

## Original Article

# Epigallocatechingallate inhibits hepatocellular carcinoma progression *in vitro*

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**Abstract:** Hepatocellular carcinoma (HCC) is one of the most mortal diseases, the metastasis and recurrence are the most important factors that influence the treatment efficacy of HCC. At present, the treatment for HCC was still not greatly improved. This study investigated the role of epigallocatechingallate (EGCG) in HCC progression *in vitro* using flow cytometry, western blot and BrdU assay. Our data indicated that EGCG promoted the HCC cell apoptosis and inhibited cell migration. Furthermore, EGCG did not inhibit the proliferation of HCCLM9 cells, a cell line of HCC with high metastatic potential, and it promoted HCCLM9 cell apoptosis through activating caspase-3. In summary, we concluded that EGCG can inhibit the progression of HCC *in vitro*, and it provided a foundation for the further study on the effective treatment of HCC.

**Keywords:** EGCG, HCC, progression, apoptosis, migration

## Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer related mortality in the world, and the second cause in China for the high incidence of hepatitis B virus (HBV) infection [1, 2]. Approximately, 600 thousands of people died for this disease worldwide each year and Chinese patients may account for over fifty percent of the total cases [3, 4]. Actually, the epidemiological study indicates that the most HCC cases occur in sub-Saharan Africa in and Eastern Asia [3, 5, 6], however, the incidence in the western countries is expected to significantly increase over the next decades [7]. Recently, surgery is still the main treatment of HCC, the recurrence rate is around 70% within 5 years [8], hence, metastatic recurrence is deemed as the main barrier to improve the treatment efficacy [2].

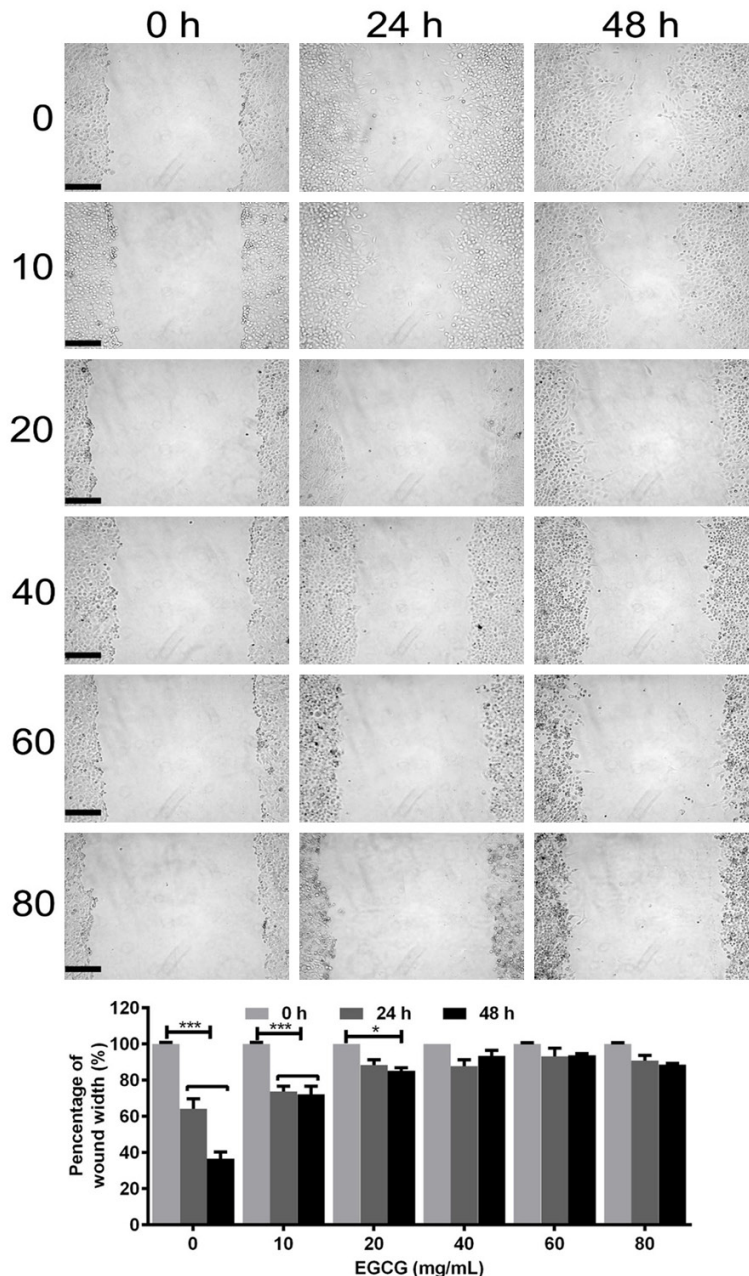
For a better insight into the treatment of HCC, HCCLM9 cells, with high metastatic potential, are repeatedly screened *in vivo* and remain in a similar genetic background [9, 10], providing a

great model to investigate the molecular mechanism of HCC metastasis *in vitro* [2, 11, 12].

Epigallocatechingallate (EGCG) is a chemical extraction from green tea, and it shows many functions as described previously, including improving endothelial function, insulin sensitivity and reducing blood pressure [13]. It is widely used to treat many diseases in the past decades, including HIV and cancer. Additionally, EGCG can be obtained easily either from green tea or chemical synthesis.

It has been reported that EGCG can inhibit the progression of many carcinomas, including colon cancer [14] and pancreatic cancer [15]. However, the role of EGCG in HCC progression is still need to be explored. Hence, we are trying to study the effect of EGCG on HCC cell apoptosis and its migration *in vitro*.

This study investigated the role of EGCG in HCC progression, especially in HCC cell migration and apoptosis *in vitro*. Our findings suggested that EGCG can significantly inhibit HCCLM9 cell migration and promote its apoptosis, suggest-



**Figure 1.** EGCG inhibits HCCLM9 cell migration *in vitro*. HCCLM9 cells were treated with 0, 10, 20, 40, 60 and 80 mg/mL of EGCG for 0, 24 and 48 h. EGCG inhibited HCCLM9 cell migration in dose and time dependent manners. \* $P < 0.05$  and \*\*\* $P < 0.001$ .

ing that EGCG can be used as a candidate small molecular drug for the treatment for HCC.

## Materials and methods

### Cell culture

The cloned cell line, HCCLM9, was a gift from Professor Yan Li (Wuhan University, Wuhan, China). These cells were cultured in RPMI 1640

(GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA) and 100 units/ml streptomycin/penicillin (Thermo Scientific, Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. SMMC7721 and LO2 cells were cultured in DMEM culture medium (GIBCO, Grand Island, NY, USA) containing 10% FBS.

### Migration assay

A wound-healing assay was used to detect the ability of cell migration as described previously [16]. The cells (10<sup>5</sup> cells/well) were seeded in a six-well cell culture plate. After one day of culture, the monolayer cells were wounded by scratching a line with a 10 µL pipette tip. The status of the wound line was recorded by microscopy at 0, 24, 48 h post-scratch, respectively.

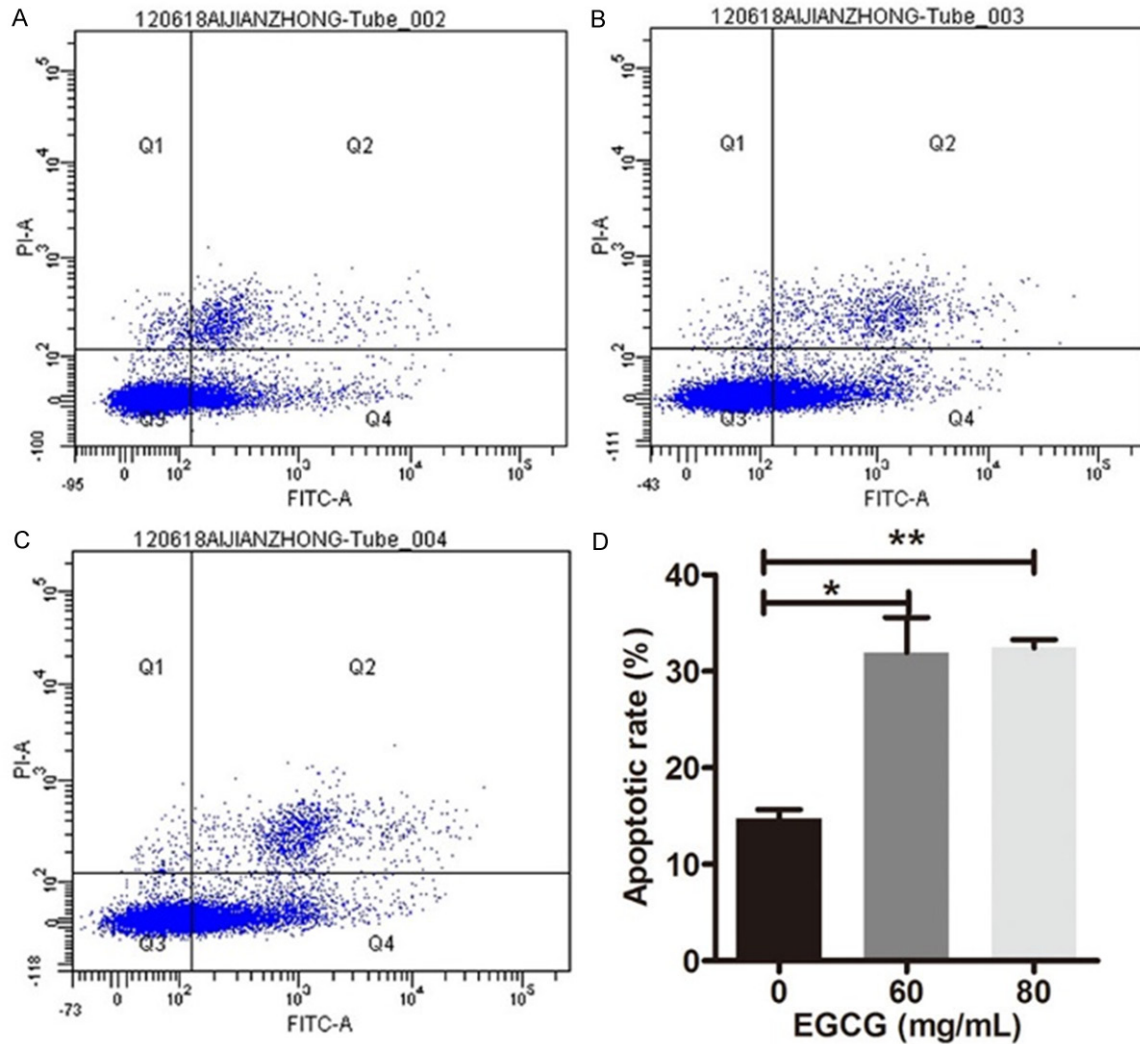
### Proteins extraction

First of all, the cell culture medium was removed, and the cells were harvested by trypsin digestion. The cells were collected by centrifuging at 1500 g for 10 min with phosphate buffer solution (PBS), and then the supernatant was discarded. The cells were washed for 3 times, and the cell number was counted using a hemocytometer (Z359629, Sigma, Shanghai, China). Then 1×10<sup>6</sup> cells were lysed by 60-100 µL of lysis

buffer. Cells were frozen and thaw for 3 times in liquid nitrogen repeatedly, and the mixture was centrifuged at 14,000 g for 1 hour (h) at 4°C. The supernatant was obtained and stored at -70°C.

### Bradford assay

Staining solution was diluted at a ratio of 1 (Bradford staining solution): 4 (water), and BSA



**Figure 2.** EGCG promotes HCCLM9 cell apoptosis. HCCLM9 cells were treated with 0 (A), 60 (B) and 80 (C) mg/mL of EGCG, and the cells were stained with PI. The apoptotic cells were detected using flow cytometry. (D) The statistical diagram indicated that EGCG significantly promoted the apoptosis of HCCLM9 cells. \* $P < 0.05$  and \*\* $P < 0.01$ .

was diluted to a concentration gradient of 0, 0.28, 0.56, 0.84, 1.12, 1.4 mg/mL. Next, 100  $\mu$ L of diluted standard protein and samples were added into EP tubes independently with 5 mL diluted staining solution. Finally, the absorbance at 595 nm was detected by Multiskan-Spectrum (51119300, Thermo Scientific, Waltham, MA), a microplate reader for ELISA. After making a standard curve of the proteins, the precise concentration of samples can be calculated using the formula of the standard curve.

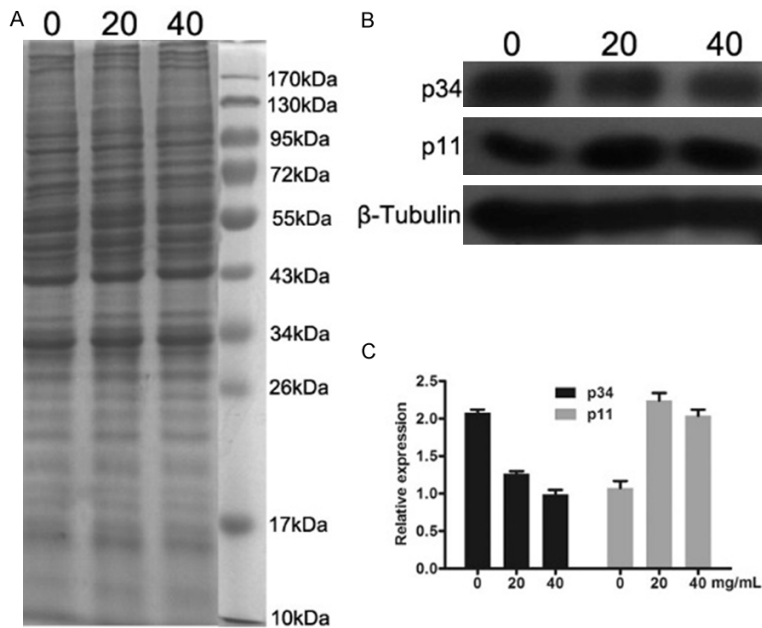
#### SDS-PAGE analysis

Proteins were prepared with 5 $\times$  and 1 $\times$  protein loading buffer, and the samples were loaded

onto a 10% SDS-PAGE gel. The gel was analyzed at 60 voltages (V) for 10 min and 100 V for 1.5 h. Gel was stained with coomassie brilliant blue (CBB) for 2 h.

#### Western blot

Total proteins (20  $\mu$ g) were analyzed using a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a polyvinylidenedifluoride (PVDF) membrane. The membrane was blocked with tris-buffered saline tween-20 (TBS-T) containing 5% (w/v) bovine serum albumin (BSA) for 1 hour at room temperature (RT). The membrane was then incubated with the



**Figure 3.** EGCG increases cleaved caspase-3 expression. Cells were also treated with different concentrations of EGCG, and the total proteins of cells were extracted. The one-dimensional SDS-PAGE gel was used to check the protein concentration (A). Caspase-3 expression was detected using western blotting (B), and the gray scale was statistically presented in (C). Here, p34 and p11 represent the pro- and cleaved-forms of Caspase 3 proteins.

primary antibodies of polyclonal rabbit anti-human caspase-3 (H-277) (1:200 dilution, Cat. No: sc-7148, Santa Cruz Biotechnology, Inc., Dallas, USA) and polyclonal rabbit anti-human beta tubulin (1:200 dilution, Cat. No: ab134185, Abcam, Cambridge, USA) overnight at 4°C. The membrane was finally incubated with the polyclonal goat-anti rabbit HRP-conjugated secondary antibodies (1:2000 dilution, ab6721, Abcam, Cambridge, MA, USA) for 1 hour at RT. Experiments were performed in triplicate.

#### Caspase-3 activity assay

The caspase-3 activity was measured using a caspase-3 activity assay kit from KeyGEN Biotech (KGA204, Nanjing, China). Briefly,  $3 \times 10^6$  cells treated with EGCG were harvested, and the total proteins were extracted using the lysis buffer provided by the kit. Then 50  $\mu$ L (100-200  $\mu$ g) of protein were incubated with 50  $\mu$ L of 2 $\times$  reaction buffer and 5  $\mu$ L of caspase-3 substrate at 37°C for 4 h in dark. Finally, the absorbance at 405 nm was measured by a Multi-Mode Microplate Reader (Synergy 2, BioTek, Winooski, VT, USA), and the absorbance represented the caspase-3 activity.

#### BrdU assay

Cells were treated with 0, 10, 20, 40, 60 and 80 mg/mL of EGCG for 24 h, and then cells were fixed with 4% paraformaldehyde *in vitro* for 30 minutes at 4°C, incubated in HCl (1 N) for 10 minutes on ice to break the DNA structure of the labelled cells. Samples were incubated with 0.1 M PBS (pH 7.4) + 1% TritonX100 + Glycine (1 M) + 5% normal goat serum (1 hr), and then incubated at RT overnight with rabbit polyclonal anti-BrdU-primary antibodies (Cat. No: ab152095, 1:200 dilution, Abcam, Cambridge, MA, USA). Samples were treated with Donkey Anti-Rabbit IgG H&L (Alexa Fluor 647) secondary antibodies (Cat. No: ab150075) to visualize the labelled cells.

#### Flow cytometry

Cells were treated with 0, 60 and 80 mg/mL of EGCG for 24 h, and harvested by centrifuging at 600 g for 5 min. Totally,  $1 \times 10^5$  cells were suspended using 500  $\mu$ L of binding buffer. Sequentially, 5  $\mu$ L of annexin V-FITC and 5  $\mu$ L of propidium iodide were mixed with cells. The mixture was incubated at room temperature for 15 min in dark. Finally, the cells were detected using flow cytometry (Millipore, Billerica, MA), and the apoptotic rate of cells can be obtained.

#### Statistical analysis

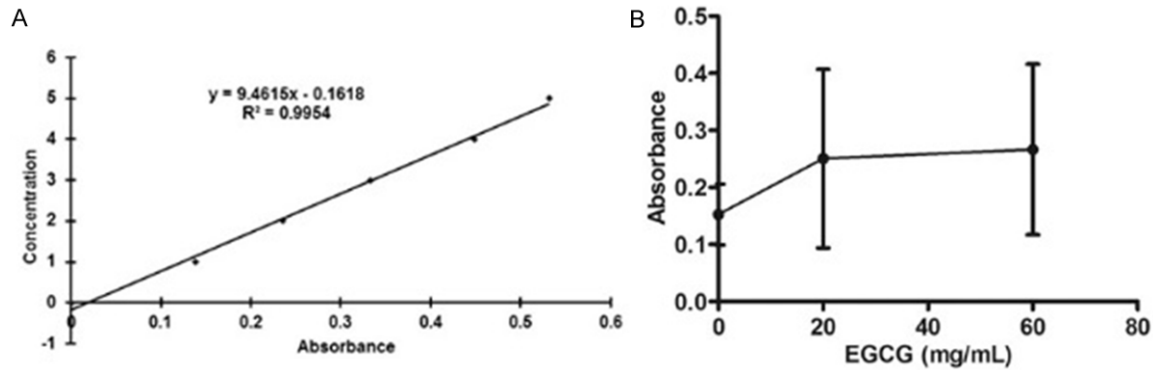
Data are presented as the means  $\pm$  SD or means  $\pm$  SEM. Statistical significances for comparisons between groups and among multiple groups ( $> 3$ ) were determined using a Student's paired t-test and analysis of variance (ANOVA) in Prism 6.0 and  $P < 0.05$  was taken as the level of significance.

## Results

#### EGCG inhibits HCCLM9 cell migration

To investigate the role of EGCG in HCC cell migration *in vitro*, a wound-healing assay was





**Figure 4.** EGCG promotes caspase-3 activation. Firstly, the protein concentration was measured using a Bradford assay, and the standard curve was shown in (A). Then the caspase-3 activity was evaluated using enzyme-substrate reaction. Data showed that EGCG indeed elevated the activity of caspase-3 (B). Prior to activity detection, the protein concentration was measured to normalize the loading protein.

employed to study the migration of HCCLM9 cells. The cells were treated with 0, 10, 20, 40, 60 and 80 mg/mL of EGCG for 0, 24 and 48 h. The real-time images of the healing wound were obtained after 0, 24, 48 h of the scratch using a 10  $\mu$ L pipette tip. As shown in **Figure 1**, EGCG can inhibit the migration of HCCLM9 cells in dose and time dependent manners (\* $P < 0.05$  and \*\*\* $P < 0.001$ ).

#### *EGCG promotes HCCLM9 cell apoptosis*

Besides the inhibitory effect of migration, our study further investigated the role of EGCG in HCCLM9 cell apoptosis using flow cytometry and western blotting assays. Firstly, the cells were treated with 0, 60 and 80 mg/mL of EGCG for 24 h, and the cells were harvested and stained with PI. Then the cell mixture was subjected to the flow cytometry assay. As shown in **Figure 2**, EGCG indeed promoted the apoptosis of HCCLM9 cells in a dose dependent manner (\* $P < 0.05$ , \*\* $P < 0.01$ ). Moreover, we explored the signaling pathway that involved in HCCLM9 cell apoptosis, hence, the caspase-3 protein expression was further detected using western blotting. The expression of cleaved caspase-3, namely the active caspase-3, was increased in the cells that were treated with EGCG (**Figure 3**). Furthermore, the activity of caspase-3 was analyzed in cells treated with EGCG using an activity detection kit from Keygen (Nanjing, China). As shown in **Figure 4**, the caspase-3 activity was elevated in cells treated with EGCG. These findings indicated that EGCG promoted the apoptosis at least in part through cas-

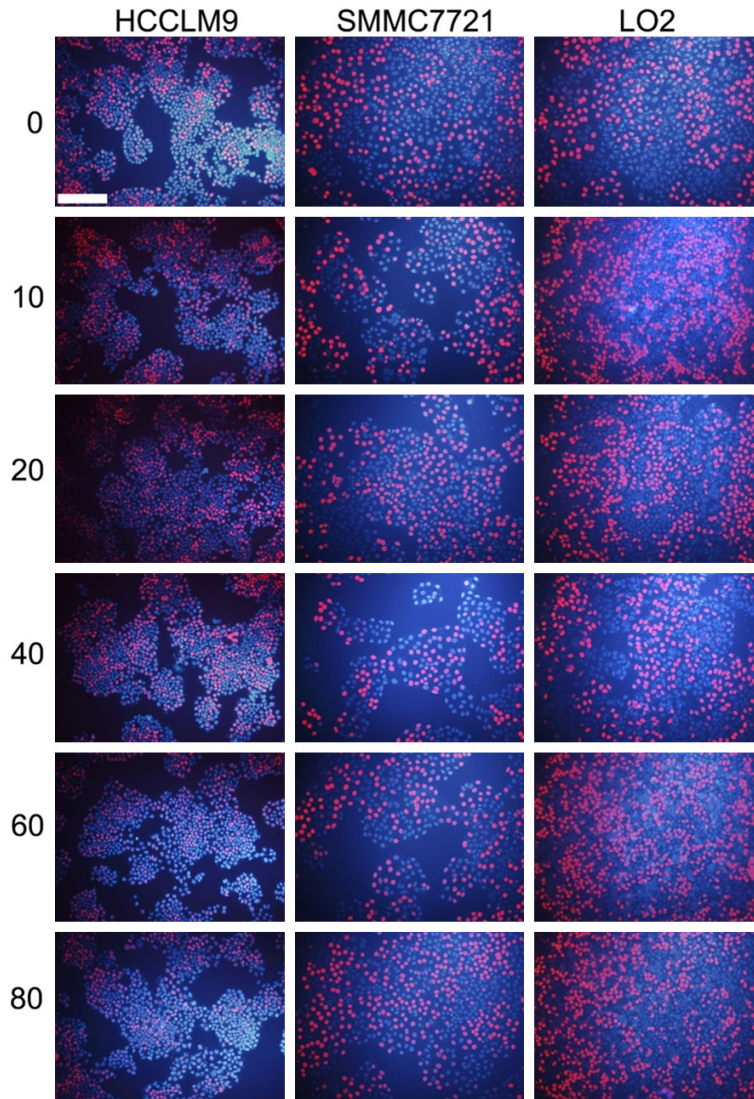
pase-3 pathway. On the other hand, the proliferation of HCCLM9, SMMC7721 and LO2 cells was evaluated using BrdU assay. As illustrated in **Figure 5**, the different concentrations of EGCG did not influence the proliferation of HCCLM9 cells and the other two cell lines. Taken together, our study demonstrated that EGCG inhibited HCC progression via promoting cell apoptosis rather than inhibiting the cell proliferation.

#### **Discussion**

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide [1], and the incidence is expected to increase over the next decades mainly for the increased HBV and HCV infection [3, 7]. However, the incidence of metastatic recurrence for HCC is still maintaining at a high level even through the curative therapies exists for early-stage and localized HCC [6].

Hence, the treatment of HCC is still a great challenge for researchers and clinical scientists [17]. The survival time and life quality of patients with HCC were still not significantly improved. So far, many small chemical molecules and monoclonal antibodies were developed to effectively treat HCC, nevertheless, the efficacy is still need to be improved.

As reported previously, EGCG is extracted from green tea, and it plays roles in many diseases and physiological functions, including cancer [18] and HIV infection [19, 20]. EGCG can inhib-



**Figure 5.** EGCG did not affect HCCLM9 cell proliferation. HCCLM9, SMMC77-21 and LO2 cells were also treated with 0, 10, 20, 40, 60 and 80 mg/mL of EGCG for 24 h. The proliferation of these cells was not markedly influenced.

it prostate and bladder carcinomas by blocking anti-apoptotic proteins, such as Bcl-xl [21-23]. Hence, we expected to explore the role of EGCG in HCC.

As reported previously, EGCG can be used to treat many carcinomas by promoting apoptosis [18]. This study also detected the effect of EGCG on promoting HCCLM9 cell apoptosis. Data from flow cytometry indicated that EGCG indeed promoted HCCLM9 cell apoptosis. Furthermore, a well-known interactor of the apoptotic signaling, caspase-3 expression and its activity were detected. Results suggested

that the cleaved caspase-3 expression were increased in HCCLM9 cells treated with EGCG. Moreover, the active caspase-3 level was also increased in these treated cells. These data partially showed that EGCG promoted HCCLM9 cell apoptosis via caspase-3 pathway. Additionally, EGCG had no significant effect on the proliferation of HCCLM9 cells. Similarly, studies also reported that EGCG could inhibit the progression, including apoptosis, invasion, angiogenesis and metastasis, of colon cancer and pancreatic cancer through AMPK and MAPK pathways [24, 25].

In summary, our study demonstrated that EGCG inhibited HCC progression by inhibiting cell migration and promoting cell apoptosis through caspase-3 pathway. These findings provided a foundation for the effective treatment of HCC, and EGCG presented a treatment potential for patients with HCC after the chemical optimization.

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#### Disclosure of conflict of interest

None.

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