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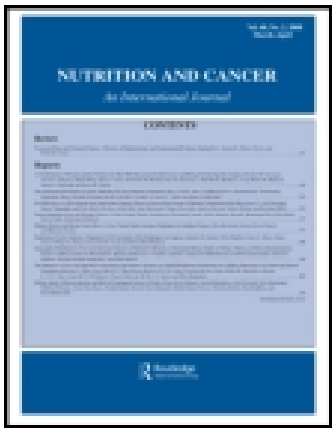

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Epigallocatechin-3-Gallate Inhibits Hepatoblastoma Growth by Reactivating the Wnt Inhibitor SFRP1

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Activation of Wnt signaling plays a central role in the formation of hepatoblastoma (HB), the most common pediatric liver cancer. Blocking this pathway with specific inhibitors is currently the target of various research endeavours. This study provides evidence that the naturally occurring flavonoid epigallocatechin-3-gallate (EGCG) is highly effective against HB growth through inhibition of Wnt signaling. We demonstrate that EGCG has a strong cytotoxic effect on HB cells in a time- and dose-dependent manner by impinging on cell viability, while leaving normal fibroblasts unaffected. Apoptotic features, including morphological changes, caspase 3 activity, and proteolytic cleavage of poly(ADP-ribose) polymerase, were frequently found in EGCG-treated HB cells, thereby suggesting involvement of the mitochondrial intrinsic apoptotic pathway. We furthermore show that EGCG effectively inhibits Wnt signaling, as evidenced by down-regulation of Wnt-responsive reporter gene activity and expression of the Wnt target genes *MYC* and *CCND1*. Interestingly, EGCG induced reexpression of the tumor suppressor gene *SFRP1*, which is transcriptionally silenced in HB cells and known to down-regulate Wnt signaling. Considering the lack of toxic effects on normal cells, EGCG should be preclinically validated as an adjuvant therapy *in vivo* with the ultimate goal of determining its efficacy in human trials.

INTRODUCTION

Hepatoblastoma (HB) is the most common liver cancer in children with an incidence of 0.7 to 1 case per million children (1). By combining neoadjuvant chemotherapy with surgical resection, the outcome of children with hepatic tumors has significantly improved over the past several years. However, especially in high-risk patients [unresectable tumors associated with extrahepatic extensions, invasion of large hepatic veins, distant metastases, and very high (>1.000.000 μg/l) alpha-fetoprotein serum-levels] there are still many cases in which children do not adequately respond to the standard therapy, even if they receive aggressive multiagent treatment (2–4). Thus, the evaluation of new treatment strategies is essential. Particularly in the early postoperative phase, in which children are weak and treated with chemotherapy, it appears worthwhile to use substances that prevent tumor relapse without further harming the weakened body. Here, we see the possibility to use nontoxic naturally occurring substances like epigallocatechin-3-gallate (EGCG) in combination with the standard therapy.

HB originates from immature liver precursor cells and presents morphologic features that mimic normal liver development. The identification of distinct histopathological subtypes and further molecular biological information derived from liver ontogenesis and growth regulation of hepatic tumors has recently helped to pave the way for a more comprehensive classification system for this disease (5). Among others, it became evident that the Wnt signaling pathway plays an important role in the development of HB. The Wnt signaling network is known to regulate diverse processes during tissue development such as cell fate determination, structural remodelling, cell polarity and morphology, cell adhesion, and growth (6). HB presents with a high rate (50–90%) of mutations in the proto-oncogene *β-catenin*, which codes for the main effector protein of Wnt signal transduction (7). Moreover, rare mutations in the *AXIN1* gene, a known antagonist of β-catenin, have been found in HB

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that may contribute to the etiology of HB in which *β-catenin* mutations have not been identified (8, 9). Studies on other components of the Wnt signaling pathway have demonstrated overexpression of the genes coding for Dickkopf-1, Nkd-1 and beta-TrCP, known antagonists of the Wnt pathway that might be activated by a negative feedback mechanism resulting from increased β-catenin (10,11).

Another group of extracellular antagonists of the Wnt signaling pathway that prevent ligand-receptor interactions belong to the family of secreted frizzled-related proteins (SFRP). These proteins have been shown to impede Wnt signaling by direct binding to the extracellular Wnt receptor complex (12). SFRP1 is a member of the SFRP family that contains a cysteine-rich domain homologous to the putative Wnt-binding site of frizzled proteins. The role of SFRP1 as a tumor suppressor has been proposed in many cancers based either on its frequent loss or inactivation by methylation-induced silencing (13,14).

The flavonoid epigallocatechin-3-gallate (EGCG) is primarily a nontoxic substance that belongs to the family of catechins and is thought to be the main active component of green tea leaves (15). Black tea leaves also contain EGCG, but in much lower quantities because of fermentation. EGCG contains 3 phenol rings and has very strong antioxidant properties, being about 25–100 times more potent than vitamins C and E (16,17). In the experimental and preclinical setting, tea flavonoids have already been shown to play an important role in treating many different cancers by inducing cell cycle arrest (18) and apoptosis (19,20), or inhibiting cell invasion and metastasis (21), as well as neoangiogenesis (22). Interestingly, EGCG has been found to inhibit Wnt signaling in a dose-dependent manner in breast cancer, lung cancer, colon cancer, and in normal cells, in which Wnt signaling was hyperactivated (23–26).

Because of the importance of Wnt signaling in the development of HB and the proven effects of EGCG on this pathway, we aimed at investigating the effect of EGCG on HB growth in our current study.

MATERIALS AND METHODS

Tumor Cell Lines

We used the 4 human hepatoblastoma cell lines HUH6 (Japanese Collection of Research Bioresources, JCRB, Osaka, Japan), HepT1 (27), HepT3 (28), and HepG2 (29), as well as normal fibroblasts. All cell lines were maintained according to supplier recommendations.

Cell Viability Assay

Cells were seeded at a density of 5×10^4 cells/12-well plate (Nunc, Wiesbaden, Germany) and after overnight attachment treated for the indicated time with 0–100 μM EGCG (Calbiochem, Merck, Darmstadt, Germany) dissolved in DMSO. For assessment of the cell viability the cells were trypsinized and stained with 0.5% Trypan Blue Solution (Biochrom, Berlin, Germany; 1:1 in media). Cells were counted in a counting chamber (Kova Glasstic Slide 10 with Grids, HYCOR Biomedical, Indianapolis, IN) using a Zeiss inverted phase-contrast microscope and living and dead cells were discriminated. The percentage of living cells was calculated using the following formula: % living cells = (unstained living cells × 100) / (unstained living cells + stained dead cells). Experiments have been tripled for statistical analysis.

Apoptosis Analyses

Morphological changes of incubated cells were documented with a Zeiss inverted phase-contrast microscope equipped with a Canon PowerShot G6 digital device. For immunofluorescent detection of cleaved caspase 3, cells were seeded at a density of 1×10^4 cells per well onto chamber slides, cultured for 48 h in the presence or absence of 37.5 μM EGCG and then fixed for 20 min with fresh 4% paraformaldehyde at room temperature. Cells were permeabilized in 0.1% Triton-X100/0.1% sodium citrate, blocked in 5% bovine serum albumin, and incubated overnight at 4°C with a polyclonal rabbit antihuman cleaved caspase 3 antibody (1:500, Cell Signaling Technology, Danvers, MA). Cells were then incubated for 30 min with fluorescein isothiocyanate-conjugated sheep antirabbit immunoglobulin G (1:320, Sigma-Aldrich, Seelze, Germany) at room temperature. Nuclear staining was performed with Vectashield containing 4,6-diamidino-2-pheylindole (Vector Laboratories Inc., Burlingame, CA). Cleaved caspase 3-positive cells were counted at 200× magnification in at least 3 randomly selected microscope fields with at least 250 cells. Experiments have been tripled for statistical analysis.

For Western blot analysis, 20 μg protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ). The membranes were incubated with phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk to block nonspecific binding. Membranes were incubated over night with rabbit antihuman poly(ADP-ribose) polymerase (PARP), or rabbit antihuman-actin (Cell Signaling Technology) antibodies and thereafter for 1 h with horseradish peroxidase-conjugated goat antirabbit immunoglobulin G secondary antibody (DakoCytomation, Hamburg, Germany). Signals were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ).

Reporter Assay

Cells were seeded the day before transfection at a density of 5×10^4 cells per well in 12-well plates. Cells were then transfected with 900 ng of the Wnt-responsive reporter plasmid pTOPFLASH or the nonresponsive control plasmid pFOPFLASH (kindly provided by Dr. Kolligs) and 100 ng of the reference plasmid pRL-TK using FuGene 6 transfection reagent (Roche Diagnostics). Forty-eight hours after transfection, cells were lysed and reporter gene activity was determined using the Dual-Glo Luciferase Reporter Assay System (Promega,

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Madison, WI). Firefly luciferase activity was normalized to Renilla luciferase activity. All reporter assay experiments were repeated 3 times and transfections done in duplicate.

Real-Time Reverse Transcription-PCR (RT-PCR)

Total RNA was extracted from tumor cell lines in Trizol (Invitrogen, Carlsbad, CA), depleted from DNA and subsequently purified using DNase set and RNeasy Mini Kit, respectively (Qiagen, Hilden, Germany). Reverse transcription of total RNA was performed using random hexamers (Roche Diagnostics) and SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA). PCR amplifications of the respective genes were carried out with 40 ng of cDNA, 500 nM forward and reverse primer and iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) in a final volume of 20 μl. PCR reactions were run for 40 cycles consisting of 15 sec denaturation at 95°C, primer annealing for 15 sec at 55°C, and extension for 30 sec at 72°C. We used the following primer pairs (5′–>3′ orientation): *MYC*, CACCACCAGCAGCGACTCT, CAGACTCTGACCTTTTGCCAGG; *Cyclin D1* (*CCND1*), TCACACGCTTCCTCTCCAGA, AGGCTTGACTCCAGCAGGG; *SFRP1,* CATGACGCCGCCCAAT, GATGGCCTCAGATTTCAACTCG; *TATA -Box-binding-Protein* (*TBP*), GCCCGAAACGCCGAATAT, CCGTGGTTCGTGGCTCTCT. *Real-time* RT-PCR experiments and data analysis was performed as described earlier (30).

Statistical Analysis

Data were presented as bar graphs, indicating mean + SD. Statistical analysis was performed using Student's unpaired *t*-test. A level of $P < 0.05$ was considered to be significant.

RESULTS

Epigallocatechin-3-Gallate Decreases Viability of HB Cells in Time- and Dose-Dependent Manner

Several lines of evidence indicate that EGCG significantly inhibits growth of tumor cells by inducing apoptosis in a variety of human cancers (18,20). By applying EGCG concentrations (0–100 μM) comparable to those used in previous studies we observed a dose-dependent decrease in cell viability in all HB cell lines (Fig. 1, upper panel). Of note, this effect was selective for tumor cells, because normal fibroblasts were unaffected by the EGCG treatment. The half-maximal cytotoxic concentration (IC50) of EGCG at 48 h was 24.1 μM, 36.5 μM, 9.2 μM, 26.5 μM, and > 100 μM for HepT1, HepT3, HUH6, HepG2, and normal fibroblasts, respectively. The cytotoxic effect of EGCG was already apparent after 12 h of incubation and reached maximal cytotoxicity after 72 h (Fig. 1, lower panel). Altogether, these data demonstrate a strong inhibitory effect of EGCG against excessive growth of HB cells.

Epigallocatechin-3-Gallate Induces Apoptosis in HB Cells

In a next step we analyzed whether EGCG can induce apoptotic characteristics in HB cells. The first signs of apoptosis

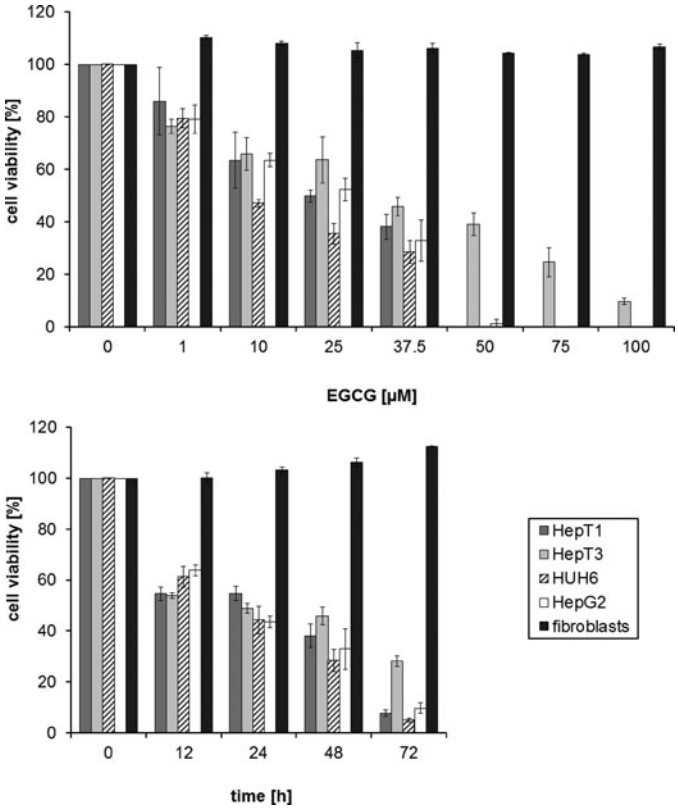

FIG. 1. Decreased viability of hepatoblastoma (HB) cells upon epigallocatechin-3-gallate (EGCG) treatment. Four HB cell lines and normal fibroblasts were treated for 48 h with 0–100 μM (upper panel) and for 0–72 h with 37.5 μM (lower panel) EGCG. Cell viability was analyzed by Trypan Blue staining and the percentage of living cells was calculated. Values represent the mean percentages ± standard deviations of 3 independent experiments.

in HB cells after EGCG treatment became evident by specific morphological changes such as cell shrinkage, nuclear fragmentation, and formation of apoptotic bodies (Fig. 2, left panel). EGCG treatment resulted in a strong increase of apoptosis in all tested HB cell lines, but not in normal fibroblasts. In the second step we examined a later stage of apoptosis characterized by proteolytic cleavage of caspase 3 and its known downstream target poly(ADP-ribose) polymerase (PARP). In the presence of EGCG cleaved caspase 3-positive cells were frequently observed in HB cell lines (32–46%), as evidenced by immunofluorescence, whereas fibroblasts lack the activated form (Fig. 2, right panel). Using Western blot analysis, we detected increased levels of the activated form of PARP in the HB cell lines (Fig. 3). Altogether, these findings suggest that EGCG treatment selectively induces apoptosis in HB cells, but not nonmalignant cells.

Epigallocatechin-3-Gallate Inhibits Wnt Signaling

Because Wnt signaling has been found to be inhibited by EGCG in different cancers, we sought to determine whether EGCG has any impact on the signaling cascade in HB cells. First, we investigated whether EGCG is able to inhibit activity

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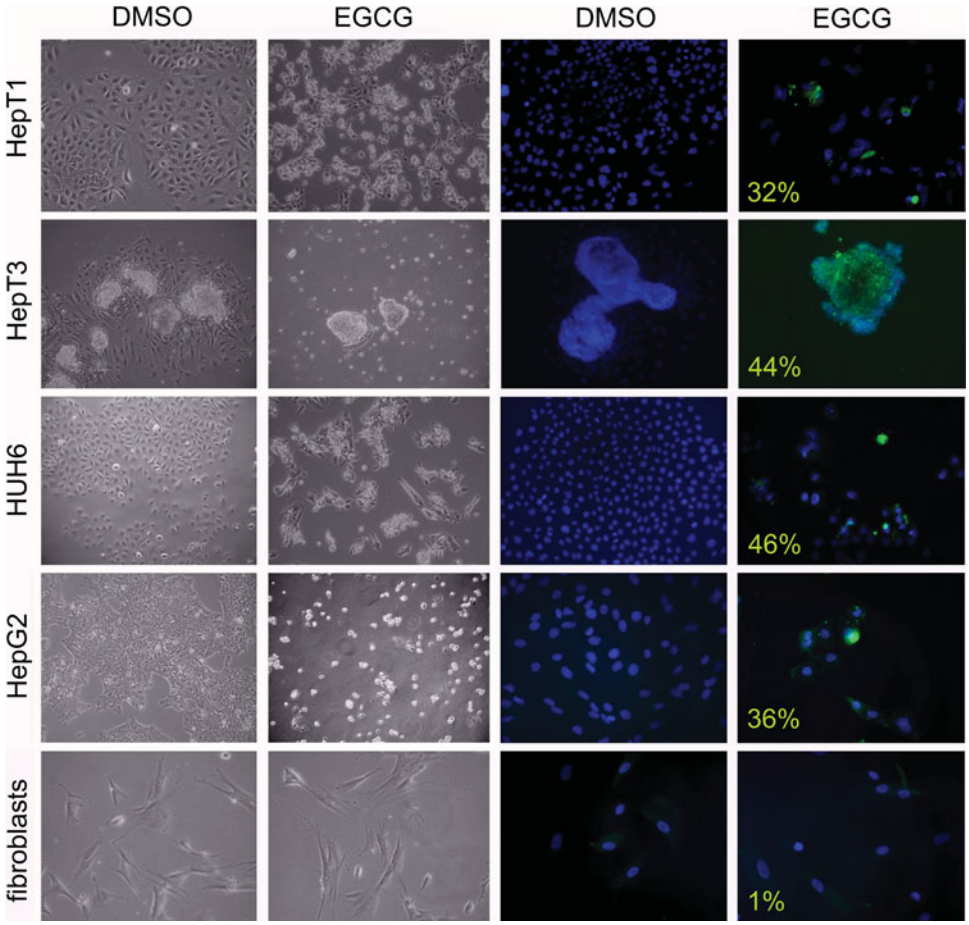

FIG. 2. Epigallocatechin-3-gallate (EGCG)-induced apoptosis in hepatoblastoma (HB) cell lines. Morphology (left panel). HB cells were treated for 48 h with or without 37.5 μM EGCG. Typical morphological changes after apoptosis induction were observed in all 4 HB cell lines, but not in normal fibroblasts. Activation of caspase 3 (right panel). Apoptotic cells were counted under a fluorescence microscope after a 48 h-treatment with 37.5 μM EGCG using immunofluorescent detection of cleaved caspase 3 (green) and nuclear counterstaining (blue). Percentages of cleaved caspase 3-positive cells in relation to total cells are depicted (color figure available online).

of a Wnt-responsive reporter plasmid (31). Consistent with previous data (11), all 4 HB cell lines harboring *ß-catenin* mutations led to an activation of the TOP reporter containing Tcf binding sites, whereas the FOP reporter containing mutant binding motifs was not transcribed. However, EGCG treatment resulted in a dramatic downregulation of the Wnt-responsive TOP reporter, thereby demonstrating a specific inhibitory potential of this compound on Wnt signaling (Fig. 4, upper panel). Using real-time RT-PCR analysis we found a significant reduction of mRNA levels for the Wnt target genes *MYC* and *Cyclin D1* (*CCND1*) in all HB cells after EGCG treatment (Fig. 4, lower panel). These results suggest that EGCG is able to inhibit the Wnt signaling pathway on the transcriptional level.

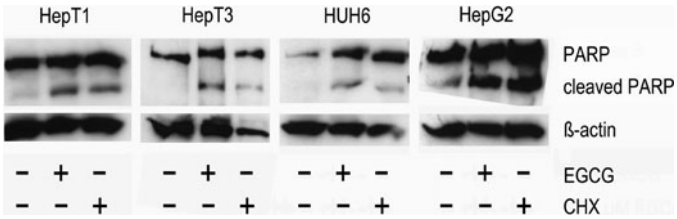

FIG. 3. Proteolytic cleavage of poly(ADP-ribose) polymerase (PARP). Hepatoblastoma (HB) cells were treated for 48 h with 37.5 μM EGCG and apoptosis-specific activation of PARP was detected by Western blot analysis, showing full length and cleaved PARP products as indicated. 100 μg/ml cycloheximide (CHX) was used as a positive control for apoptosis induction. Immunodetection of beta-actin served as a standard for equal protein loading of the gel. EGCG = epigallocatechin-3-gallate.

Epigallocatechin-3-Gallate Induces Reexpression of the Transcriptionally Silenced *SFRP1* Gene

Transcriptional suppression of the gene encoding the soluble inhibitor of Wnt signaling SFRP1 by aberrant promoter methylation is a frequent finding in a variety of human cancers (13). Because EGCG has been described to reactivate epigenetically silenced genes by inhibiting DNA methyltransferases (32), we analyzed the expression of *SFRP1* after the treatment with

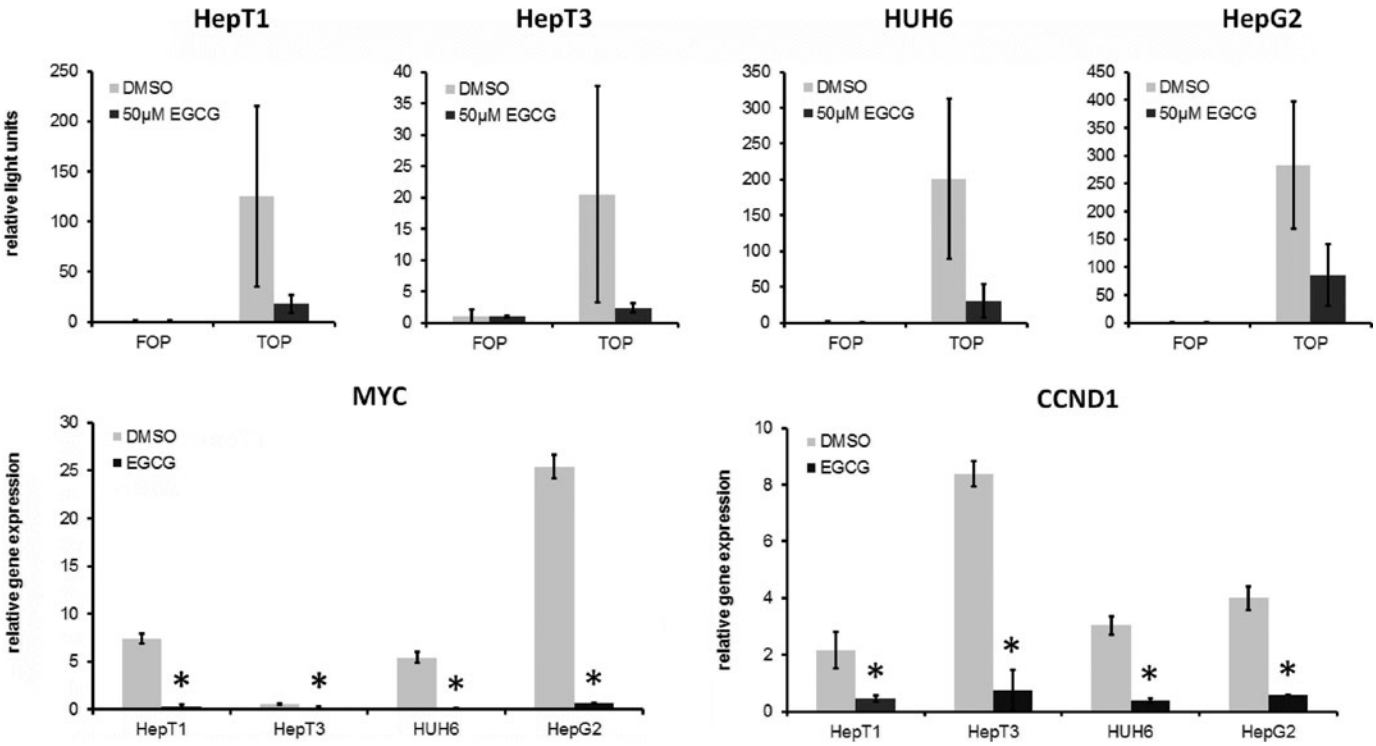

FIG. 4. Inhibition of Wnt signaling activity. Reporter activity (upper panel). Cells were transfected with the pTOPFLASH or pFOPFLASH reporter plasmids and pRL-TK as a reference and treated for 48 h with vehicle (grey bars) or 37.5 μM epigallocatechin-3-gallate (EGCG) (black bars). Wnt reporter activity was determined 48 h after transfection, normalized to Renilla luciferase, and is depicted in arbitrary units. Reporter assay experiments were repeated 3 times and transfections done in duplicate. Wnt target gene expression (lower panel). Hepatoblastoma (HB) cell lines were treated for 48 h with vehicle (grey bars) or 37.5 μM EGCG (black bars). Quantitative real-time reverse transcription-PCR for *MYC* and *CCND1* mRNA was performed and compared to the level of the housekeeping gene *TBP*. Statistical significant difference versus vehicle: $^{*}P < 0.05$; $^{**}P < 0.001$ (unpaired Student's *t*-test).

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EGCG. Using an EGCG concentration of 37.5 μM that effectively inhibits proliferation and induces apoptosis in all HB cell lines (see above), we found no obvious effects on the *SFRP1* transcription rate, which is very low in all HB cell lines (Fig. 5). However, when we lowered the EGCG concentration to 20 or even 10 μM, a significant increase of the originally suppressed *SFRP1* gene was detected in all HB cell lines. These data suggest that the antitumorigenic effects on HB cells elicited by EGCG might be caused at least in part by the reestablishment of the *SFRP1* expression and thereby inactivation of Wnt signaling.

DISCUSSION

Although HB treatment has dramatically improved over the years by combining chemotherapy regimens with surgery, there is still an urgent need for further beneficial therapeutic substances to prevent relapse of tumor growth or the metastatic spread of the disease. The application of natural occurring pleiotropic substances might be a hopeful new strategy for adjuvant treatment of HB preventing relapses in long-term therapy. Here, we present data that the green tea catechin EGCG leads to a significant inhibition of growth and induction of apoptosis in human HB cells, while leaving normal fibroblasts unaffected, even at very high concentrations of up to 100 μM. Moreover, EGCG treatment led to the inhibition of the Wnt signaling pathway, which is constitutively activated in HB cells through mutations in the *ß-catenin* gene. Thus, our data advocate EGCG to be further tested in the preclinical setting with the ultimate goal of using it as an agent to treat HB patients.

Our data clearly document a strong cytotoxic effect of EGCG on HB cells, which is in line with many studies in a variety of cancers. An epidemiological study on the cancer-preventive effect of green tea in a Japanese population of 8,552 individuals has shown that drinking more than 10 cups a day is associated with later onset of cancer (33). Accordingly, an inverse relationship between green tea consumption and the development of gastrointestinal cancers has been described (34). However, as many of the beneficial effects of green tea are related to the activities of EGCG, which is a major component of green tea catechins, pure drinking of green tea would probably not be sufficient. Pietta and colleagues detected in the plasma of 6 healthy volunteers after bolus administration of 300 mL green tea [cultivar "Sencha" (Japan); infusions of 11 g tea leaves and 600 ml of boiling water; brewing time 5 min] a maximum EGCG concentration of 2 μM after 2 h (35). The required concentration of EGCG for our in vitro treatment of HB cells was not much

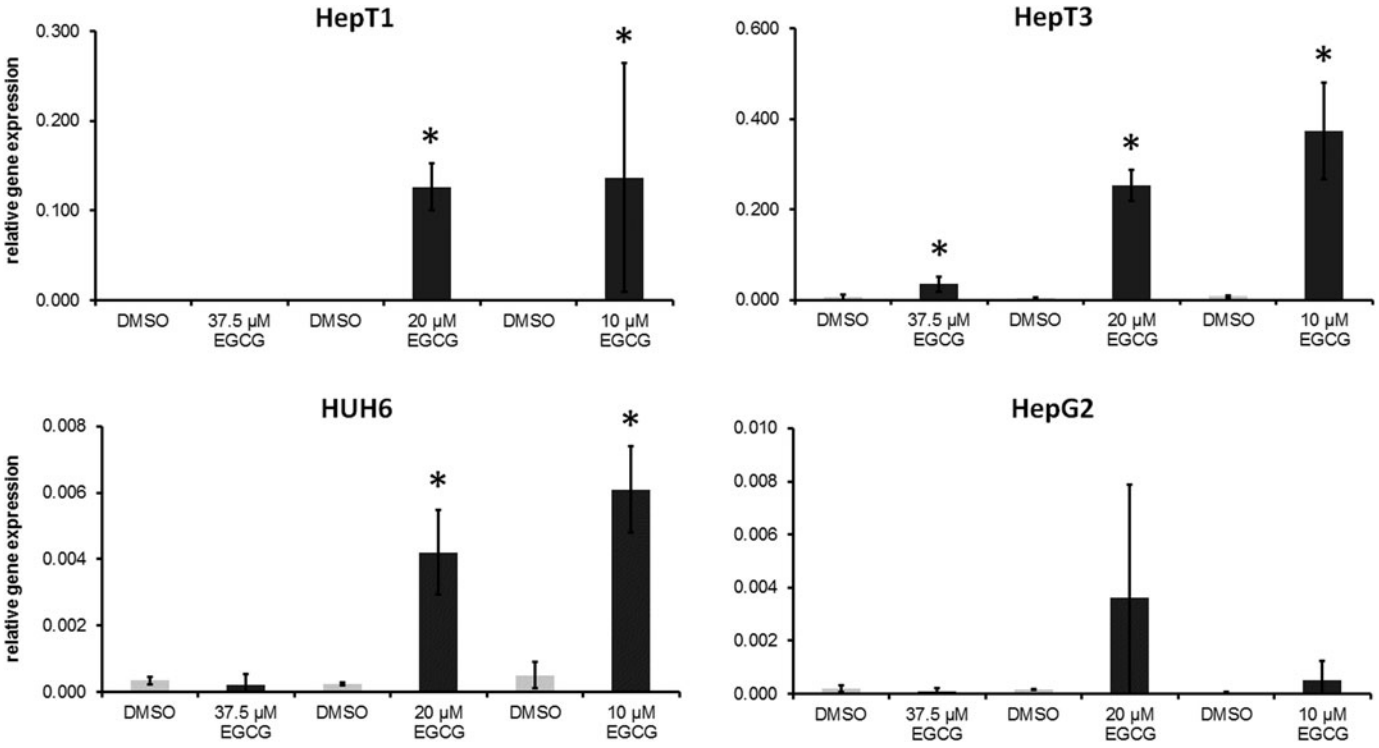

FIG. 5. Reactivation of *SFRP1* expression. Hepatoblastoma (HB) cells were treated for 48 h with vehicle (grey bars) or decreasing amounts of epigallocatechin-3-gallate (EGCG) (black bars). *SFRP1* mRNA level was measured and compared to the level of the housekeeping gene *TBP* by quantitative real-time reverse transcription-PCR. Statistical significant difference versus vehicle: $^{*}P < 0.05$; $^{**}P < 0.001$ (unpaired Student's *t*-test).

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higher, as the IC50 ranged from 9.2 to 36.5 μM. However, formulated preparations such as capsules are already available in different doses and are also cheap with high bioavailability. Nevertheless, further studies are necessary to elucidate if tea extracts or capsules with high EGCG content as a dietary supplement are suitable for HB treatment in patients.

Another beneficial aspect of green tea and EGCG is that they have no appreciable side effects in humans. In a study of healthy males it has been reported that single oral doses up to 1600 mg EGCG are safe and is well tolerated (36). Chow and colleagues conducted a 4-wk pharmacokinetics and safety study of green tea polyphenols with daily oral administration of 800 mg of EGCG in healthy volunteers (37). In our study we also found that normal cells were nearly unaffected by EGCG, even at very high doses of 100 μM. It is well documented that cancer cells are more susceptible to apoptosis induced by EGCG than their normal counterparts (38,39). It has been speculated that normal cells express larger amounts of several EGCG-binding, Fas-like decoy proteins on the cell surface than cancer cells, leading to a diminution in the concentration of EGCG available to bind Fas, resulting in resistance to apoptosis (40). The EGCG-induced change in the redox state of cancer cells (41) or a preferential induction of reactive oxygen species (42) may also be involved in this mechanism. Nevertheless, whatever the molecular mechanism for the tumor-specificity of EGCG might be, this effect could be of high importance for treating HB patients in the early postoperative phase, in which the children are weak and treated with chemotherapy. Of note, EGCG is already occasionally used for adjuvant treatment of cancer in humans and well tolerated (43).

Numerous observations have indicated that constitutively activated Wnt signaling is a key factor of cancer development. The important role of the Wnt pathway in HB pathogenesis has been proven by the identification of frequent *ß-catenin* and rare *AXIN1* and *APC* mutations (7–9). Thus, Wnt signaling is considered to be a possible therapeutic target in HB therapy. A number of existing drugs and natural compounds have been identified as inhibitors and/or modulators of Wnt signaling (44), including EGCG (23–26). In line with this we demonstrated that EGCG effectively blocks Wnt signaling in HB cells by the inhibition of Tcf binding to a reporter plasmid with a corresponding significant reduction of mRNA levels of the Wnt target genes *MYC* and *CCND1*. Most importantly, low doses of EGCG also induced reexpression of the *SFRP1* gene, which has been described to be transcriptionally silenced in HB cells by promoter methylation (45) thereby preventing its physiological role to downregulate the Wnt signaling pathway. An explanation for the failure of high EGCG doses to reestablish *SFRP1* expression might be deduced from the strong inhibitory effect of EGCG on DNA methyltransferases activity (46). It has been shown that

high drug concentrations of azacytidine, another demethylating agent that directly impacts on DNA methyltransferase activity, leads to a G2 phase arrest and pronounced cell death, whereas low doses robustly demethylate DNA (47). Thus, it might be speculated that low doses of EGCG have a more pronounced impact on DNA methylation, whereas higher doses rather stops replication and induces apoptosis, which is in line with our data. However, other Wnt inhibitory mechanisms have been shown in lung cancer cells, in which EGCG treatment resulted in the upregulation of the Wnt inhibitor factor 1 (*WIF1*) gene (24). As *SFRP1* expression is either repressed or absent in approximately half of HB primary tumors (Dr. Eichenmüller, personal communication), a phenomenon also seen in other tumor types like breast cancer (48), it might be assumed that the antitumorigenic effects of EGCG are caused at least in part by the derepression of the Wnt inhibitor SFRP1.

Altogether, our data convincingly demonstrate the high efficacy of EGCG to selectively inhibit HB growth by inducing apoptosis and inhibiting Wnt signaling through reactivation of SFRP1. The data suggest that further testing of EGCG as an adjuvant therapy should be pursued in animal models with the ultimate goal of determining its efficacy in human trials.

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