Prostanoid EP₁ receptor as the target of (−)-epigallocatechin-3-gallate in suppressing hepatocellular carcinoma cells in vitro

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Aim: To investigate the effects of (−)-epigallocatechin-3-gallate (EGCG), an active compound in green tea, on prostaglandin E₂ (PGE₂)-induced proliferation and migration, and the expression of prostanoid EP₁ receptors in hepatocellular carcinoma (HCC) cells.

Methods: HCC cell line HepG2, human hepatoma cell lines MHCC-97L, MHCC-97H and human hepatocyte cell line L02 were used. Cell viability was analyzed using MTT assay. PGE₂ production was determined with immunoassay. Wound healing assay and transwell filter assay were employed to assess the extent of HCC cell migration. The expression of EP₁ receptor and Gq protein were examined using Western blot assay.

Results: PGE₂ (4-40,000 nmol/L) or the EP₁ receptor agonist ONO-DI-004 (400-4000 nmol/L) increased the viability and migration of HepG2 cells in concentration-dependent manners. EGCG (100 μg/mL) significantly inhibited the viability and migration of HepG2 cells induced by PGE₂ or ONO-DI-004. HepG2 cells secreted an abundant amount of PGE₂ into the medium, and EGCG (100 μg/mL) significantly inhibited the PGE₂ production and EP₁ receptor expression in HepG2 cells. EGCG (100 μg/mL) also inhibited the viability of MHCC-97L cells, but not that of MHCC-97H cells. Both EGCG (100 μg/mL) and EP₁ receptor antagonist ONO-8711 inhibited PGE₂- and ONO-DI-004-induced growth and migration of HepG2 cells. Both EGCG (100 μg/mL) and ONO-8711 (20 μmol/L) inhibited PGE₂ and ONO-DI-004-induced EP₁ expression. EGCG and ONO-8711 had synergistic effects in inhibiting EP₁ receptor expression. PGE₂ and ONO-DI-004, ONO-8711, and EGCG had no effects on Gq expression in HepG2 cells, respectively.

Conclusion: These findings suggest that the anti-HCC effects of EGCG might be mediated, at least partially, through the suppressing EP₁ receptor expression and PGE₂ production.

Keywords: hepatocellular carcinoma; epigallocatechin-3-gallate; prostaglandin E₂; prostanoid EP₁ receptor

Introduction

Hepatocellular carcinoma (HCC), one of the most common malignancies worldwide[1], often arises in the background of chronic liver inflammation and cirrhosis. At this time, no effective chemotherapeutic or chemopreventive treatments are available. HCC is a growing health problem, and innovative treatment approaches are urgently needed.

(−)-Epigallocatechin-3-gallate (EGCG, structure shown in Figure 1), one of the most abundant bioactive components in leaves of green tea, has received increasing attention for its various physiological activities, such as antioxidant activity[2, 3] and anti-tumor properties[4–6]. The effects of EGCG on tumor cell proliferation and apoptosis have been well documented[7].

PGE₂ is the prostaglandin that is abundantly present in HCC. Studies have established the important role of the PGE₂ synthesis pathway as a potential target for the treatment and/or prevention of HCC[8, 9]. PGE₂ exerts its biological activities primarily via G-protein-coupled prostaglandin receptors (EP₁-₄), which belong to the highly conserved superfamily of...
7-transmembrane-spanning proteins. Among these four EP receptors, studies have shown EP2 to be the most important in tumor development. EP3, through activation of epidermal growth factor receptor (EGFR)/c-Met signaling, plays an important role in tumor cell invasion[10]. A selective EP1 agonist increased the phosphorylation of EGFR, which suggests that it might enhance the invasion of tumor cells[10]. Moreover, an EP1 antagonist reduced the viability of HCC cells and increased their apoptosis[11]. The EP1 receptor is of major importance in colon cancer development as well. For example, in one study, EP1 receptor deficiency inhibited colon cancer development[13]. In addition, a selective EP1 antagonist suppressed tongue carcinogenesis in rats, notably reduced the number of tumors in UV-induced mouse skin cancer[12] and inhibited the COX-2 and PGE2-induced migration and viability of human chondrosarcoma[13]. These reports suggest that the EP1 receptor might play a key role in the PGE2-induced tumor process.

Studies have demonstrated the anti-inflammatory and anti-oxidant activity of EGCG, which is mediated via the inhibition of COX-2[14] and microsomal prostaglandin E2 synthase-1 (mPGES-1)[15]. Although previous studies have suggested that EGCG downregulates COX-2 and mPGES-1 expression, whether the antitumoral effects of EGCG on HCC are mediated via regulation of EP1 and PGE2 has not been established. We hypothesized that EGCG might exert an anti-HCC effect by virtue of its suppressive action on both PGE2 production and EP1 expression.

Materials and methods

Drugs
The EP1-receptor selective antagonist ONO-8711 and EP2-receptor selective agonist ONO-DI-004 were kindly provided by ONO Pharmaceutical Co., Osaka, Japan. EGCG and PGE2 were purchased from Sigma (St Louis, MO, USA). EP1-receptor-antibody and Gq-receptor-antibody were obtained from Cayman Chemical, Ann Arbor, MI, USA.

Cell culture
The human hepatoma cell line MHCC-97L and MHCC-97H was obtained from the Liver Cancer Institute of Zhongshan Hospital, Fudan University, Shanghai, China. The HepG2 cell line was obtained from the Shanghai Cell Bank of Chinese Academy of Sciences. Human hepatocyte cell line L02 was obtained from the Shanghai Institute of Cell Research. All of the cell lines were cultured at 37 °C in a 5% CO2 environment in DMEM from Gibco BRL Life Technologies Inc (Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS, Sigma).

Cell proliferation assay
Proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (EZ4U; Bio-medica, Vienna, Austria). To assess the effects of PGE2 (4×10^{-6} mol/L), ONO-8711 (210 nmol/L and 10 μmol/L), ONO-DI-004 (400 nmol/L) and EGCG (12.5, 25, 50, and 100 μg/mL) on cell proliferation, HepG2, MHCC-97L and MHCC-97H cells in an exponential growth phase were cultured at a density of 8×10^{3} cells/well in a 96-well plate and incubated overnight in DMEM containing 10% FBS. The plates were then serum starved for 24 h before being treated with the compounds. After treatment with various concentrations of reagents, MTT solution (5.0 mg/mL in PBS) was added (20.0 μL/well), and the plates were incubated for another 4 h at 37 °C. The purple formazan crystals were dissolved in 150.0 μL of Dimethyl Sulfoxide (DMSO) per well. After 10 min, the plates were read on a microplate reader (American Bio-Tek) at 490 nm. The cells without drugs were used as a control. The assays were performed in three independent experiments. The inhibition of cell proliferation was determined using the following formula: inhibition of cell proliferation (%)=[1–(OD of the experimental samples/OD of the control)]×100% (n=3, mean±SD).

Transwell filter cell migration assay
Boyden chambers containing polycarbonate filters with 8 μm pore size (Costar, Bodenheim, Germany) were employed. Cells were seeded at a density of 5×10^{5} cells per milliliter. To initiate the chemotaxis assay, cells (5×10^{5}) in 100 μL of DMEM without FCS were added to the inner chamber, and the lower chamber was filled with 600 μL of DMEM with 10% FCS, as well as PGE2 (4×10^{-6} mol/L) or ONO-DI-004 (400 nmol/L) as an inducer of cell migration. The cells were allowed to migrate for 12 h at 37 °C in an atmosphere of 95% air/5% CO2. Cells on the filter were first stained with hematoxylin-eosin staining, and cells that remained on the upper surface of the filter were removed using a cotton swab. The cells that migrated onto the lower surface of the filter were examined by microscope after mounting them onto a slide. A total of six random high-power microscopic fields (HPF) (100×) per filter were photographed, and the number of cells was directly counted. Experiments were carried out in triplicate and were repeated three times with consistent results.

Wound healing assay
Cell migration was examined using the wound-healing assay. HepG2 cells were cultured to a confluent monolayer in a 6-well plate at 37 °C in an atmosphere of 95% air/5% CO2. Thereafter, a scratch (wound) was introduced in the confluent cell layer using a yellow pipette tip. The cells were washed three times with phosphate-buffered saline (PBS) to remove detached cells. The cells were then incubated with different compounds for 24 h, and pictures of a defined wound spot were made with a computer-aided phase contrast microscope at 0 and 24 h. The area of the wound in the microscopic pictures was measured using Image J software (National Institutes of Health, MD) at different time points. The relative distance of wound closure (%)=(distance at 0 h-distance at 24 h)/relative distance of control×100%.

Enzyme-linked immunosorbent assay
The HCC cell line HepG2 was plated into six-well plates and grown to 70% confluence. After washing with PBS, the cells
were treated with increasing concentrations of EGCG (0, 12.5, 25, 50, and 100 μg/mL) in serum-free DMEM for 24 h. The cell supernatants were then collected, and the PGE_{2} levels were measured using a commercial PGE_{2} immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. A PGE_{2} standard was run in parallel to the supernatant samples. Each assay was performed using triplicate samples.

**Western-blot analysis**

The cells were treated with PGE_{2} and ONO-DI-004 for 2 h with or without a 1 h pretreatment with EGCG at 100 μg/mL. After treatment for 2 h, protein was extracted from cells in RIPA lysis buffer (50 mmol/L TRIS (tris (hydroxymethyl) aminomethane)-HCl, pH 7.4, 150 mmol/L NaCl, 10 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate). The protein concentration was determined with the Lowry Protein Assay. A protein sample was mixed with the 5×sample buffer (4:1) (Bio-Rad, Hercules, CA) and heated in boiling water for 10 min. The proteins were resolved by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA), and incubated with blocking buffer [Tris-buffered saline/Tween 20 (TBST)/5% nonfat dry milk] overnight at 4°C. Immunoblotting was performed with the mouse antibody raised against human EP1 receptor (1:1000) or Gq protein (1:1000) followed by the appropriate horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:15000). Immunodetection was performed with an enhanced chemiluminescence system (ECL, Pierce, Rockford, IL) using hydrogen peroxide and luminol as a substrate.

**Statistical analysis**

Biostatistical analyses were conducted using the SPSS 11.5 software package. All experiments were repeated at least three times. The results of multiple experiments are given as the mean±SEM. The non-parametric Kruskal-Wallis test was used to detect differences among the different experimental groups. A level of P<0.05 was accepted to indicate statistical significance.

**Results**

**EP1 expression in HCC cells**

Previous studies showed that PGE_{2} promoted tumor progression via binding to the EP1 receptor\textsuperscript{[10, 11]}. We showed that the EP1 receptor protein was expressed in the L02, MHCC-97H, MHCC-97L, and HepG2 cell lines (Figure 2). The expression of EP1 receptors was higher in HCC cell lines compared with human normal hepatocyte L02 cells.

**PGE_{2} induced HCC growth**

PGE_{2} is known to have a critical role in carcinogenesis. Therefore, we first detected the effect of PGE_{2} on the viability of HepG2 cells. PGE_{2} (0, 4, 40, 400, and 4000 nmol/L) promoted HepG2 growth in a dose-dependent way (P<0.05, Figure 3A). After treatment with 4 μmol/L PGE_{2} for 24, 48, and 72 h, the viability of HepG2 cells was increased (Figure 3B).

**Figure 2.** Expression of EP1 receptor in HCC cells and human hepatocytes L02. (A) A representative Western blot showing the expression of EP1 in HCC cell lines MHCC-97H, MHCC-97L, and HepG2. Human hepatocyte LO2 cell line was used as control. (B) Bar graphs show quantitative evaluation of EP1 expression by densitometry from triplicate independent experiments. Mean±SEM. *P<0.05, **P<0.01 compared with normal hepatocyte cell line L02.

**Figure 3.** Effect of PGE_{2} on HepG2 growth. (A) Effect of PGE_{2} (0, 4, 40, 400, and 4000 nmol/L) on cell viability after 24 h treatment. (B) Effect of 4 μmol/L PGE_{2} on cell viability after 24, 48, and 72 h. Data were expressed as mean±SEM of two separate experiments which was performed in duplicate. *P<0.05, **P<0.01 compared with control group.
Effect of selective EP<sub>1</sub>-receptor agonist and antagonist on HepG2 growth

We next measured the response of HepG2 cells to selective EP<sub>1</sub>-receptor agonist ONO-DI-004. Cells were grown in the absence or presence of ONO-DI-004 (0, 4, 40, 400, and 4000 nmol/L), and proliferation was evaluated after 24 h. As shown in Figure 4A, both 400 nmol/L and 4 μmol/L of ONO-DI-004 significantly promoted HepG2 cell growth (P<0.05). We chose the 400 nmol/L of ONO-DI-004 to investigate its effect on cell viability at different time points (Figure 4B). The growth of cells was significantly increased after 48 h of stimulation by ONO-DI-004 (P<0.01). Subsequent experiments aimed to evaluate the potential of the selective EP<sub>1</sub>-receptor-antagonist ONO-8711 as a chemotherapeutic compound. As shown in Figure 4C, the treatment of HepG2 cells with ONO-8711 (210 nmol/L, 1, 5, and 10 μmol/L) significantly reduced cell viability as compared with controls (cells treated with serum-free DMEM) (P<0.01).

EGCG inhibits PGE<sub>2</sub> production

After showing that PGE<sub>2</sub> stimulated HepG2 cell growth and knowing that mPGES expression is inhibited by EGCG,[15] we aimed to determine the inhibitory effect of EGCG (12.5–100 μg/mL) on the production of PGE<sub>2</sub> in HCC cell lines. EGCG inhibited PGE<sub>2</sub> production as compared with controls (cells treated with serum-free DMEM). HepG2 cells secreted an abundant amount of PGE<sub>2</sub> into the growth medium, which was significantly reduced after treatment with 100 μg/mL of EGCG (Figure 5).

EGCG inhibits HCC cell growth

First, we aimed to investigate the mechanisms by which EGCG exerts its anti-tumor activity. HCC cell lines HepG2, MHCC-97L and MHCC-97H were treated with EGCG (12.5–100 μg/mL) for 24 or 48 h. As shown in Figure 6, at a concentration of 50 μg/mL, the reduction of cell viability was significant in MHCC-97L and HepG2 cells (P<0.01), but MHCC-97H cell growth had no change. The extent of viability-reduction differed between HCC cell lines. In addition, EGCG might inhibit MHCC-97H cell migration or invasion or induce cell apoptosis but not cell growth. The exact mechanism would need to be further determined.

EGCG inhibits selective EP<sub>1</sub> receptor agonist-induced growth of HepG2 cells

Our results suggested that EGCG inhibited PGE<sub>2</sub> expression and HepG2 cell growth. We next tested the effect of EGCG (100 μg/mL) on PGE<sub>2</sub>- and ONO-DI-004-induced cell growth. As shown in Figure 7, EGCG 100 μg/mL significantly inhibited PGE<sub>2</sub>- and ONO-DI-004-induced growth of HepG2 cells (P<0.01).

Figure 4. Selective EP<sub>1</sub> receptor agonist ONO-DI-004 and antagonist ONO-8711 on viability of HepG2 cells. (A) Effect of ONO-DI-004 (0, 4, 40, 400, and 4000 nmol/L) on cell viability after 24 h. (B) Effect of 400 nmol/L of ONO-DI-004 on cell viability after 24, 48, and 72 h. (C) Effect of 210 nmol/L, 1, 5, and 10 μmol/L of ONO-8711 on viability of HepG2 cells after 24 h. Data were expressed as the relative inhibitory ratio in untreated cells and the mean±SEM of two separate experiments, each of which was performed in duplicate. *P<0.05, **P<0.01 compared with control group.

Figure 5. Effect of EGCG 12.5–100 μg/mL on production of PGE<sub>2</sub> by HepG2 cells after 48 h. Data were expressed as mean±SEM of two separate experiments, each of which was performed in duplicate. *P<0.01 compared with control group.

Figure 6. EGCG inhibits HCC cell growth. A, B, C: Cell viability of HepG2, MHCC-97L, and MHCC-97H cells treated with EGCG (12.5–100 μg/mL) for 24 or 48 h. Data were expressed as the relative inhibitory ratio in untreated cells and the mean±SEM of two separate experiments, each of which was performed in duplicate. *P<0.05, **P<0.01 compared with control group.

EGCG inhibits selective EP<sub>1</sub> receptor agonist-induced migration of HepG2 cells

We next investigated how PGE<sub>2</sub>, ONO-DI-004 and ONO-8711 influenced the migratory behavior of HepG2 cells by performing wound healing assays. Consistent with their role as positive regulators of cell growth, cells treated with PGE<sub>2</sub> or ONO-DI-004 closed the wound faster than the control, and EGCG and ONO-8711 inhibited cell migration. PGE<sub>2</sub> and ONO-DI-004 had an additive effect on cell migration (Figure 8A, 8B). We further investigated the inhibitory effects of EGCG on PGE<sub>2</sub>- and ONO-DI-004-induced migration using transwell filter assays. EGCG (100 μg/mL) significantly inhibited HepG2
cell migration induced by PGE\(_2\) (4 \(\mu\)mol/L) or ONO-DI-004 (400 nmol/L) \((P<0.01\), Figure 8C).

**EGCG inhibits EP\(_1\) expression in HepG2 cells**

We further examined whether EP\(_1\) receptor expression was altered after treatment with EGCG in HepG2 cells by Western blot assay. As shown in Figure 9A, when the cells were treated with PGE\(_2\) or ONO-DI-004, an increase was observed in the expression of EP\(_1\) receptors, which were inhibited by EGCG. In general, EP\(_1\) receptors are coupled to Gq proteins, which are important for EP\(_1\) signaling. Therefore, we also tested the expression of Gq proteins. There was no significant suppression of Gq expression after treatment with EGCG or ONO-8711 (Figure 9A). The results suggested that EGCG inhibited proliferation and migration of HepG2 induced by PGE\(_2\) partially by suppressing the expression of the EP\(_1\) receptor but not the Gq protein. We tested the effect of ONO-8711 or EGCG on PGE\(_2\) and ONO-DI-004-induced EP\(_1\) expression (Figure 9B). We found that both ONO-8711 and EGCG inhibited the PGE\(_2\) and ONO-DI-004-induced EP\(_1\) expression. PGE\(_2\) and ONO-DI-004 had an synergistic effect on EP\(_1\) expression.

**Discussion**

The increased expression of COX-2 and concomitant increased production of PGE\(_2\) are considered to be a major cause of the pathological changes seen in cancers. Studies have indicated that COX-2-mediated production of PGE\(_2\) increases cancer cell growth, which can be suppressed by several COX-2 inhibitors\([16, 17]\). Because of the unfavorable cardiovascular safety profile of selective COX-2 inhibitors, there is an interest in using PG receptor-specific compounds as novel agents in the prevention and treatment of certain cancers\([18]\). PGE\(_2\) exerts its biological activities primarily via EP\(_1\) receptors. Indeed, the EP\(_1\) receptor has previously been shown to play a key role in the progression of various carcinomas, including colon cancer and skin cancer\([10, 12, 18, 19]\). Suppression of synthesis of PGE\(_2\) and expression of its receptors is a compelling rationale in the treatment of HCC. Since the effects of EGCG on the migration and proliferation of HCC as induced by PGE\(_2\) and EP\(_1\) agonists was unknown, we decided to investigate this topic.

EGCG exerts an inhibitory effect in many cancers, such as prostate cancer\([20, 21]\) and liver cancer\([22]\). Previous studies have shown that EGCG decreased the expression of COX-2\([23]\) and mPGES\([15]\). However, the exact mechanism of the inhibitory...
The inhibitory effect of EGCG on HCC cells is not well understood. We examined HCC cell proliferation after treatment with EGCG. After incubation with 50 or 100 μg/mL of EGCG, a significant reduction in cell proliferation in HepG2 and MHCC-97L cells, but not MHCC-97H cells was observed (Figure 6). To evaluate the effect of EGCG on PGF2α production in HCC cells, we detected PGF2α expression in HepG2 cells. As shown in Figure 5, EGCG decreased PGF2α expression in a dose-dependent way. We found that EGCG produced a strong inhibitory effect on the growth of HCC cells and significantly downregulated PGF2α production.

Previous studies demonstrated that PGF2α binding to the
EP1 receptor up-regulated survivin, which accelerated HCC cell growth and metastasis[19], but the important role of EP1 receptors in tumor progression remained to be elucidated. To specifically determine whether the EP1 receptor is functionally active in HCC cells, we used a selective EP1 receptor agonist and antagonist to analyze their effects on cell viability and migration.

Previous studies showed that the EP1 receptor antagonist AH6809 inhibited anchorage-independent cell growth and reduced the viability of HCC cells[24]; however, ONO-8711 significantly inhibited breast cancer and HCC development, presumably via induction of apoptosis[1, 25]. Our data showed that the selective EP1 receptor agonist ONO-DI-004 dramatically increased cell viability and migration in HCC cells in a dose-dependent manner and that the EP1 receptor antagonist ONO-8711 exerted an inhibitory effect. In addition, ONO-8711 significantly inhibited PGE2-induced HepG2 cell migration. Furthermore, we detected the effect of PGE2 and the selective EP1 receptor agonist on EP1 expression by Western blot assay. The results showed that both of them up-regulated EP1 receptor expression. Taken together, these data provide direct evidence that EP1 expression as well as proliferation and migration of HCC cells are increased by both PGE2 and the selective EP1 receptor agonist. By contrast, the selective EP1 receptor antagonist inhibited PGE2-induced cell migration. These findings demonstrate that the EP1 receptor appears to be the predominant receptor that regulates PGE2-induced HCC cell growth and metastasis.

Our study focuses on the inhibitory effect of EGCG on PGE2 and EP1 receptor agonist-induced HCC development. First, we observed that both PGE2 and the EP1 receptor agonist stimulated HCC progression. To elucidate the effects of EGCG on PGE2/EP1 agonist-induced tumor development, we investigated cell proliferation, migration and EP1 receptor expression. Cell proliferation and migration were significantly reduced by EGCG at 100 μg/mL compared with the control (P<0.01). In PhIP-induced breast cancers, COX-2 and PGE2 are closely related to estrogen biosynthesis through the aromatase gene (CYP), and these members may be involved in mammary gland carcinogenesis through the EP1 receptor[26]. COX-2 acts as an oncogene under certain circumstances, leading to the production of PGE2 which could then act in a paracrine or autocrine way to induce signaling via EP receptors, in particular the EP1 receptor[27]. In our study, we found that expression of the EP1 receptor was significantly decreased in the HCC cell line HepG2 after treatment with EGCG (Figure 9). EP1 receptors are coupled to Gq proteins, but no significant difference in Gq protein expression was observed after treatment with EGCG or ONO-8711. The level of Gq expression was unchanged in our cell system, suggesting that although no significant change occurred in Gq production, the activity of Gq might be already changed or that the level of Gq expression may depend on specific cell types. The exact mechanism should be further studied. We are attempting to determine whether the activity of the Gq protein changed upon treatment with specific chemicals or whether the level of Gq expression was changed in other cell lines.

Figure 9. EGCG inhibited EP1 receptor expression in human HepG2 cells. Effect of PGE2, 4 μmol/L, ONO-DI-004 400 nmol/L, ONO-8711 210 nmol/L, and EGCG 100 μg/mL alone on EP1 receptor expression (A) and Gq expression (B). (C) EGCG and ONO-8711 on PGE2 and ONO-DI-004-induced EP1 receptor expression. The data were expressed as mean±SEM of three independent experiments. *P<0.05, **P<0.01 compared with control groups. †P<0.01 vs single PGE2- or ONO-DI-004- treated group, respectively. ‡P<0.01 vs single EGCG- or ONO-8711-treated group, respectively.
level in HCC cells compared with normal human hepatocytes. EGCG significantly inhibited PGE\(_2\)/EP\(_1\) agonist-induced HCC development through suppression of cell viability and migration. We also found that EGCG demonstrated prominent inhibition of PGE\(_2\) synthesis and EP\(_1\) receptor expression. These findings suggest that EGCG may be effective in managing HCC and may help in the development of new therapeutic strategies for both the prevention and treatment of HCC.

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Author contribution
Wei WEI designed research. Juan JIN and Yan CHANG performed research and wrote the manuscript; Yi-fu HE contributed new reagents or analytic tools. Shan-shan HU, Di WANG, and Yu-jing WU performed research.

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