells that were given different concentrations of CGA (10 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL) were set as experimental groups, and blank control wells were given the same volume of medium. After 24 h incubation, the collected cells were washed with 0.01 mol/L cold phosphate buffered saline. The cells were centrifuged at 111.8 × g and 25°C. The supernatant was discarded. The cells were re-suspended using 100 μL 1 × binding buffer and transferred to the detecting tubes. Each tube was mixed with 5 μL 7AAD and 5 μL PE Annexin

V and incubated for 15 min at room temperature in a dark place. Then each tube was mixed with 400 μL 1 × binding buffers. Detection by flow cytometry was finished within 1 h. Each sample was repeatedly detected three times. Cell fluorescence was detected by flow cytometry, with an excitation wavelength of 488 nm and the detection wavelengths of 530 nm and 575 nm. The results of flow cytometry were obtained, and the Q3 area of **Figure 1** showed the cell apoptosis (**Figure 1B-F**).

### qRT-PCR detection

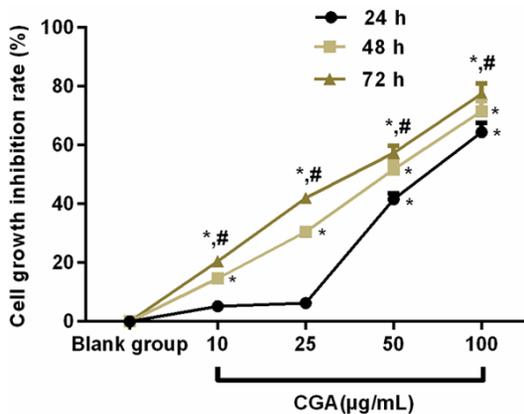
Total RNA of the collected cells was extracted by using the EasyPure miRNA kit. The purity, concentration and integrity of total RNA were measured by using ultraviolet spectrophotometer and agarose gel electrophoresis. Total RNA of 2 μL was used to prepare cDNA according to the instruction of the kit. Reverse transcription reaction solution: 42°C for 60 min, and 95°C for 5 min. The synthesized cDNA sample was stored at -20°C for subsequent use. U6 was used as the reference gene, and total volume of reaction solution was 20 μL, including 10 μL PCR Premix, 2 μL forward primer (10 ×), 2 μL reverse primer (10 ×), and 6 μL dd water (Rnase and Dnase

free). Conditions of PCR amplification circulation: 90°C for 5 min, 90°C for 5 s, 60°C for 30 s, and 72°C for 5 s, for 40 circles. Amplification data were analyzed by software in the ABI PRISM 7500 fluorescent quantitative PCR. The result was shown as 2<sup>-ΔCT</sup>.

### Western blot detection

HepG2 cells in each group were mixed with 60-80 μL RIPA lysis buffers. The lytic HepG2 cells were transferred to the centrifuge tube. Centrifugation was performed at 12,000 × g

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**Figure 2.** Effect of different concentrations of CGA on HepG2 cell growth inhibition rate. CGA: chlorogenic acid. Compared with the blank group, \* $P < 0.05$ ; compared with 24 h incubation at the same concentration of CGA, # $P < 0.05$ .

and 4°C for 10 min. The supernatant was collected as protein samples. Protein concentration was determined by BCA method. Protein samples were diluted by lysis buffer and inactivated at 95°C for 5 min to prepare 20 mg/mL protein. The 8% separation gel, 5% spacer gel, SDS-PAGE and PVDF transmembrane were prepared. The PVDF membrane was sealed with 5% skim milk for 1 h. Primary antibodies rabbit anti-human SIRT1 polyclonal antibody (1:1,000), rabbit anti-human HO-1 monoclonal antibody (1:1,000), mouse anti-human PCNA monoclonal antibody (1:1,000), rabbit anti-human Bax monoclonal antibody (1:1,000), mouse anti-human Bcl-2 monoclonal antibody (1:1,000), and  $\beta$ -actin (1:3,000) were added and sealed overnight at 4°C. The membrane was washed to remove primary antibodies. Horseradish peroxidase-labeled goat anti-mouse secondary antibody (1:5,000) was added and incubated at 37°C for 1 h. The membrane was rinsed with tris buffered saline tween three times, 5 min each time. The solution in the membrane was filtered with filter paper, and the membrane was put into ECL solution and developed in the darkroom. Protein bands were scanned, and the gray values were analyzed by Quantity One (Molecular Devices Corp, the Bay Area, CA, USA). Relative expression of protein = gray value of target protein band/gray value of  $\beta$ -actin protein band.

### Statistical analysis

Statistical analysis was performed by using SPSS 20.0 (IBM Corp, Armonk, NY, USA). The

data were drawn using GraphPad Prism 7. The measurement data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm sd$ ). The measurement data between groups were compared by independent sample t test. Comparison among several time points was performed by using repeated measures analysis of variance. Mean among groups was compared by using one-way analysis of variance, and pairwise comparison was performed by Dunnett-t test. There was a significant difference at  $P < 0.05$ .

## Results

### Effect of different concentrations of CGA on HepG2 cell growth inhibition rate

The results of MTT detection showed that after 24 h of treatment, compared with the blank group, there was no significant change in the cell growth inhibition rates of HepG2 cells that were treated with 10  $\mu\text{g/mL}$  and 25  $\mu\text{g/mL}$  CGA ( $P > 0.05$ ), and the cell growth inhibition rates of HepG2 cells that were treated with 50  $\mu\text{g/mL}$  and 100  $\mu\text{g/mL}$  CGA significantly increased ( $P < 0.05$ ). After 48 h and 72 h treatment, the cell growth inhibition rates of HepG2 cells that were treated with the four concentrations of CGA significantly increased as compared to the blank group ( $P < 0.05$ ). As the increase of CGA concentration and the extension of incubation time, the cell growth inhibition rates of HepG2 cells significantly increased (**Figure 2**).

### Effect of different concentrations of CGA on HepG2 cell apoptosis

The results of flow cytometry showed that compared with the blank group, the apoptosis of HepG2 cells that were treated with the four concentrations of CGA significantly increased ( $P < 0.05$ ). With the increase of drug concentration, the apoptosis gradually increased (**Table 2; Figure 1**).

### Effect of different concentrations of CGA on miR-181b expression of HepG2 cells

After 24 h treatment, the relative expression of miR-181b in HepG2 cells that were treated with 25  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$  and 100  $\mu\text{g/mL}$  CGA significantly decreased as compared to the blank group and those treated with 10  $\mu\text{g/mL}$  CGA ( $P < 0.05$ ). With the increase of drug concentration, the relative expressions of miR-181b in HepG2 cells significantly decreased (**Figure 3**).

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**Table 2.** Effect of different concentrations of CGA on HepG2 cell apoptosis ( $\bar{x} \pm sd$ )

Group	Apoptosis (%)
Blank group	2.19±0.26
10 µg/mL CGA	8.47±0.89*
25 µg/mL CGA	14.32±1.37* <sup>#</sup>
50 µg/mL CGA	24.27±2.27* <sup>#</sup>
100 µg/mL CGA	29.08±2.58* <sup>#</sup>
F	126.100
P	<0.001

Note: CGA: chlorogenic acid. Compared with the blank group, \*P<0.05; compared within groups, <sup>#</sup>P<0.05.

### Effect of different concentrations of CGA on SIRT1/HO-1 expression of HepG2 cells

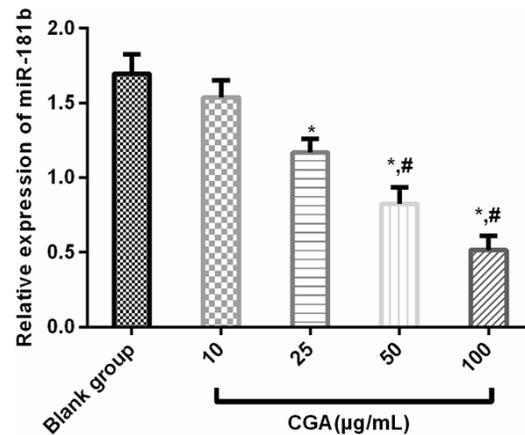
After 24 h treatment, the relative expression of SIRT1 and HO-1 in HepG2 cells that were treated with 25 µg/mL, 50 µg/mL and 100 µg/mL CGA significantly decreased as compared to the blank group and those treated with 10 µg/mL CGA (P<0.05). With the increase of drug concentration, the relative expression of SIRT1 and HO-1 of HepG2 cells significantly decreased (Figure 4).

### Effect of different concentrations of CGA on Bax, Bcl-2 and PCNA expression of HepG2 cells

After 24 h treatment, the relative expression of Bax in HepG2 cells that were treated with 25 µg/mL, 50 µg/mL and 100 µg/mL CGA significantly increased (P<0.05), and the relative expressions of Bcl-2 and PCNA significantly decreased (P<0.05), as compared to the blank group and those treated with 10 µg/mL CGA. With the increase of drug concentration, the relative expression of Bax in HepG2 cells significantly increased, and the relative expressions of Bcl-2 and PCNA significantly decreased (Figure 5).

## Discussion

Hepatic carcinoma is a malignant tumor that occurs in the liver, with an extremely high incidence and fatality rate [13]. Most patients with hepatic carcinoma are in an advanced stage at diagnosis and treated with surgery combined with chemotherapy. However, some commonly used drugs in clinical practice have severe side effects and strong drug resistance, which are

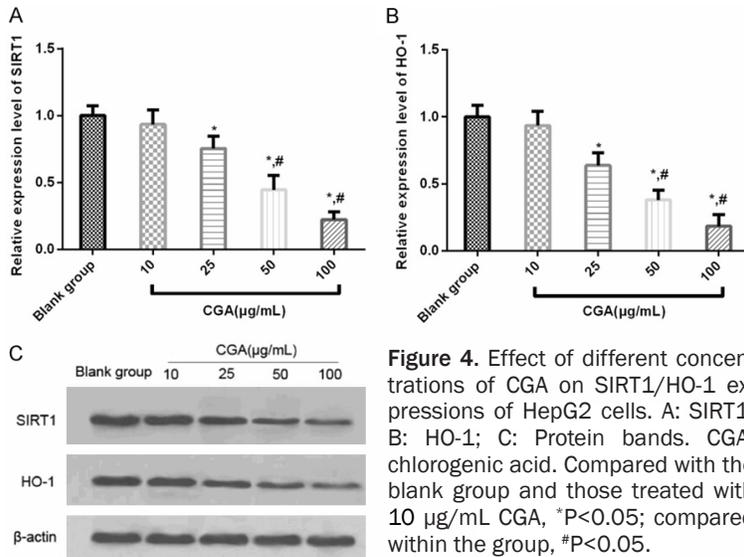


**Figure 3.** Effect of different concentrations of CGA on miR-181b expressions of HepG2 cells. CGA: chlorogenic acid. Compared with the blank group and those treated with 10 µg/mL CGA, \*P<0.05; compared within the group, <sup>#</sup>P<0.05.

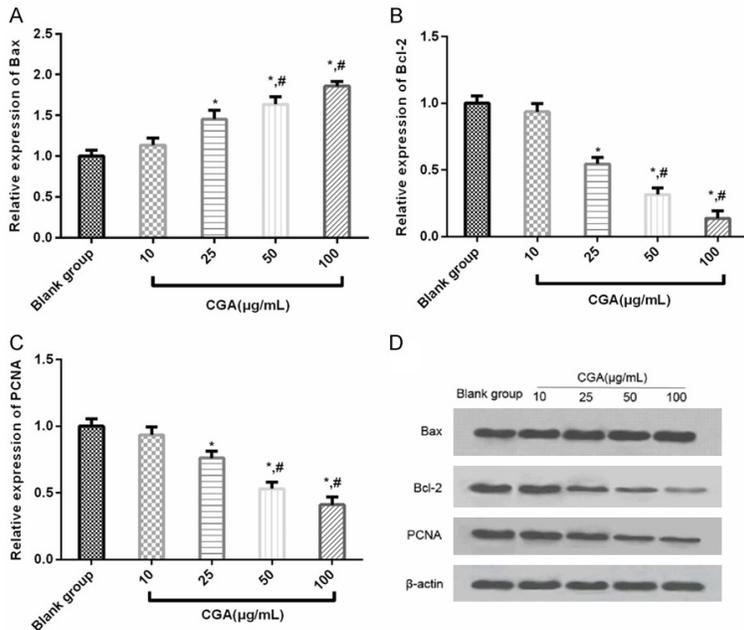
prone to cause toxic side effects in the body. However, natural anti-tumor drugs are able to reduce the toxic side effects in the body [14]. Therefore, it is particularly important to investigate natural anti-tumor drugs for the treatment of hepatic carcinoma.

CGA is a kind of natural anti-tumor drug and plays roles in various tumors, such as the inhibition of cancer cell growth in lung cancer, colon cancer and breast cancer [15]. Yamagata et al. found that CGA inhibited the proliferation of lung cancer A549 cells and reduced the expression of tumor associated markers NANOG, POU5F1 and SOX2 [16]. The study by Sadeghi et al. confirmed that CGA inhibited the viability of human colon cancer HCT116 and HT29 cells through inducing the generation of reactive oxygen species [17]. However, the effect and mechanism of CGA on the proliferation and apoptosis of hepatocellular carcinoma HepG2 cells are still unclear. In this study, we found that the cell growth inhibition rate of HepG2 cells significantly increased with the increase of CGA concentration and the extension of incubation time, indicating that CGA was able to significantly inhibit the proliferation of HepG2 cells. The results of flow cytometry showed that HepG2 cell apoptosis gradually increased with the increase of CGA concentration, suggesting that CGA was able to promote the apoptosis of HepG2 cells. In the study by Yan et al., 5-fluorouracil inhibited the proliferation of hepatocellular carcinoma cells; moreover, the combination

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**Figure 4.** Effect of different concentrations of CGA on SIRT1/HO-1 expressions of HepG2 cells. A: SIRT1; B: HO-1; C: Protein bands. CGA: chlorogenic acid. Compared with the blank group and those treated with 10 µg/mL CGA, \*P<0.05; compared within the group, #P<0.05.



**Figure 5.** Effect of different concentrations of CGA on Bax, Bcl-2 and PCNA expressions of HepG2 cells. A: Bax; B: Bcl-2; C: PCNA; D: Protein bands. CGA: chlorogenic acid. Compared with the blank group and those treated with 10 µg/mL CGA, \*P<0.05; compared within the group, #P<0.05.

of 5-fluorouracil and CGA significantly enhanced the inhibition, increased the generation of reactive oxygen species, and inactivated extracellular signal-regulated kinase [18]. Therefore, CGA may have a potential to act as a chemosensitizer in the treatment of hepatic carcinoma.

miRNAs are well-conserved small non-coding RNAs found in eukaryotes and play an impor-

tant role in the occurrence and progression of malignant tumors [19]. In the study by Wang et al., miR-181b promoted the proliferation of hepatic stellate cells by regulating cell cycle, and cell cycle regulatory factor p27 was a direct target of miR-181b in hepatic stellate cells [20]. SIR2 has an important effect on cell biological function such as cell survival and apoptosis, and SIRT1 and SIR2 demonstrate high homology in human beings [21]. SIRT1 is involved in cellular pathways such as gene transcription and apoptosis and is significantly expressed in various cancer tissues or cells; it participates in the occurrence and progression of multiple malignant tumors and mainly plays a tumor-promoting role [22, 23]. HO-1, mainly existing in mammals, is a rate-limiting enzyme that catalyzes heme catabolism *in vivo* [24]. HO-1 has multiple biological functions such as the protection of tissues, cells and organs and is highly expressed in various malignant tumors; it promotes cell proliferation and anti-apoptosis function in tumors [25]. Bax and Bcl-2 of the Bcl-2 family proteins play important roles in the signaling pathway of apoptosis [26]. PCNA, a protein with a molecular weight of 36KD that can participate in DNA damage repair and plays a key role in the initiation of cell proliferation; it can be used as an important indicator for the

evaluation of cell proliferation [27]. In the study by Choi et al., HO-1 induced and participated in the anti-proliferation effect of paclitaxel on vascular smooth muscle cells in rats [28]. Lee et al. indicated that the cytoprotection and apoptosis alleviation effect of lithospermic acid B on pancreatic β cells might be achieved by activating the anti-apoptosis pathways of Nrf2-HO-1 and SIRT1 [29]. In the study by Hou et al., licochalcone A inhibited the gen-

eration of matrix metalloproteinase and pro-inflammatory cytokines in abdominal aneurysm that was induced by angiotensin II and reduced the incidence of vascular smooth muscle cell apoptosis, with the possible mechanism of regulating the miR-181b/SIRT1/HO-1 signaling pathway to treat angiotensin II-induced abdominal aneurysm [30]. Therefore, the biological function of miR-181b/SIRT1/HO-1 in tumors may be a potential target for oncotherapy. In this study, the relative expression of miR-181b, SIRT1, HO-1, Bcl-2 and PCNA of HepG2 cells significantly decreased, and the relative expression of Bax significantly increased, with the increase of CGA concentration; indicating that proliferation inhibition and apoptosis promotion of CGA on HepG2 cells might be achieved by the miR-181b/SIRT1/HO-1 signaling pathway.

In this study, we confirmed the proliferation inhibition and pro-apoptosis effect of CGA on HepG2 cells. However, there were some shortcomings in this study. First, we did not investigate the cell cycle of HepG2 cells that were treated with CGA. Secondly, mitochondrial membrane potential of HepG2 cell was not detected. These shortcomings need to be resolved in a future study, and further evidence should be provided to confirm the results of this study.

In summary, CGA can inhibit the proliferation of HepG2 cells and promote their apoptosis, which may be achieved through regulation of the miR-181b/SIRT1/HO-1 signaling pathway.

### Disclosure of conflict of interest

None.

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