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Original Article

Green tea polyphenol epigallocatechin-3-gallate enhances 5-fluorouracil-induced cell growth inhibition of hepatocellular carcinoma cells

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Aim: 5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic drugs. Resistance to 5-FU is a major cause of chemotherapy failure in advanced-stage hepatocellular carcinoma (HCC). Green tea polyphenol Epigallocatechin-3-gallate (EGCG) plays a critical role in growth inhibition and apoptotic induction in HCC cell lines. The aim of this study is to investigate whether EGCG can enhance 5-FU-induced cell growth inhibition and to explore its potential mechanisms.

Methods: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell growth. Western blotting analysis was performed to detect the proteins expression in Hep3B cells. Small interfering RNA was used to suppress cyclooxygenase-2 (COX-2) expression. Furthermore, enzyme linked immunosorbent assay was used to test the prostaglandin E_2 (PGE_2) production in cell cultures.

Results: Epigallocatechin-3-gallate augmented the antitumor effect of 5-FU in Hep3B cells. Significant difference was observed between the treated groups and the control group ($P < 0.05$). EGCG (its concentrations at over 5 μmol/L) combined with 5-FU presented a synergic effect. Furthermore, the combination of EGCG and 5-FU abrogated the COX-2 overexpression and PGE_2 secretion induced by 5-FU. The upregulation of COX-2 expression decreased the phosphorylation of Akt (Thr^{308}) expression. These appeared to be followed by the AMPK hyperactivation.

Conclusion: Epigallocatechin-3-gallate sensitizes HCC cells to 5-FU antitumor activity, and the combination of EGCG and 5-FU exhibits synergism in chemo-resistant cancer cells. The results suggest potential novel therapies for the treatment of advanced-stage liver cancer.

Key words: 5-fluorouracil, epigallocatechin-3-gallate, hepatocellular carcinoma, synergism

INTRODUCTION

EPIGALLOCATECHIN-3-GALLATE (EGCG), one of polyphenol, is the main active ingredient from green tea, accounting for 10–15% of the total green tea. It is an aqueous extract, which has a potent antioxidative effect, and plays a considerable role in preventing oxidative stress-related diseases including tumors, fibrosis and cirrhosis, and cardiovascular problems.[1,2] Accordingly, EGCG is highlighted nowadays as a potential cancer-chemopreventive agent. EGCG suppresses cell growth of HCC, and induces apoptosis HCC possibly through a certain signaling pathways or altering the expression of regulatory proteins.[3,4] However, much remains to be learnt about EGCG as an antitumor agent.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China with high mortality and is a great threat to the health and life of human beings. It is the second leading cause of cancer death in China and its annual incidence increases worldwide.[5,6] Most patients are already at the advanced stage once the diagnosis is confirmed and have lost the opportunity of surgical operation. Traditional systemic chemotherapy for liver cancer has a low curative rate with many toxic side effects, and is therefore not widely accepted by

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clinical practitioners. Recently, the application of new chemotherapeutic drugs and the new combination of regimens have been proved to be effective against advanced-stage HCC, especially with portal tumor thrombi.[7] Unfortunately, inherent or acquired drug resistance remains a major obstacle to chemotherapy.[8] Therefore, exploring the alternative modalities of combination chemotherapy for advanced HCC is desirable.

Fluorouracil (5-FU) is an inhibitor of deoxynucleoside triphosphate *de novo* synthesis and is widely used to treat some tumors, including colorectal, gastric, and pancreatic cancer etc. in clinical practice.[9] Moreover, 5-FU is also commonly used in advanced-stage HCC chemotherapy either alone or in combination with other agents. However, cancer cells develop drug resistance over time, which is an obstacle for effective chemotherapy targeting advanced liver cancer.[10] Thus, it is necessary to explore novel chemotherapeutic regimens to overcome drug resistance and to understand their molecular mechanisms in the process of liver cancer therapy.

The aim of this study is to investigate the effects of the combination of EGCG and 5-FU on HCC cells and to reveal the underlying mechanisms.

METHODS

Reagents

EPIGALLOCATECHIN-3-GALLATE (EGCG), 5-FU, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), and LY294002 were purchased from Sigma Chemical Company (St Louis, MO, USA). These reagents were dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was maintained at 0.1%. Rabbit polyclonal antibody Phosphor-Acetyl-CoA Carboxylase-Ser[79] (p-ACC, 280 kDa) was obtained from Cell Signaling Technology. Rabbit polyclonal anti-human COX-2, Akt, phospho-Akt (Thr 308) primary antibodies, and horseradish peroxidase conjugated sheep anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human HCC HEP3B were kindly donated by Professor Qian Wang (Surgery Laboratory at the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD, USA), penicillin (100 U/mL) and streptomycin (100 mg/L). The cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay for cell viability

Exponentially growing cells were diluted to a concentration of 2.5×10^4 cells/mL with DMEM, planted in 96-well plates (Corning Inc, Corning, NY, USA) with 200 μL/well. After being treated with drugs and incubated for 48 h (triplicate wells for each sample), the cells were exposed to 20 μL/well MTT (5 g/L, Amresco, Solon, OH, USA). The medium was then removed after being incubated for 4 h, and DMSO (200 μL/well) was added to dissolve the formazan product. Finally, the plate was read in an enzyme-linked immunity implement (Bio-Rad 2550, Hercules, CA, USA) at 570 nm. Cell viability and growth inhibition were calculated as described below: cell viability rate = A570 value of the drug treated group/A570 value of the control untreated group × 100%; growth inhibition rate = 1 − cell viability. The interaction between two drugs was judged according to a method describe by Jin.[11] Briefly, a q-value was obtained by comparing the dose-effect curves of the growth inhibition rates by treatment with two drugs combined to the growth inhibition rates by a single drug treatment. That is, $q = E_{A+B}/(E_A + E_B - E_A \times E_B)$. Two drugs have additive effects if $0.85 \leq q \leq 1.15$, are synergic if $q > 1.15$, and are antagonistic if $q < 0.85$.

Western-blotting analysis

Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) buffer and scraped with lysis buffer and protease inhibitor cocktail (Boehringer Mannheim, Lewes, UK) for 30 min at 4°C. The supernatant was saved by centrifugation at 15 000 *g* for 20 min. The protein concentration was determined with Coomassie Brilliant Blue G-250. Cell extracts (50 μg/lane) were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidine fluoride (PVDF) membranes (Immobilon, Bedford, MA, USA). After being blocked in 20 mmol/L Tris-HCl, pH 7.6 (containing 150 mmol/L NaCl, 0.1% Tween-20, and 5% non-fat dry milk), membranes were incubated with primary antibodies (used as a sample loading control) overnight at 4°C and then incubated with horseradish peroxidase-conjugated secondary antibody. Blots were developed using an

enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Gene silencing by small interfering RNA

The siRNA targeting human COX-2 and nonspecific control siRNA were designed and synthesized by RiboBio(Guangzhou RiboBio Co. Ltd, China). Cells were plated onto 6-well plates, maintained in antibiotic medium for 24 h, and grown to about 50% confluence. COX-2 siRNA or control siRNA were transfected with the Lipofectamine 2000 reagent (Invitrogen, Madison, WI, USA). In short, oligomer-fectamine reagent was diluted at 1 : 50 in OptiMEMI reduced serum medium (Gibco, PaloAlto, Calif, USA), mixed gently and incubated for 5 min at room temperature. Subsequently, a mixture of siRNA was added and incubated for 20 min. The mixture was diluted by adding medium to each well, and the final concentration of siRNA in each well was set as 100 nmol/L. The cells were then incubated for 48 h until processed.

PGE_2 secretion measurement by ELISA assay

After cell treatment at the indicated methods for 24 h, the supernatants in cell culture media were gathered by centrifuging and then stored at −70°C. The amount of PGE_2 secretion was measured by enzyme linked immunosorbent assay (ELISA) assay kit (Jingmei Biotech Co. Ltd, Shenzhen, China) according to the manufacturer's protocol. PGE_2 standards were diluted with appropriate media. The sample was added to wells, then PGE_2 conjugate was also added into all wells except the blank. Finally, monoclonal antibody against PGE_2 was administered to the indicated wells. After incubation for 18 h at 4°C, the plates were washed four times and color reaction was developed by tetramethylbenzidine substrate. 30 min later, the reaction was quenched by the addition of 1 M sulfuric acid. Optical density was tested at 450 nm in enzyme-linked immunity implement (Bio-Rad 2550, Hercules, CA, USA).

Statistical analysis

Experimental data were expressed as mean ± standard deviation (SD) of at least three separate experiments. Differences between groups were assessed with one-way analysis of variance (ANOVA) and Student-Newman-Keuls *q*-test using SPSS 11.0 for windows (SPSS Inc, Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

RESULTS

EGCG enhanced cell growth inhibition of Hep3B cells induced by 5-FU

TO INVESTIGATE THE effect of EGCG on 5-FU-inhibited HCC cell growth, Hep3B cells were treated with EGCG in the presence or absence of 5-FU. After Hep3B cells were treated with 5, 10, 25, and 50 μmol/L EGCG for 48 h, the viability of cells was (92.94 ± 2.26)% (83.95 ± 2.78)% (47.32 ± 3.18)%, and (25.40 ± 3.06)%, respectively. Significant difference was observed between the individual EGCG-treated group and the control group ($P < 0.05$). The cell viability in 30 μmol/L 5-FU alone was (71.35 ± 3.42)%. After we co-administered 5-FU with EGCG, the cell viability decreased dramatically to be (63.21 ± 5.98)% (49.12 ± 3.15)% (14.21 ± 2.66)%, and (5.21 ± 1.34)%, respectively (Fig. 1). The corresponding *q* values were 1.09, 1.27, 1.30, and 1.16. These data indicate that a combination of 5 μmol/L EGCG and 30 μmol/L 5-FU presents additive effect, and the effects on the other concentrations (over 5 μmol/L) of EGCG combined with 5-FU are synergic.

Combination of EGCG and 5-FU modulated COX-2 expression and phosphorylation of AMPK and Akt

AMP-activated protein kinase (AMPK) is a metabolite-sensitized protein kinase, and plays an important cellular homeostasis and protective role under metabolic

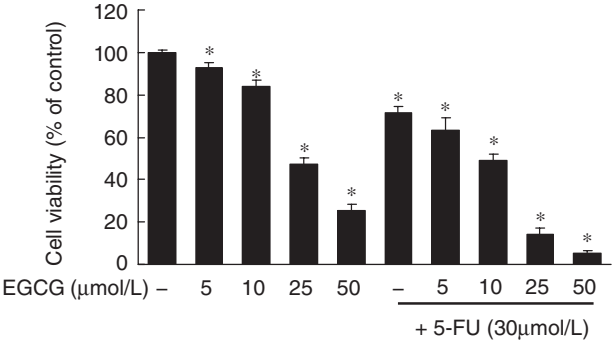

Figure 1 The cell death effect of the combination of epigallocatechin-3-gallate (EGCG) and 5-Fluorouracil (5-FU) on Hep3B cells. Hep3B cells were treated with varying concentrations of EGCG in the presence or absence of 5-FU (30 μmol/L). Cell viability was determined by MTT assay. Data are expressed as the mean ± standard deviation (SD) of three independent experiments. *$P < 0.05$ vs control group (treated with 0.1% dimethylsulfoxide [DMSO]).

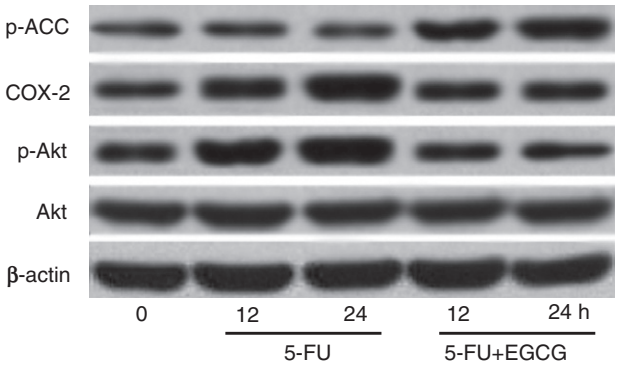

Figure 2 The effect of epigallocatechin-3-gallate (EGCG) combined with 5-Fu modulated COX-2 expression and phosphorylation of ACC and Akt. Cellular protein was isolated from Hep3B cells that were cultured with 5-Fluorouracil (5-FU) (30 μmol/L) with or without EGCG (25 μmol/L) at the indicated periods. Western blotting analysis was used to detect COX-2 expression and phospho-ACC and phospho-Akt. Results are representative of three independent experiments.

stress conditions in various cell types.[12] The AMPK activation is observed indirectly by the phosphorylation of acetyl-CoA carboxylase (p-ACC, Ser 79), which is the best characterization of phosphorylation site under the activation of AMPK.[13] Moreover, previous studies have demonstrated that COX-2 expression and Akt activation are involved in chemo-resistant cancer cells.[14,15] To examine the modulation of 5-FU alone or combined with EGCG on COX-2 expression and phosphorylation of ACC and Akt, western blotting analysis was applied to detect these protein expressions. The 5-FU treatment alone group increased COX-2 expression and the phosphorylation of Akt (Thr^{308}), and p-ACC expression levels did not change. However, the combination of EGCG and 5-FU almost completely abrogated COX-2 and phosphor-Akt (Thr^{308}) expression, and increased dramatically the phosphorylation level of ACC. Total Akt expression was not altered (Fig. 2).

AMPK activation by the combination of EGCG and 5-FU abrogated 5-FU-induced COX-2 expression and the production of PGE_2

AICAR, an AMPK activator, was administered to pretreat for 30 min before Hep3B cells was treated with 5-FU alone for 24 h, western blotting analysis was performed to detect the proteins expression efficiency. The 5-FU treatment induced COX-2 overexpression, compared with the control group (0.1% DMSO). AICAR increased the phosphorylation level of ACC and accompanied with the activation of AMPK. We further observed that 5-FU-induced COX-2 overexpression was abrogated by AMPK activation in the combination of 5-FU and EGCG or AICAR (Fig. 3a). PGE_2 is the predominant COX-2 product in cancer cells.[16] We also measured the amount of PGE_2 in the supernatants of various treatments by enzyme immunoassay. 5-FU alone promoted significantly the secretion of PGE_2, compared with the control group (0.1% DMSO) ($P < 0.05$). Whereas, significant decrease of PGE_2 production was observed by the treatment of AICAR, and 5-FU combined with EGCG or AICAR ($P < 0.05$). Therefore, the AMPK activation by AICAR or EGCG caused a significant reduction

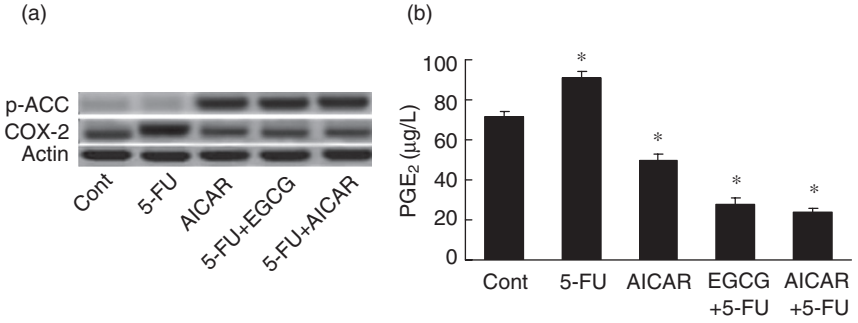

Figure 3 AMPK activation by the combination of epigallocatechin-3-gallate (EGCG) and 5-Fluorouracil (5-FU) abolished 5-FU induced COX-2 expression and PGE_2 secretion. Cellular protein was isolated from Hep3B cells that were cultured with 0.1% dimethylsulfoxide (DMSO), 5-FU (30 μmol/L), AICAR (5 mmol/L), 5-FU (30 μmol/L) plus EGCG (25 μmol/L), and 5-FU (30 μmol/L) plus AICAR (5 mmol/L) for 24 h, respectively. Western blotting analysis was used to detect COX-2 expression and phospho-ACC (a). Enzyme linked immunosorbent assay (ELISA) assay was used to measure the amount of PGE_2 production in the supernatant of the culture media (b). Results are representative of three independent experiments. *$P < 0.05$ vs. control group, Cont, cells treated with 0.1% DMSO.

of PGE_2 production in culture media (Fig. 3b). These results indicate clearly that AMPK activation by the combination of EGCG and 5-FU abrogated 5-FU-induced COX-2 expression and the production of PGE_2.

Combination of EGCG and 5-FU reduced the phosphorylation of Akt via the downregulation of COX-2 expression

To further confirm the relationship between COX-2 overexpression and Akt activation in Hep3B cells treated with 5-FU alone or the coadministration, Hep3B cells were transfected with COX-2 siRNA or scramble siRNA (nonspecific control siRNA), and endogenous COX-2 protein was completely eliminated (data not shown).[17] Then, cells were treated with 5-FU in the presence or absence of EGCG for 24 h. Since the knockout of COX-2 in Hep3B cells, the phenomenon that 5-FU alone treatment group increased COX-2 expression was not observed. Meanwhile, the phosphorylation of Akt (Thr^{308}) was also not observed. Whereas no changes were observed in the control siRNA group. The reduced COX-2 expression by COX-2 siRNA or EGCG was simultaneously accompanied with the lower phosphorylation level of Akt (Thr^{308}), indicating Akt activation through the upregulation of COX-2 expression (Fig. 4a). To evaluate the role of Akt in Hep3B cell growth, LY294002, a specific inhibitor of the PI3K/Akt pathway, was used to block the activation of Akt. Cells were incubated in the medium containing 20 μmol/L, or 40 μmol/L LY294002 with or without 5-FU (30 μmol/L) for 48 h, cell viability decreased significantly, compared with the control group ($P < 0.05$), suggesting that the inhibition of Hep3B cell growth induced by the combination of EGCG and 5-FU was mainly via the inactivation of Akt pathway (Fig. 4b). These results indicate the combination of EGCG and 5-FU suppresses COX-2 expression and Akt activation.

DISCUSSION

CHEMOTHERAPY IS ONE of the most effective ways to treat various cancers and has also been proved to be effective against advanced-staged HCC, especially for patients with non-resected HCC. Transcatheter hepatic arterial chemoemblolization (TACE) and local chemotherapeutic drugs perfusion via portal vein have been regarded as two highly effective palliative methods and are applied widely in clinical practice.[18,19] The biggest challenge for chemotherapy is the development of drug-resistance. Multiple factors are implicated in increasing resistance to chemotherapeutic agents, including reduction of intracellular drug accumulation, and DNA damage repair by the modulation of proliferative or anti-apoptotic proteins, etc.[20] 5-FU causes cell injury by inhibiting thymidylate synthesis. It has become a

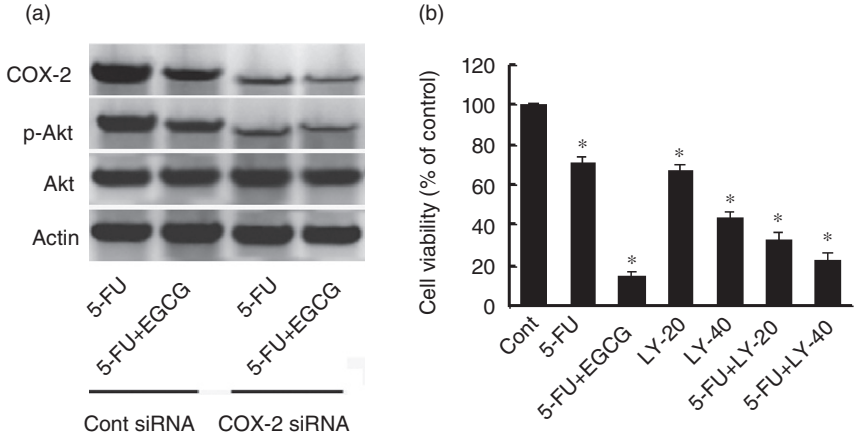

Figure 4 The combination of epigallocatechin-3-gallate (EGCG) and 5-Fluorouracil (5-FU) reduced the phosphorylation of Akt by the downregulation of COX-2 expression. After Hep3B cells were transfected with control or COX-2 siRNA for 48 h, then cells were exposed to 5-FU (30 μmol/L) in the presence or absence of EGCG (25 μmol/L) for 24 h. Western blotting analysis was used to detect the proteins expression of COX-2, phospho-Akt (Thr^{308}), and Akt (a). LY294002, a specific inhibitor of the PI3K/Akt pathway, was used to block the activation of Akt. The cell viabilities were determined by MTT assay (b). Data are expressed as the mean ± standard deviation (SD) of three independent experiments. *$P < 0.05$ vs. control group, Cont, cells treated with 0.1% DMSO; LY-20, 20 μmol/L LY294002; LY-40, 40 μmol/L LY294002.

mainstay of treatment for advanced-stage HCC. Resistance to 5-FU coming from cancer cells delays the evolution of HCC chemotherapy.[21] There is so far no effective single agent or polychemotherapeutic regimen for the treatment of resistant HCC. Therefore, it is necessary to find a new agent that enhances the effect of 5-FU on anticancer therapy.

Epigallocatechin-3-gallate is the major biologically active component of green tea and has a potent antioxidative effect.[22] It has been reported that EGCG suppresses cell growth and induces apoptosis of human HCC *in vitro* and *in vivo*, possibly through many signaling pathways.[23,24] However, the anticancer effects of EGCG on HCC have been poorly understood until now. We observed that EGCG enhanced inhibitory effect on Hep3B cell growth induced by 5-Fu, and a combination of EGCG and 5-FU presents a synergic effect. The modulatory failure of cell proliferative and apoptotic proteins are involved in increasing chemotherapeutic agents resistant to cancer treatment.[25] The synergism coming from the combination of EGCG and 5-FU will be the basis to overcome drug resistance in HCC treatment. Recent evidence has also shown that the natural compound EGCG augments the antitumor activity of doxorubicin for chemoresistant liver cancer.[26]

The chemosensitizing effect of EGCG in the present study may occur directly or indirectly involving the modulation of cell survival proteins. COX-2 is an inducible enzyme by various factors, including cytokines, growth factors, and tumor promoters.[16] It is known that overexpression of COX-2 is associated with tumor cell proliferation, cell apoptotic escape, and tumor angiogenesis.[27] It has also been proposed that COX-2 expression is increased in chemo-resistant cancer cells,[28] and its higher levels are identified in HCC, increasing PGE_2 production and cell growth.[29] Whereas COX-2 inhibitor enhances cell apoptosis in HCC cell lines.[30,31] nonsteroidal anti-inflammatory drugs (NSAIDs) are also shown to reduce the risk of sporadic colorectal, breast, prostate, and lung cancers.[32,33] In the present study, the 5-FU alone treatment group increased COX-2 expression, and the combination of EGCG and 5-FU almost completely abrogated COX-2 induced by 5-FU alone, which provided some important evidence for the synergic effect of the combined administration. There is emerging evidence that EGCG has been involved in chemotherapeutic effects by its anti-proliferation and apoptosis induction activities of cancer cells.[34]

AMPK is a metabolic sensor and plays an essential role as energy-sensor in adenosine triphosphate (ATP)-deprived conditions.[35] So, AMPK is known to play an important protective role under metabolic stressed conditions. This state is activated by a green tea polyphenol (EGCG) and shuts down the ATP-consuming metabolic pathways.[36] Moreover, AMPK has been proved to be involved in stress-induced apoptosis, and also be potential targets for cancer chemotherapy against hepatocellular carcinoma.[37] In the present study, the combination of EGCG and 5-FU increased the phosphorylation level of ACC, which is the best characterization of the AMPK activation. Under this state, COX-2 expression was downregulated, and PGE_2 production was reduced. These molecular events were also observed in AMPK activated conditions induced by AICAR(AMPK activator). These results indicate clearly that AMPK activation by the combination of EGCG and 5-FU abrogated 5-FU-induced COX-2 expression and the secretion of PGE_2. Also, EGCG that acts as an anti-cancer agent in the combination treatment is explored to be synergism. Hwang *et al*.[38] have found that apoptotic effect of EGCG in HT-29 colon cancer cells is mediated via AMPK signal pathway. Previous reports have demonstrated that Akt plays a crucial role in tumorigenesis and cancer progression by stimulating cell proliferation and inhibiting apoptosis. Akt activation needs translocation to the plasma membrance and phosphorylation at Thr308 and Ser 473.[39,40] We documented in the present study that COX-2 overexpression accompanied with Akt phosphorylation at Thr308 was induced by 5-FU, promoting cell growth. COX-2 downregulation by COX-2 siRNA or EGCG resulted in Akt inactivation, preventing HCC cell growth. After the Akt activation was blocked by LY294002, the cell death effect was also observed. So, EGCG and LY294002 had similar effects on HCC cell death in the presence or absence of 5-FU, indicating that their underlying mechanism in blocking cell growth may be similar. These results were consistent with the report of Leng *et al*.[41] Therefore, Akt inactivation in these molecular events was required for the synergic effect of EGCG combined with 5-FU.

In summary, the present study reveals that green tea polyphenol EGCG enhances fluorouracil-suppressed hepatocellular carcinoma cell growth, and the combination of EGCG and 5-FU produces the synergic effect. Furthermore, we also find that EGCG exhibits a series of molecular events in 5-FU based combination therapy in HCC cells, including AMPK activation, reduced COX-2 expression, and Akt inactivation. The observations may explain the underlying mechanisms of the synergic effect of EGCG combined with 5-FU, and constitute potential novel chemotherapies for the treatment or prevention of liver cancer.

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