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Tea polyphenols epigallocatechin gallete and theaflavin restrict mouse liver carcinogenesis through modulation of self-renewal Wnt and hedgehog pathways

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Abstract

The aim of this study is to evaluate chemopreventive and therapeutic efficacy of tea polyphenols epigallocatechin gallete (EGCG) and theaflavin (TF) on self-renewal Wnt and Hedgehog (Hh) pathways during CCl_4/N-nitosodiethylamine-induced mouse liver carcinogenesis. For this purpose, the effect of EGCG/TF was investigated in liver lesions of different groups at pre-, continuous and post initiation stages of carcinogenesis. Comparatively increased body weights were evident due to EGCG/TF treatment than carcinogen control mice. Both EGCG and TF could restrict the development of hepatocellular carcinoma at 30th week of carcinogen application showing potential chemoprevention in continuous treated group (mild dysplasia) followed by pretreated (moderate dysplasia) and therapeutic efficacy in posttreated group (mild dysplasia). This restriction was associated with significantly reduced proliferation, increased apoptosis, decreased prevalence of hepatocyte progenitor cell (AFP) and stem cell population (CD44) irrespective of EGCG/TF treatments. The EGCG/TF could modulate the Wnt pathway by reducing β-catenin and phospho-β-catenin-Y-654 expressions along with up-regulation of sFRP1 (secreted frizzled-related protein 1) and adenomatosis polyposis coli during the restriction. In case of the Hh pathway, EGCG/TF could also reduce expressions of glioma-associated oncogene homolog 1 (Gli1) and SMO (smoothened homolog) along with up-regulation of PTCH1 (patched homolog 1). As a result, in Wnt/Hh regulatory pathways decreased expressions of β-catenin/Gli1 target genes like CyclinD1, cMyc and EGFR/phospho-EGFR-Y-1173 and up-regulation of E-cadherin were seen during the restriction. Thus, the restriction of liver carcinogenesis by EGCG/TF was due to reduction in hepatocyte progenitor cell/stem cell population along with modulation of Wnt/Hh and other regulatory pathways.

Keywords: EGCG; TF; Hepatocellular carcinoma; CD44; β-Catenin; Gli1

1. Introduction

Hepatocellular carcinoma (HCC) is one of the deadliest cancers world wide due to high recurrence rate after surgical resection and lack of effective chemotherapeutic drugs [1]. Recent evidences indicated that the chemo-radiation resistance of HCC might be due to prevalence of cancer stem cells (CSC) and deregulation of self-renewal pathways like Wnt, hedgehog etc. [2]. It was evident that expression of cluster of differentiation 44 (CD44), one of the most common stem cell markers, is strongly correlated with α-fetoprotein (AFP) expression, poor survival and resistance in liver cancer [3]. Thus, targeting the CD44-positive CSC population might be important in prevention of HCC. It was evident that activation of self-renewal Wnt and hedgehog (Hh) pathways and their interplay might be necessary not only to maintain the CSC population but also in regulation of multiple cellular pathways leading to HCC progression [4–6]. In case of Wnt pathway, activation of effector gene β-catenin along with inactivation of some Wnt pathway inhibitors like sFRPs, adenomatosis polyposis coli (APC), etc. were found to be associated with liver carcinogenesis [7–11]. Different studies also indicated activation of self-renewal Hh pathway in HCC patients and cell lines due to aberrant expression of the effector gene glioma-associated oncogene homolog 1 (Gli1) along with other key regulatory genes including patched homolog (PTCH), smoothened homolog (SMO) [12–14]. In addition, it was evident that activated β-catenin and Gli1 could control expressions of multiple down stream genes like Cyclin D1, cMyc, epidermal growth factor receptor (EGFR), E-cadherin etc. during carcinogenesis resulting alterations in associated cellular pathways like cell cycle, cell signaling, epithelial-to-mesenchymal transition etc. [15–20]. Thus, inhibition of the self-renewal pathways might be important to inhibit the CSCs and to prevent HCC.

Abbreviations: HCC, hepatocellular carcinoma; NDEA, N-nitrosodiethyl amine; CD44, cluster of differentiation 44; sFRP1, secreted frizzled-related protein 1; APC, adenomatosis polyposis coli; Hh, hedgehog pathway; Gli1, glioma-associated oncogene homolog 1; SMO, smoothened homolog (Drosophila); PTCH1, patched homolog 1; EGFR, epidermal growth factor receptor.

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Recently, chemoprevention with dietary chemopreventive agents has received much attention for the treatment of such aggressive cancers. Several studies indicated the beneficial roles of tea polyphenols particularly, epigallocatechin 3-gallate (EGCG) and theaflavin (TF) against several types of cancers including HCC [21,22]. Different *in-vitro* and *in vivo* studies indicated that tea polyphenols EGCG could inhibit CSC population and inhibit self-renewal Wnt/Hh pathways in different cancers [5,23]. Few studies indicated the inhibitory role of TF on Wnt/β-catenin signaling [24,25]. However, the modulatory roles of EGCG and TF on both the self-renewal pathways during *in vivo* liver carcinogenesis are not known clearly. Moreover, comparative analysis of the effects of both the polyphenols during liver carcinogenesis are unknown.

Thus, in this study the effect of tea polyphenols EGCG and TF was evaluated on self-renewal Wnt and Hh pathways in established mouse liver carcinogenesis model induced by CCl_4/N-nitosodiethylamine (NDEA) at pre-, continuous and post initiation stages. Our study has been focused on the analysis of following aspects in liver lesions at different stages of carcinogenesis, i.e., (i) status of AFP and CD44-positive population (ii) expressions of some key regulatory genes in the Wnt and Hh pathways and (iii) expressions of some downstream target genes of Wnt/Hh pathways. This study indicates that both the tea polyphenols could act similarly to restrict liver carcinogenesis at mild/moderate dysplastic stages with reduction in AFP and CD44-positive population along with modulation in the self-renewal Wnt, Hh pathways and their regulatory pathways.

2. Materials and methods

2.1. Reagents

Epigallocatechin gallete (EGCG; 95%) and theaflavin (TF, >80%) were obtained from Sigma-Aldrich, St. Louis, MO, USA. CCl_4 was purchased from Sisco Research Laboratories, Mumbai, Maharashtra, India. *N*-nitrosodiethyl amine (NDEA) was purchased from Sigma-Aldrich. 5-bromo-2-deoxyuridine (BrdU) labeling and detection kit II and In situ Cell Death Detection Kit II, POD kit were procured from Roche Molecular Biochemicals, Manheim, Germany. RPMI 1640 medium was purchased from Life Technology/Thermo Fisher Scientific, Waltham, Massachusetts. TRIzol reagent was purchased from Invitrogen/Life technology. Primary antibody, HRP-conjugated secondary antibody and luminol reagents were purchased from Santa Cruz Biotechnology, Inc, Dallas, TX, USA.

2.2. Experimental animals

Female Swiss albino mice were obtained from animal house of Chittaranjan National Cancer Institute, Kolkata, India. Animals were maintained at 25±5°C temperature, with alternating 12 h light/dark cycle and 45–55% humid conditions. Food pellets and drinking water were provided routinely. Mice were under observation for their well being, body weight, toxicity and survival. All the animal experiments were carried out in accordance with institutional ethical committee.

2.3. Experimental design

Mice (5–6 weeks) with average body weight 25 g were divided into following experimental groups, containing 12 mice in each groups.

Group I or normal control group: Mice without any treatment.

Group II or carcinogen control group: Mice in this group received intraperitoneal injection (i.p.) of CCl_4 (50 μl/kg body weight in liquid paraffin) for 4 days successively followed by i.p. injection of NDEA: 75 mg/kg body weight weekly for 3 successive weeks and 100 mg/kg body weight for another 3 successive weeks [26].

Group III (pre-treatment group): Mice of this group received oral administration of tea polyphenols (EGCG/TF) daily for 15 days prior to carcinogen administration and not continued thereafter.

Group IV (continuous treatment group): Mice of this group received oral administration of EGCG/TF daily, 15 days prior to carcinogen administration and continued in that way till end of the experiment.

Group V (post treatment group): Mice in this group received oral administration of EGCG/TF daily after completion of carcinogen application and continued till end of the experiment.

Doses of EGCG (8 μg/kg body weight; aqueous solution) and TF (10 μg/kg body weight; aqueous solution prepared from stock 1 mg/ml containing 25% ethanol) were selected based on toxicity analysis (Supplementary Document 1, Supplementary Fig. 1). Vehicle control group for TF was omitted in this study as there were no remarkable changes in liver histology and toxicity with respect to Group I as seen during toxicity analysis (data not shown). Mice from different experimental groups were sacrificed at 10th, 20th and 30th weeks of first carcinogen administration. At each time point three animals were sacrificed from each group. All the experiments were repeated once. After sacrifice, liver was dissected out from each mouse followed by histopathological analysis and among the samples, three samples from prevalent/advanced histological stages were used for different analysis.

2.4. Tissue processing and histopathological analysis

Liver tissue samples from different groups were fixed in 10% phosphate buffered formalin and were embedded in paraffin. About 4-μm-thick sections were stained by hematoxylin and eosin for routine histopathological analysis according to standard protocol [26].

2.5. In situ cell proliferation assay

Cellular proliferation in the liver sections was determined using BrdU labeling and detection kit II. Dissected liver tissue was washed with 1× phosphate-buffered saline (PBS) (pH 7.4) and immediately incubated into pre-warmed (37°C) RPMI 1640 medium at 37°C for $1^1/_2$ h containing BrdU (1: 200). After washing with PBS tissues were fixed in 10% phosphate buffered formalin followed by paraffin embedding and sectioning (4 μm). Then the tissue sections were processed for BrdU assay according to manufacturers' protocol as described previously [22]. Proliferation assay was performed in three samples per group. Percentage of BrdU-positive cells was determined from labeled nuclei with respect to the total number of nuclei counted at 5–10 randomly chosen microscopic fields of the liver lesions.

2.6. In situ cell death detection using TUNEL assay

Cellular apoptosis was detected in the formalin-fixed and paraffin-embedded liver sections (4 μm) by the terminal deoxyneucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method using *in situ* cell death detection kit according to manufacturers' protocol as described previously [22]. TUNEL assay was performed in three samples per group. Percentage of apoptotic cells was determined from labeled nuclei with respect to the total number of nuclei counted at 5–10 randomly chosen microscopic fields of the liver lesions.

2.7. Quantitative RT-PCR analysis

Total tissue RNA was extracted from liver lesions by TRIzol reagent according to the manufacturer's protocol. cDNA was synthesized from the 5 μg of total RNA with Super Script III Reverse Transcriptase (Invitrogen/Life technology, USA) in accordance with the manufacturer's protocol. Gene expression was carried out by real-time PCR (ABI Prism 7500; Life Technology, MA, USA) using specific primers (Supplementary Table 1) and Power SYBR Green PCR Master Mix (Applied Biosystems, Life technology, USA). Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method [26]. Mouse β2-microglobulin gene (B2M) was used as an endogenous control and for target gene normalization. Expression analysis was performed in three samples per group. Each sample was loaded in triplicate. Relative expression was graphically represented.

2.8. Protein extraction and Western blot analysis

Protein was extracted from liver tissues and western blotting was performed as described previously [26]. Equal amount of proteins were separated by 10–12% SDS-PAGE and then transferred to polyvinylidine difluoride membrane (Millipore, MA). Membranes were incubated with 3–5% nonfat dry milk for 1–2 h at room temperature for blocking followed by overnight incubation at 4 °C with desired primary antibodies (1:500–1:1000; Supplementary Table 2) and then with corresponding HRP-conjugated secondary antibodies (1:2000–1:10000; Supplementary Table 2). The target protein bands were then visualized using luminol reagent and autoradiographed on X-ray film (Kodak, Rochester, NY, USA). All the immuno-blotting experiments were performed in three samples per group. The band intensities were quantified using densitometric scanner (Bio Rad GS-800, Hercules, CA, USA). Peak densities of the proteins of interest were normalized using peak density of loading control α-tubulin.

2.9. Immunohistochemical (IHC) analysis:

About 4-μm-thick paraffin sections were processed for deparaffinization and rehydration followed by antigen retrieval with 10 mM citrate buffer (pH 6.0) at 85°C for 40 min. Then, the slides were incubated with 3–5% blocking solution of bovine serum albumin for 1 h at room temperature (RT) followed by incubation with desired primary antibody (1:50–1:100; Supplementary Table 2), overnight at 4°C and with HRP-conjugated secondary antibody (1:500–1:1000; Supplementary Table 2) for 2 h at RT. For colour substrate reaction diaminobenzidine was used after incubation with secondary antibody followed by counterstaining with hematoxylin. Negative controls slides were prepared in the same process as describe above except with PBS instead of primary antibody. Protein expressions were analyzed in three samples per groups. The protein expression pattern in the liver lesions was scored according to the Perrone et al. [27] with some modifications as described before [26]. The staining intensity of a protein in the experimental groups was compared with Group I.

2.10. Statistical analysis

Data obtained from Group II was compared with Group I and data obtained from Groups III–V was compared with Group II. Statistical analysis was performed using one way analysis of variance followed by post hoc Tukey test with the help of critical difference or least significant difference at 5% and 1% level of significance to compare the mean values. $P<.05$ was considered as statistically significant. Data were expressed as mean with S.D.

3. Results

3.1. Effect of EGCG and TF on mice body weight during carcinogenesis

The first visible changes were seen in mice body weights due to carcinogen and tea polyphenol treatments (Supplementary Fig. 2). In Group II mice, body weight decreased gradually up to 30th week compared to Group I. In case of EGCG and TF-treated groups, a slight decrease in body weights were seen at 6th–10th weeks, then it increased and remained quite stable in the following weeks, indicating their protective effects during carcinogenesis (Supplementary Fig. 2).

3.2. Analysis of histopathological changes of liver in different experimental groups

Visible macroscopic changes were evident in the livers of Group II due to CCl_4/NDEA treatment (data not shown). Normal smoothness of liver was lost in Group II compared to Group I and it became very rough in texture at 10th week and foci formation was observed in the subsequent weeks. Whereas, no remarkable macroscopic changes were seen in the livers of EGCG and TF-treated groups (Group III–V), though slight rough liver surface was seen at 30th week in Group III and at 10th week in Group V (data not shown).

Histological analysis revealed moderate dysplasia (100%), severe dysplasia (66.7%) and HCC (60%) at 10th, 20th and 30th weeks of carcinogen application respectively in Group II (Table 1, Supplementary Fig. 3) as reported previously [26]. On the other hand, EGCG and TF pre-treatment protocol (Group III) could restricted the carcinogenesis at mild dysplastic stages up to 20th week followed by moderate dysplastic changes at 30th week (Table 1, Supplementary Fig. 3). Interestingly, EGCG and TF continuous treatment protocol (Group IV) showed better effect with mild dysplastic changes in livers at all the time points, indicating their importance in chemoprevention. In case of post treatment group (Group V), moderate dysplasia was seen at 10th week followed by restriction of the carcinogenesis at mild dysplastic stages up to 30th week, indicating therapeutic efficacy of EGCG and TF against liver carcinogenesis (Table 1, Supplementary Fig. 3).

Table 1
Histopathological observations of livers in different experimental groups at different time points

Experimental groups		Histological observations		
		10th week	20th week	30th week
Carcinogen control group	Group II	Mod [6/6; 100%]	Sev [4/6; 66.7%], Mod [2/6; 33.3%]	HCC [3/5; 60%], Sev [2/5; 40%]
EGCG-treated groups	Group III	Mild [6/6; 100%]	Mild [5/6; 83.3%], Mod [1/6; 16.7%]	Mod [4/6; 66.7%], Mild [2/6; 33.3%]
	Group IV	Mild [6/6; 100%]	Mild [6/6; 100%]	Mild [6/6; 100%]
	Group V	Mod [6/6; 100%]	Mild [4/6; 66.7%], Mod [2/6; 33.3%]	Mild [5/6; 83.3%], Mod [1/6; 16.7%]
TF-treated groups	Group III	Mild [6/6; 100%]	Mild [4/6; 66.7%], Mod [2/6; 33.3%]	Mod [6/6; 100%]
	Group IV	Mild [6/6; 100%]	Mild [6/6; 100%]	Mild [5/6; 83.3%], Mod [1/6; 16.7%]
	Group V	Mod [6/6; 100%]	Mod [4/6; 66.7%], Mild [2/6; 33.3%]	Mild [4/6; 66.7%], Mod [2/6; 33.3%]

Mild: mild dysplasia; Mod: moderate dysplasia; Sev: severe dysplasia; HCC: hepatocellular carcinoma.

3.3. Determination of cellular proliferation and apoptosis status during restriction of liver carcinogenesis by EGCG and TF

Cellular proliferation and apoptosis status was determined in the liver lesions during the EGCG and TF mediated restriction of the liver carcinogenesis. In situ BrdU incorporation assay revealed significantly high ($P<.001$) percentage of proliferating cells in the liver lesions of Group II at 10th week (>40%) followed by gradual increase in the subsequent weeks up to 30th week (>80%) than normal liver (Group I) (Fig. 1A, Supplementary Fig. 3). The BrdU-positive cells were found to localize through out the liver lesions of Group II during the carcinogenesis compared to portal vain centric localization in normal liver (Group I). Interestingly, in comparison with Group II, EGCG and TF treatment could significantly reduce ($P<.05$, $P<.001$) the percentage of proliferating cells in all the treated groups at different time points during the restriction, except at 10th week in Group V (Fig. 1A, Supplementary Fig. 3). IHC analysis of proliferation marker proliferating cell nuclear antigen in the liver lesions validated the BrdU incorporation in different groups (Fig. 1B, Supplementary Fig. 3).

On the other hand, in situ TUNEL assay showed gradual decrease in percentage of apoptotic cells in the liver lesions of Group II from 10th week (14%) to 30th week (5%, $P<.05$) during the carcinogenesis than normal liver (Group I) (Fig. 1C, Supplementary Fig. 3). During the

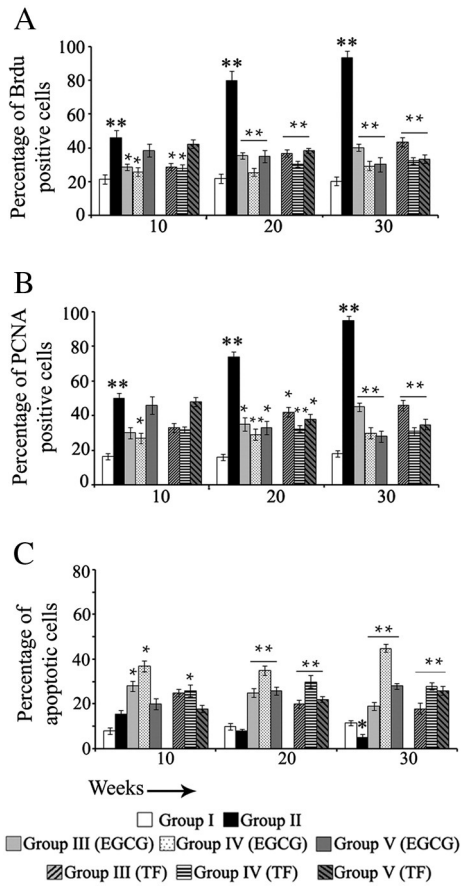

Fig. 1. Effect of EGCG and TF on cellular proliferation and apoptosis during liver carcinogenesis. Graphical representation of (A) percentage of Brdu incorporated cells (B) PCNA-positive cells and (C) apoptotic cells in different groups at different time points. Data presented as mean±S.D. *Represents significant P value in Group II with respect to Group I and * in Group III–V with respect to Group II (*/*$P<.05$ and **/**$P<.001$).

restriction, EGCG and TF treatment could significantly increase the percentage of apoptotic cells ($P<.05$, $P<.001$) in all the treated groups (Group III–V) compared to Group II, except in TF-treated Group III and Group V (EGCG/TF) at 10th week (Fig. 1C, Supplementary Fig. 3). Thus, the restriction of liver carcinogenesis by tea polyphenols might be due to modulation in cellular proliferation and apoptosis.

3.4. Status of α-fetoprotein (AFP) and CD44 expressions during restriction of liver carcinogenesis by EGCG and TF

In IHC analysis, cytoplasmic expression of AFP was found to be increased significantly ($P<.001$) in the liver lesions of Group II at 10th week (~70%) followed by gradual increase in the subsequent weeks up to 30th week (~90%) than normal liver (Group I) (Fig. 2A and B). Interestingly, in the liver lesions of Group III–V, both the polyphenols could significantly reduce ($P<.001$) the AFP expression during restriction of the carcinogenesis up to 30th week than Group II (Fig. 2A and B). However, among the different treated groups AFP expression was comparatively high in Group V than Group III and IV at 10th week. Similarly, at 30th week the AFP expression was significantly high in Group III than Group IV and V.

In case of CD44, the Western blot analysis showed significant increased expression ($P<.001$) in the liver lesions of Group II at different time points than Group I (Fig. 2C, Supplementary Fig. 4). Interestingly, both the tea polyphenols could significantly reduce ($P<.001$) its expression in all the treated groups (Group III–V) at different time points, except in Group V at 10th week (Fig. 2C, Supplementary Fig. 4). Similarly in IHC analysis, significant ($P<.001$)

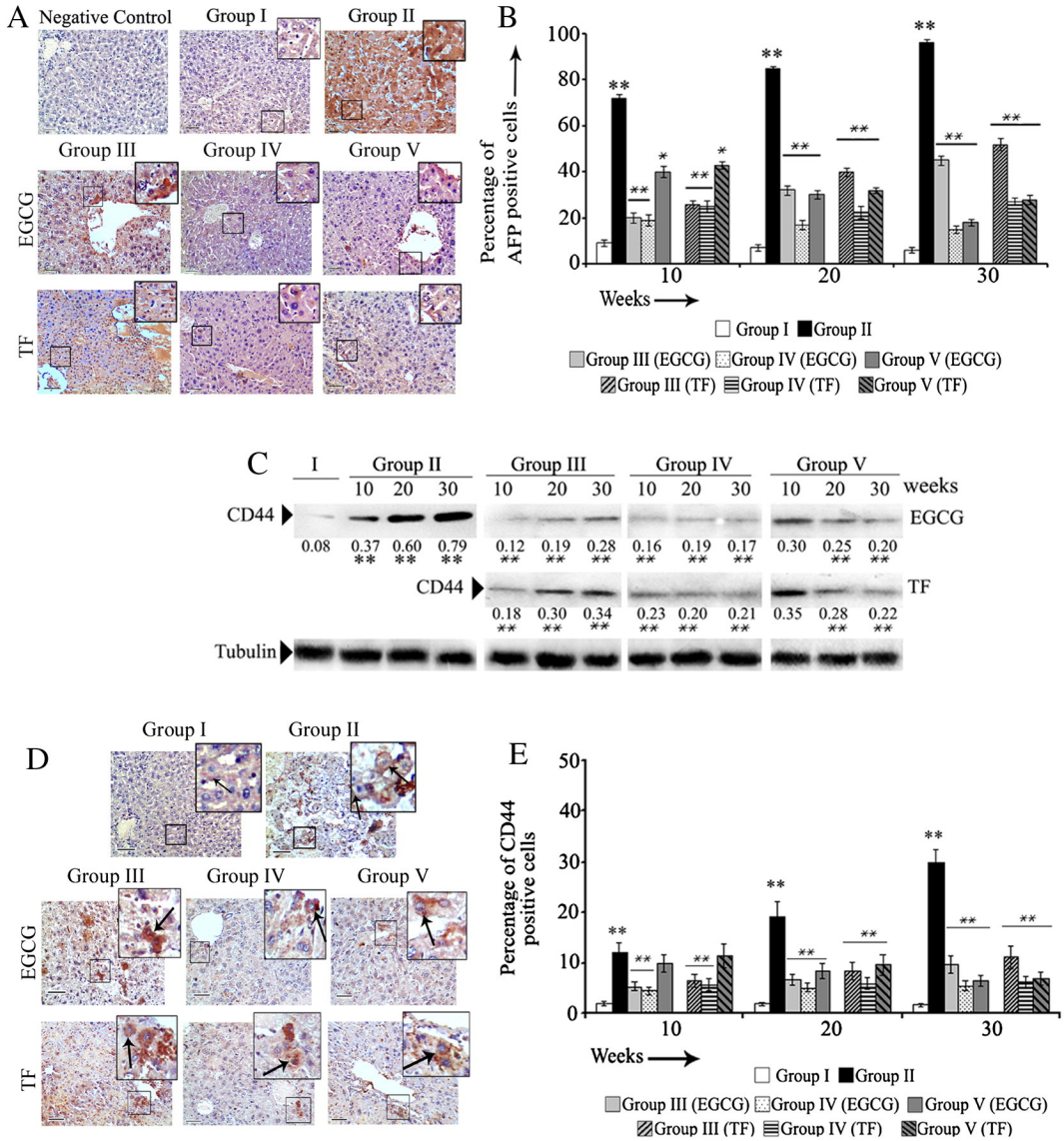

Fig. 2. Effect of EGCG and TF on expressions of α-fetoprotein (AFP) and CD44 during liver carcinogenesis. (A) Representative photographs of immunohistochemical expressions of α-fetoprotein (AFP) in liver sections of different groups with/without EGCG and TF at 30th week along with negative control (without primary antibody). Magnifications 20×. Inset magnifications 40×. Scale bar represents 50 μm. (B) Graphical representation of percentage of AFP-positive cells observed by IHC analysis in different groups at different time points. Data presented as mean±S.D. (C) Western blot analysis of CD44 in liver lesions of different groups at 10th, 20th and 30th weeks. α-Tubulin (Tubulin) used as loading control. Relative peak density was normalized with loading control. (D) Representative photographs of immunohistochemical expressions of CD44 in liver sections of different groups with/without EGCG and TF at 30th week. Magnifications 20×. Inset magnifications 40×. Scale bar represents 50 μm. Arrows indicate positive cells. (E) Graphical representation of percentage of CD44-positive cells observed by IHC analysis in different groups at different time points. Data presented as mean±S.D. *Represents significant P value in Group II with respect to Group I and * in Group III–V with respect to Group II (*/*$P<.05$ and **/**$P<.001$).

high membrane/cytoplasmic expression of the CD44 was evident in hepatocytes and non-hepatocytes cells of the liver lesions in Group II at 10th week (~12%) followed by gradual increase up to 30th week (~30%) during the carcinogenesis than normal liver (Group I) (Fig. 2D and E). Whereas, EGCG and TF treatment could reduce its expression significantly ($P<.001$) in all the treated groups (Group III–V) at different time points compared to Group II (Fig. 2D and E), except in Group V at 10th week. Thus, this indicates that EGCG and TF treatment could modulate the AFP-positive cells/hepatocyte progenitor cells and CD44-positive stem cell population in restriction of the liver carcinogenesis.

3.5. Effect of EGCG and TF on expressions of key regulatory genes in Wnt pathway during restriction of the liver carcinogenesis

To understand the effect of EGCG and TF on Wnt pathway in restriction of the liver carcinogenesis, the expression of β-catenin, effector gene of this pathway and the key antagonists like secreted frizzled-related protein 1 (sFRP1) and APC were analyzed in the liver lesions of different groups. In Group II, the expression of β-catenin (mRNA) was significantly increased at 10th week followed by gradual increase up to 30th week than Group I (Fig. 3A). Whereas, EGCG treatment could significantly reduce its expression at all the time points in Group III-V than Group II, except at 10th week in Group V. On the other hand, TF treatment could significantly reduce the mRNA expression only at 20th and 30th week in all the treated groups than Group II (Fig. 3A). In Western blot analysis, similar trend of β-catenin expression was seen in the liver lesions during carcinogenesis (Group II) as well as due to EGCG and TF treatment (Group III–V) (Fig. 3B, Supplementary Fig. 4). Similarly, expression of activated β-catenin (phospho-β-catenin Y-654) was increased significantly during liver carcinogenesis in Group II than Group I and significantly decreased at different time points due to EGCG and TF treatment (Group III–V) than Group II, except at 10th week in TF-treated Group V (Fig. 3B, Supplementary Fig. 4).

IHC analysis showed significantly high ($P<.05$) nuclear and membrane/cytoplasmic expression of β-catenin in the liver lesions of Group II at 10th week followed by gradual increased expression up to 30th week compared to the low expression in normal liver (Group I)

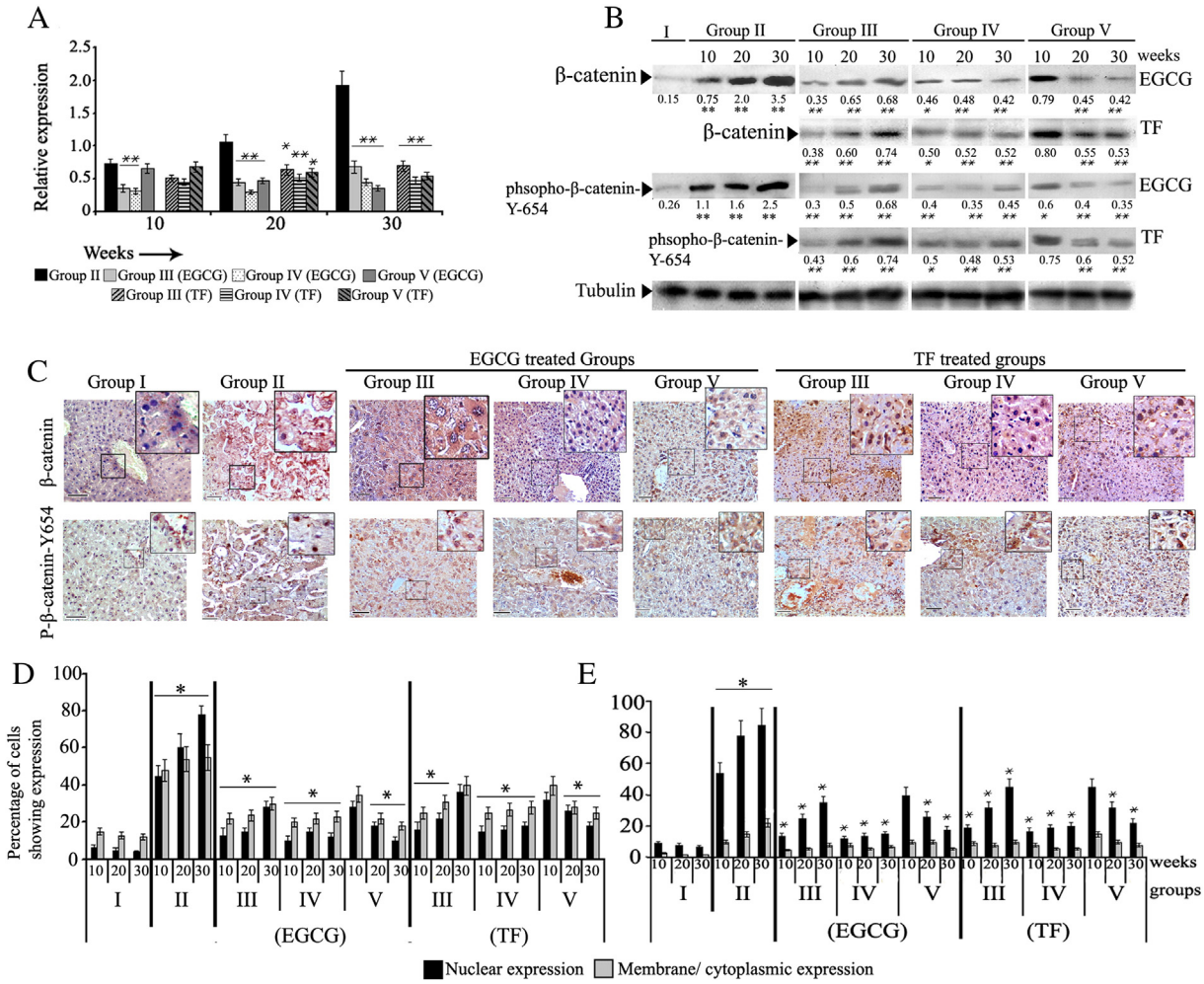

Fig. 3. Effect of EGCG and TF on Wnt pathway effector gene β-catenin and activated β-catenin (phospho-β-catenin-Y-654) expressions during liver carcinogenesis. (A) Relative mRNA expression of β-catenin in different groups at different weeks analyzed by quantitative RT-PCR. Mouse B2M was used as endogeneous control and for target gene normalization. Data presented as mean±S.D. (B) Western blot analysis of β-catenin and phospho-β-catenin-Y-654 proteins in different groups at 10th, 20th and 30th weeks. α-Tubulin (Tubulin) used as loading control. Relative peak density was normalized with loading control. (C) Representative photographs of immunohistochemical expressions of β-catenin and phospho β-catenin (Y-654) in liver sections of different groups with/without EGCG and TF at 30th week. Magnifications 20×. Inset magnifications 40×. Scale bar represents 50 μm. (D) Graphical representation of percentage of cells showing membrane/cytoplasmic and nuclear expressions of β-catenin and (E) phospho-β-catenin-Y-654 observed by IHC analysis in the liver tissues of different groups at different weeks. Data presented as mean±S.D. *Represents significant *P* value in Group II with respect to Group I and * in Group III–V with respect to Group II (*/*$P<.05$ and **/**$P<.001$).

Table 2
Immunohistochemical scores of some regulatory proteins of Wnt, hedgehog (Hh) and their associated pathways

				EGCG-treated			TF-treated		
		Gr I	Gr II	Gr III	Gr IV	Gr V	Gr III	Gr IV	Gr V
β-Catenin	10th	L	H	L	L	M	L	L	H
	20th	L	H	L	L	L	M	L	M
	30th	L	H	M	L	L	M	L	L
p-β-Catenin (Y654)	10th	L	H	L	L	M	L	L	H
	20th	L	H	L	L	L	M	L	M
	30th	L	H	M	L	L	M	L	L
sFRP1	10th	H	L	H	H	L	M	H	L
	20th	H	L	M	H	H	M	H	L
	30th	H	L	L	H	H	L	H	M
APC	10th	H	M	H	H	M	M	M	L
	20th	H	L	H	H	H	M	H	M
	30th	H	L	L	H	H	L	H	H
Gli1	10th	L	H	L	L	H	L	L	H
	20th	L	H	L	L	L	M	L	M
	30th	L	H	M	L	L	M	L	L
SMO	10th	L	M	L	L	M	L	L	M
	20th	L	H	L	L	L	M	L	L
	30th	L	H	M	L	L	M	L	L
PTCH1	10th	H	M	H	H	M	M	H	M
	20th	H	M	H	H	H	M	H	M
	30th	H	L	M	H	H	L	H	H
Cyclin D1	10th	L	H	L	L	M	L	L	M
	20th	L	H	L	L	L	M	L	L
	30th	L	H	M	L	L	M	L	L
cMyc	10th	L	H	L	L	M	L	L	M
	20th	L	H	L	L	L	M	L	M
	30th	L	H	M	L	L	M	L	L
EGFR	10th	L	H	L	L	M	L	L	M
	20th	L	H	L	L	L	L	L	M
	30th	L	H	M	L	L	M	L	L
p-EGFR (Y1173)	10th	L	H	L	L	M	L	L	M
	20th	L	H	L	L	L	L	L	L
	30th	L	H	M	L	L	M	L	L
E-cadherin	10th	H	M	H	H	M	H	H	M
	20th	H	L	H	H	H	M	H	H
	30th	H	L	M	H	H	M	H	H

Low (L), moderate (M) and high (H) expressions of different proteins in majority of samples per groups (Gr; Group I–V) at 10th, 20th and 30th weeks.

(Table 2, Fig. 3C and D). However, EGCG and TF treatment showed low nuclear and membrane/cytoplasmic expression in Group III–IV at 10th week ($P<.05$) with moderate to high expression in Group V. In subsequent weeks, β-catenin expression remained low in Group IV but gradually increased in Group III and decreased in Group V (Table 2, Fig. 3C and D). Similar trend in nuclear expression of phospho-β-catenin (Y-654) was evident in the liver lesions during carcinogenesis (Group II) and due to EGCG and TF treatment (Group III–V) (Table 2, Fig. 3C and E).

In parallel, gradual decrease in relative mRNA expressions of sFRP1 and APC were evident in the liver lesions of Group II during carcinogenesis (Fig. 4A). Whereas, EGCG and TF treatment could significantly ($P<.001$) increase their expressions in different groups (Group III–V) at different time points than Group II, except in Group V at 10th week (Fig. 4A). Similarly, western blot analysis showed gradual and significant decrease ($P<.001$) in sFRP1 and APC expressions in Group II during carcinogenesis than Group I and increased expressions in Group III–V due to EGCG/TF treatment at different time points (significant increase in Group IV at all the time points and in Group III and V only at 20th and 30th week than Group II) (Fig. 4B, Supplementary Fig. 4).

Similarly, IHC analysis showed high cytoplasmic expressions of sFRP1 and APC in the normal liver (Group I) (Table 2, Fig. 4C). During carcinogenesis, low expression of sFRP1and moderate expression of APC was seen in Group II at 10th week followed by low expressions of these proteins in the subsequent weeks than Group I. Whereas, in EGCG-/TF-treated groups, moderate to high expressions of sFRP1 and APC were seen in Group III–IV at 10th week along with low to moderate expressions in Group V (Table 2, Fig. 4C). In the subsequent weeks, their expressions remained high in Group IV, but decreased gradually in Group III and increased in Group V irrespective of polyphenols. Thus, this indicates that EGCG and TF might modulate the Wnt pathway by down-regulating β-catenin/activated β-catenin expressions along with up-regulation in expressions of sFRP1 and APC in restriction of the carcinogenesis.

3.6. Effect of EGCG and TF on expression of the key regulatory genes in Hedgehog pathway (Hh) during restriction of the liver carcinogenesis

To analyze the effect of EGCG and TF on Hh pathway, the expression of effector gene Gli1 along with its up-stream key regulatory genes like SMO and PTCH1 were studied in the liver lesions of different groups. Significant increase in relative mRNA expression of Gli1 was seen in the liver lesions of Group II at 10th week followed by gradual increase in the subsequent weeks (Fig. 5A). Interestingly, EGCG treatment could significantly decrease ($P<.001$) its expression in all the treated groups (Group III–V) at different time points than Group II, except in Group V at 10th week. On the other hand, TF treatment could significantly decrease ($P<.05$, $P<.001$) its expressions in different groups (Group III–V) only at 20th and 30th weeks than Group II (Fig. 5A). Similarly, western blot analysis of Gli1 showed concordance with its mRNA expression during carcinogenesis (Group II) and in Group III-V due to EGCG and TF treatment during restriction of the carcinogenesis (Fig. 5B, Supplementary Fig. 4).

Likewise, IHC analysis revealed significantly high ($P<.05$) nuclear/cytoplasmic expression of Gli1 in the liver lesions of Group II at 10th week and in the subsequent weeks during the carcinogenesis compared to low expression in normal liver (Group I) (Table 2, Fig. 5C and D). However, low Gli1 expression was seen in the liver lesions of Group III–IV at 10th week along with high expression in Group V (Table 2, Fig. 5C and D). In the following weeks, Gli1 expression was unchanged in Group IV but increased gradually in Group III and decreased gradually in Group V (Table 2, Fig. 5C and D).

Like Gli1, significant increased mRNA expression of SMO was evident in Group II during carcinogenesis and reduced expression was seen during the restriction in Group III–V due to EGCG and TF treatment (Fig. 6A). On the other hand, decreased mRNA expression of PTCH1 was evident in the liver lesions of Group II at 20th and 30th week (Fig. 6A). Whereas, EGCG and TF treatment could significantly ($P<.001$) increase its expression in all the treated groups (Group III-V) at different time points than Group II, except in Group V (EGCG/TF) at 10th week (Fig. 6A). Western blot analysis of these proteins in different groups showed concordance with their respective mRNA expressions in different time points (Fig. 6B, Supplementary Fig. 4).

Similarly in IHC analysis, moderate cytoplasmic expression of SMO and PTCH1 were seen in the liver lesions at 10th week of Group II (Table 2, Fig. 6C). However, during progression of carcinogenesis, the expression of SMO gradually increased whereas, PTCH1 expression gradually decreased. On the other hand, in EGCG-/TF-treated groups, low SMO expression and moderate to high PTCH1 expression were evident in Group III–IV at 10th week along with moderate expressions of both the proteins in Group V (Table 2, Fig. 6C). In the subsequent weeks, their expressions were unchanged in Group IV but SMO expression gradually increased and PTCH1 expression decreased gradually in Group III. Whereas, in case of Group V, gradual reduction in SMO expression and increase in PTCH1 expression were evident in the subsequent weeks irrespective of polyphenols (Table 2, Fig. 6C). Thus, this indicates that both the polyphenols might modulate the Hh

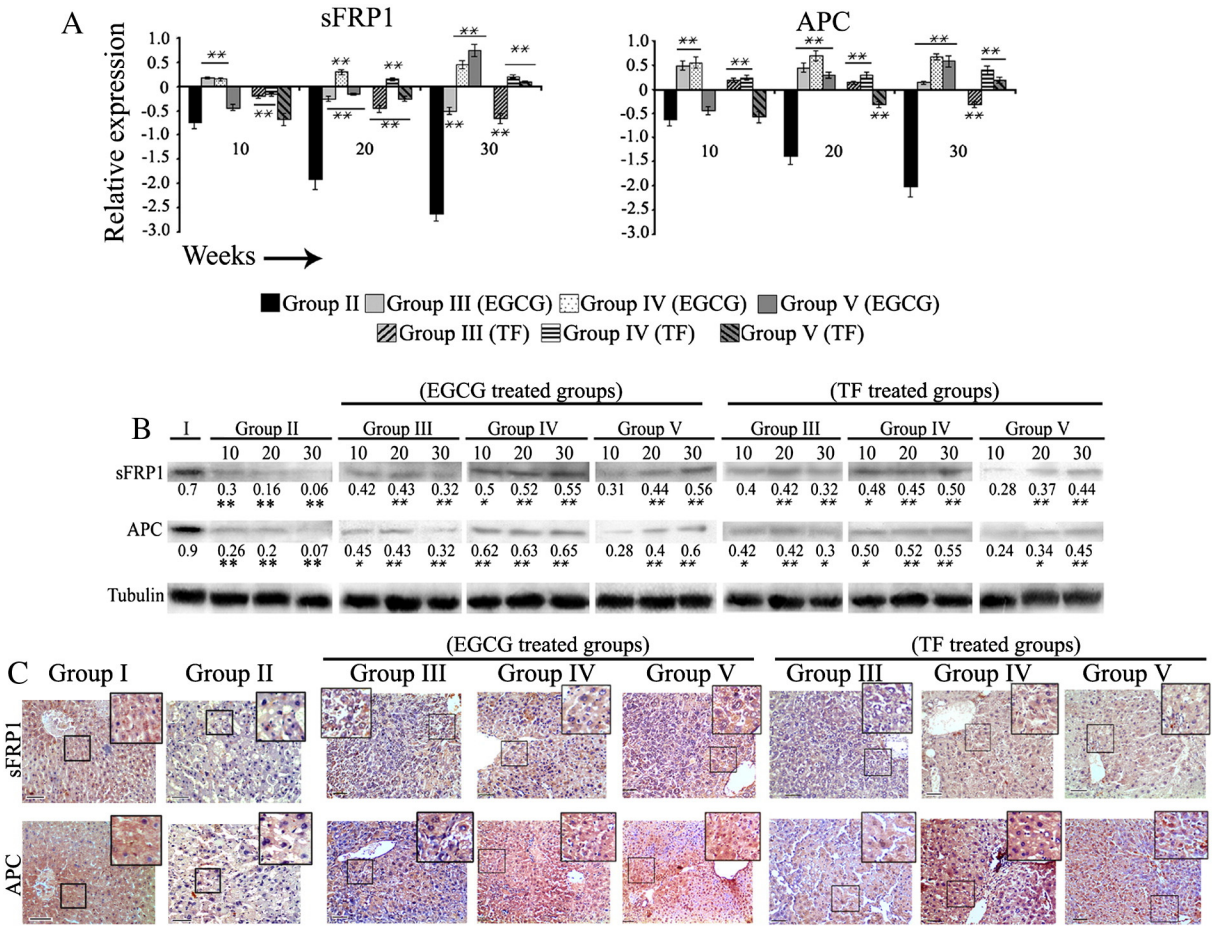

Fig. 4. Effect of EGCG and TF on expressions of Wnt pathways antagonists' sFRP1 and APC during liver carcinogenesis. (A) Relative mRNA expressions of sFRP1 and APC in different groups at different weeks analyzed by quantitative RT-PCR. Mouse B2M was used as an endogeneous control and for target gene normalization. Data presented as mean±S.D. (B) Western blot analysis of sFRP1 and APC in different groups at 10th, 20th and 30th weeks. α-Tubulin (Tubulin) used as loading control. Relative peak density was normalized with loading control. Data presented as mean±S.D. (C) Representative photographs of immunohistochemical expressions of sFRP1 and APC in the liver sections of different groups with/without EGCG and TF at 30th week. Magnifications 20×. Inset magnifications 40×. Scale bar represents 50 μm.*Represents significant *P* value in Group II with respect to Group I and * in Group III–V with respect to Group II (*/**P*<.05 and **/***P*<.001).

pathway by reducing expressions of Gli1 and SMO along with up-regulation of PTCH1 in restriction of liver carcinogenesis.

3.7. Effect of EGCG and TF on expressions of some Wnt and Hh pathways associated genes during restriction of liver carcinogenesis

To understand the effect of EGCG and TF on some Wnt and Hh pathways associated genes, the expressions of Cyclin D1 and cMyc (trans-activated by both β-catenin and Gli1) followed by EGFR (activated by β-catenin) and E-cadherin (down-regulated by Gli1) were analyzed in the liver lesions of different groups.

Significant up-regulation in relative mRNA expressions of Cyclin D1 and cMyc were evident in the liver lesions of Group II at 10th week followed by gradual increase up to 30th week (Fig. 7). Interestingly, EGCG treatment could significantly decrease their expressions in all the treated groups (Group III–V) at all the time points than Group II, except in Group V at 10th week. On the other hand, TF treatment could significantly decrease their mRNA expressions in all the treated groups only at 20th and 30th weeks than Group II (Fig. 7). Similar trend in mRNA expression of EGFR was also evident in the liver lesions of Group II during carcinogenesis and in Group III–V due to EGCG/TF treatment at different time points (Fig. 7). On the other hand, significant decreased mRNA expression of E-cadherin was seen in the liver lesions of Group II at 10th week followed by gradual decrease in the subsequent weeks during the carcinogenesis (Fig. 7). Whereas, EGCG and TF treatment could significantly increase its mRNA expression in all the treated groups (Group III–V) during the restriction than Group II, except in Group V at 10th week (Fig. 7).

IHC analysis of these proteins showed concordance with their respective mRNA expressions (Table 2, Supplementary Fig. 5). During carcinogenesis in Group II, high nuclear/cytoplasmic expressions of Cyclin D1 and cMyc along with high cytoplasmic expressions of EGFR and activated EGFR (phospho-EGFR-Y-1173) were seen at 10th week and in subsequent weeks than low expression in normal liver (Group I) (Table 2, Supplementary Fig. 5). Whereas, low expressions of these proteins were seen in the liver lesions of Group III–IV and moderate expressions was observed in Group V at 10th week (Table 2, Supplementary Fig. 5). In the following weeks, the expressions of these proteins did not change in Group IV but gradually increased in Group III and reduced gradually in Group V up to 30th week irrespective of the polyphenols. On the other hand, moderate membrane/cytoplasmic expression of E-cadherin was seen in the liver lesions of Group II at 10th week followed by low expression in the subsequent weeks compared to high expression in normal liver (Table 2, Supplementary Fig. 5). However, high E-cadherin expression was seen in the liver lesions of Group III–IV at 10th week with moderate expression in Group V (Table 2, Supplementary Fig. 5). In the following weeks, its expression remained high in Group IV, with moderate expression in Group III and

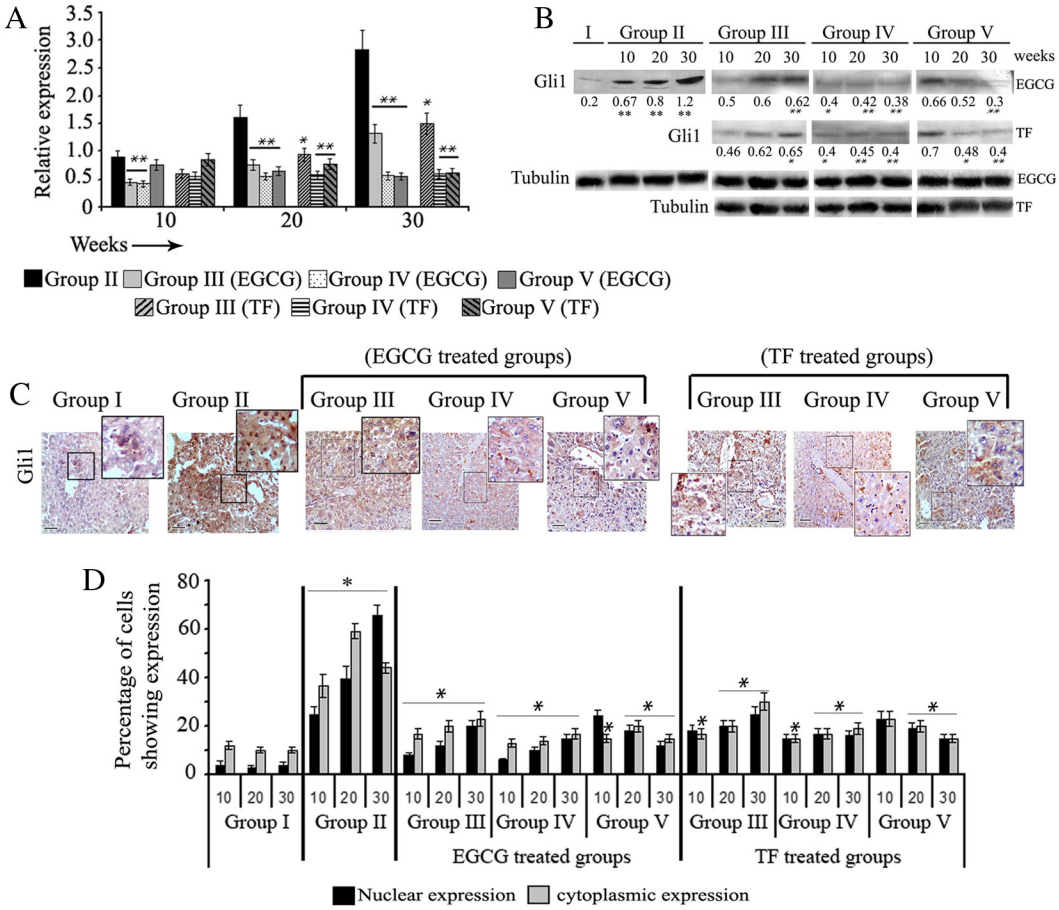

Fig. 5. Effect of EGCG and TF on expressions of Hedgehog pathway effector gene Gli1 during liver carcinogenesis. (A) Relative mRNA expression of Gli1 in different groups at different weeks analyzed by quantitative RT-PCR. Mouse B2M was used as an endogeneous control and for target gene normalization. Data presented as mean±S.D. (B) Western blot analysis of Gli1 in different groups at 10th, 20th and 30th weeks. α-Tubulin (Tubulin) used as loading control. Relative peak density was normalized with loading control. (C) Representative photographs of immunohistochemical expressions of Gli1 in the liver sections of different groups with/without EGCG and TF at 30th week. Magnifications 20×. Inset magnifications 40×. Scale bar represents 50 μm. (D) Graphical representation of percentage of cells showing cytoplasmic and nuclear expressions of Gli1 observed by IHC analysis in different groups at different weeks. Data presented as mean±S.D. *Represents significant *P* value in Group II with respect to Group I and * in Group III–V with respect to Group II (*/**P*<.05 and **/***P*<.001).

gradual increased expression in Group V. Thus, it indicates that modulation in expression of the Wnt and Hh pathways associated genes might have importance in restriction of liver carcinogenesis.

4. Discussion

The aim of this study is to understand the molecular mechanism of restriction of liver carcinogenesis by EGCG/TF in the CCl_4/NDEA induced mouse model. For this purpose, to analyze the chemopreventive and therapeutic efficacy of the polyphenols non-toxic doses were selected for regular oral administration of EGCG and TF in pre-, continuous and post treatment schedule. First protective effect of the polyphenols was visualized in mouse body weight changes where quite stable body weights were seen in the treated groups compared to decreasing body weight in carcinogen control group (Supplementary Fig. 2). Histopathological analysis indicated the potential chemopreventive effect of EGCG/TF with mild dysplasia in continuous treated Group IV and partial chemopreventive effect in pretreated Group III with mild dysplasia (10th week) to moderate dysplasia (30th week). This might be due to the withdrawal of EGCG/TF application before carcinogen administration in Group III than continuous application in Group IV. On the other hand, the post treatment protocol (Group V) showed potential therapeutic effect of the polyphenols in restriction of the liver carcinogenesis from moderate dysplasia (10th week) to mild dysplastic changes (30th week) (Table 1, Supplementary Fig. 3). It seems that EGCG/TF treatment could modulate the tumor initiating clones to be in the early pre-malignant lesions. This restriction of liver carcinogenesis might be associated with reduction in cellular proliferation and induction of apoptosis, as seen in our analysis (Fig. 1, Supplementary Fig. 3). Different *in-vitro* and *in-vivo* studies also indicated the important roles of the tea polyphenols in reduction of cellular proliferation and induction of apoptosis in prevention of different cancers [22,28]. In addition, significant reduction in expressions of AFP and CD44 in different treated groups indicates that EGCG/TF could reduce the proliferation of hepatocyte progenitor cells and stem cells respectively in restriction of the liver carcinogenesis (Fig. 2). It was evident that CD44 expression was strongly correlated with AFP expression in HCC [3]. Studies also indicated that AFP producing pancreatic cancer cells showed high self-renewal efficacy, increased tumorigenic potential as well as increased drug resistance than AFP non producing cells [29]. On the other hand, studies indicated that EGCG either alone or in combination with chemotherapeutic drug like docetaxel could inhibit CD44-positive stem cell population and self-renewal capacity resulting reduction in cell proliferation and enhance drug sensitivity in oral cancer and prostrate cancer cells [30,31]. This indicates that EGCG/TF treatment could modulate hepatocyte progenitor cell and stem cell population in restriction of liver carcinogenesis.

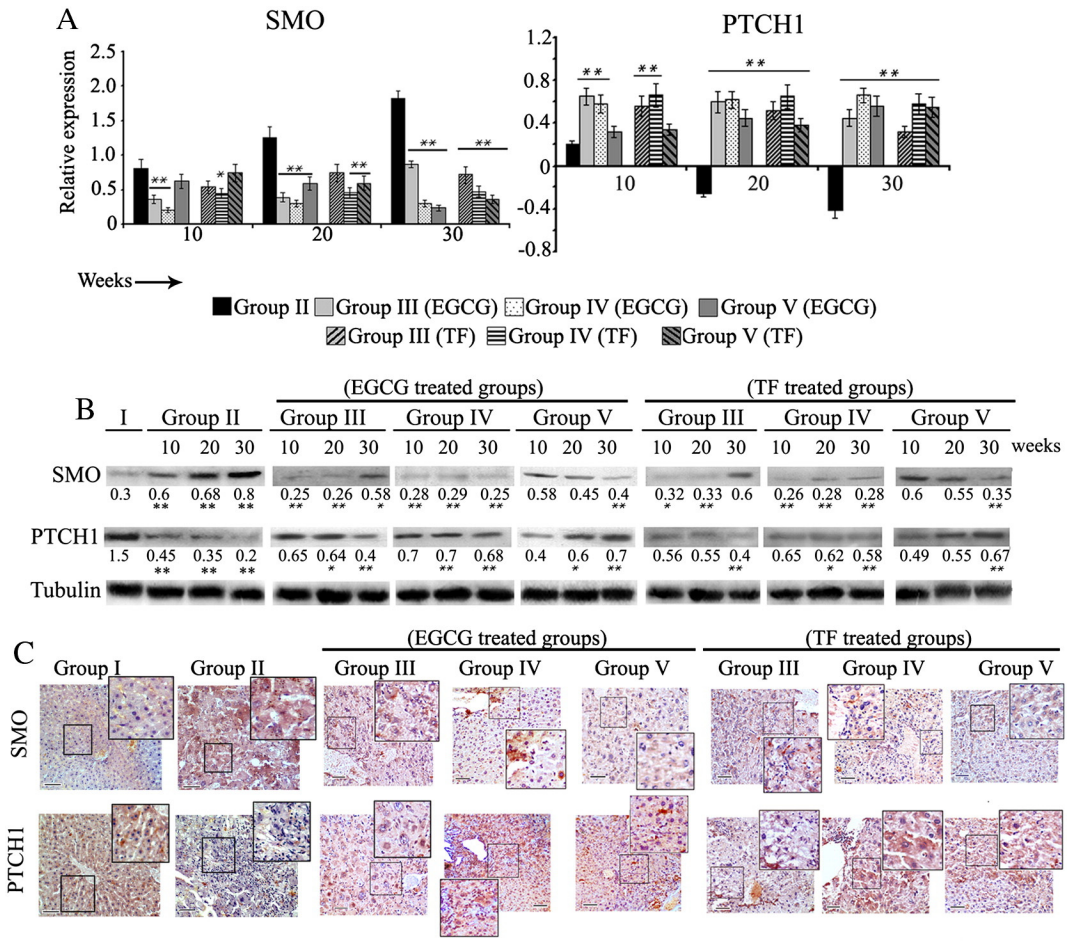

Fig. 6. Effect of EGCG and TF on expressions of Hedgehog pathway key regulatory genes SMO and PTCH1 during liver carcinogenesis. (A) Relative mRNA expression of SMO and PTCH1 in different groups at different weeks analyzed by quantitative RT-PCR. Mouse B2M was used as an endogeneous control and for target gene normalization. Data presented as mean±S.D. (B) Western blot analysis of SMO and PTCH1 in different groups at 10th, 20th and 30th weeks. α-Tubulin (Tubulin) used as loading control. Relative peak density was normalized with loading control. (C) Representative photographs of immunohistochemical expressions of SMO and PTCH1 in the liver sections of different groups with/without EGCG and TF at 30th week. Magnifications 20×. Inset magnifications 40×. Scale bar represents 50 μm. *Represents significant *P* value in Group II with respect to Group I and * in Group III–V with respect to Group II (*/**P*<.05 and **/***P*<.001).

The low prevalence of the hepatocyte stem cells might be due to the changes in self-renewal pathways particularly Wnt and Hh pathways. In Wnt pathway, the effector protein β-catenin regulates the expression of CD44 [32]. Down-regulation (RNA/protein) of β-catenin was seen due to EGCG/TF treatment in different groups (Fig. 3). The reduced mRNA expression of β-catenin might be due to transcriptional modulation or inhibition of mRNA stabilization by EGCG/TF [33,34]. On the other hand, the reduced β-catenin protein expression might be due to its cytoplasmic destabilization by up-regulation of antagonists' sFRP1 and APC as seen in our study (Fig. 4). It was evident that ectopic over expression of sFRP1/APC or restoration of their expressions from hypermethylated states by different DNA methylation inhibitory drugs could reduce β-catenin protein expression in different cancers including HCC [7,10,11,35]. Moreover, studies also indicated that EGCG could up-regulate sFRP1 expression resulting inhibition of Wnt signaling in hepatoblastoma cells [36]. Thus, up-regulation of sFRP1/APC by EGCG and TF might be important in restriction of the carcinogenesis. In parallel, the reduced nuclear expression of activated β-catenin (phospho β-catenin-Y654) by EGCG/TF seen in different treated groups (Fig. 3B, C and E) might be due to destabilization of β-catenin or inhibition of β-catenin phosphorylation by down-regulation of EGFR expression as evident in our study during the restriction (Fig. 7, Supplementary Fig. 5, Table 2) [37,38]. Thus, both the polyphenols might modulate the Wnt pathway by regulating β-catenin/activated β-catenin expression in restriction of the liver carcinogenesis.

The changes in Wnt pathway due to EGCG/TF treatment might affect the self-renewal Hedgehog pathway (Hh) for their co-operative signaling as evident in development and cancers [5,39–42]. Besides different other studies with EGCG in different cancers [5,23], our study could clearly indicate the down-regulation of the effector genes Gli1 expression (mRNA/protein) by both EGCG and TF during restriction of the liver carcinogenesis (Fig. 5). Studies indicated that targeted inhibition of Gli1 by siRNA or small molecule inhibitors could inhibit Hh signaling resulting growth inhibition, and increased apoptosis in pancreatic and prostrate cancer models [43,44]. However, the reduced Gli1 expression by EGCG/TF might be due to modulation of its upstream signaling molecules as evident with decreased SMO expression and increased PTCH1 expression during the restriction (Fig. 6). It was evident that, inactivation of SMO by cyclopamine or re-expression of PTCH1 from its hypermethylated state by promoter demethylating agent could inhibit Hh pathway resulting down-regulation of Gli1 expression [45,46]. Moreover, studies also indicated that EGCG could inhibit Hh pathway components like SMO, Gli1 as well as Gli1 transcriptional activity resulting reduced proliferation, increased apoptosis and decreased tumorigenic potential of pancreatic

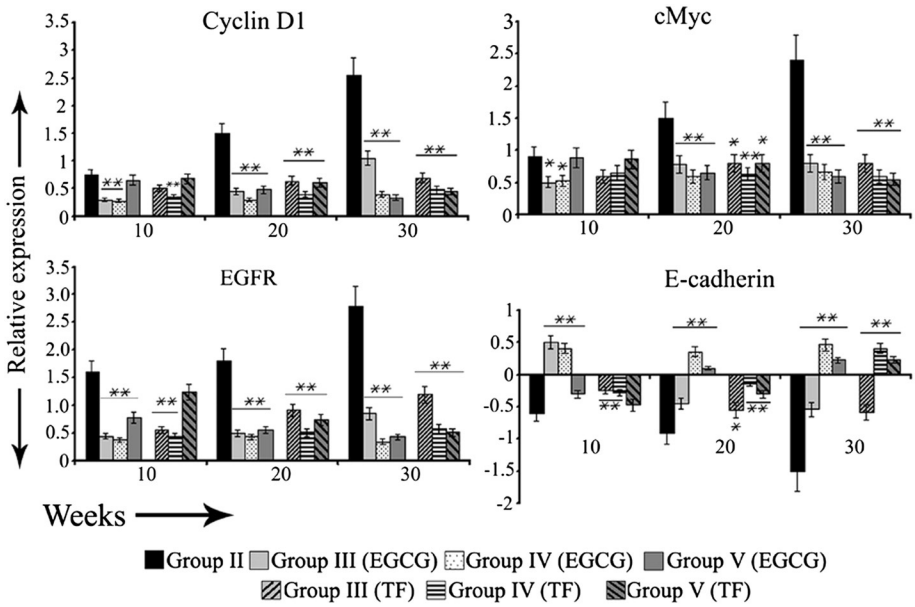

Fig. 7. Effect of EGCG and TF on expressions of some Wnt and hedgehog (Hh) pathways associated genes during liver carcinogenesis. Relative mRNA expression of Cyclin D1, cMyc, EGFR and E-cadherin in different groups at different weeks analyzed by quantitative RT-PCR. Mouse B2M was used as an endogeneous control and for target gene normalization. Data presented as mean±S.D. *Represents significant *P* value in Group III–V with respect to Group II (*$P<.05$ and **$P<.001$).

cancer cells [23]. In addition, reduced Gli1 expression by EGCG/TF might be due to its mRNA destabilization associated with β-catenin expression [39,42]. Moreover, the down-regulation of Gli1 might be associated with reduced expression of EGFR (Fig. 7) in SMO independent pathway [47,48]. Thus, down-regulation of Gli1 is an important key event in restriction of liver carcinogenesis by EGCG/TF.

It seems that the reduced expressions of Wnt/Hh pathways target genes like Cyclin D1, cMyc, EGFR/activated EGFR (phospho-EGFR-Y-1173) along with up-regulation of E-cadherin seen during the restriction (Fig. 7, Supplementary Fig. 5, Table 2) might be due to reduced expression of β-catenin and Gli1 by EGCG/TF. Similar to our study, different studies also indicated the importance of tea polyphenols mainly EGCG on expressions of these genes in different cancers, resulting modulation in their respective signaling pathways like cell cycle, cell signaling, epithelium to mesenchymal transition etc. [28]. Thus, the modulation of Wnt and Hh pathways by both EGCG and TF might be important not only in regulation of stem cell population, but also in regulation of different cellular pathways to restrict the liver carcinogenesis.

Overall, our data indicate that EGCG/TF continuous treatment followed by pre-treatment showed potential chemopreventive effect and post treatment protocol showed therapeutic efficacy to restrict CCL_4/NDEA induced mouse liver carcinogenesis. This restriction was associated with decreased cellular proliferation, increased apoptosis along with reduced prevalence of hepatocyte progenitor cell (AFP) and stem cell population (CD44) irrespective of EGCG and TF treatment. Importantly, EGCG and TF treatment could modulate the expressions of Wnt/Hh pathway effector genes, i.e., β-catenin and Gli1 (respectively), to regulate the expressions of their target genes associated with different cellular pathways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.08.016.

Conflicts of interest

The authors declared that they have no conflict of interest.

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