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Tea polyphenols EGCG and TF restrict tongue and liver carcinogenesis simultaneously induced by N-nitrosodiethylamine in mice

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ABSTRACT

The aim of this study is to understand the molecular mechanisms of N-nitrosodiethylamine (NDEA) induced multi-organ carcinogenesis in tongue and liver of the same mouse and restriction of carcinogenesis by Epigallocatechin gallate (EGCG) and Theaflavin (TF), if any. For that purpose, cellular proliferation/apoptosis, prevalence of CD44 positive stem cell population and expressions of some key regulatory genes of self renewal Wnt and Hedgehog (Hh) pathways and some of their associated genes were analyzed in the NDEA induced tongue and liver lesions in absence or presence of EGCG/TF. Chronic NDEA exposure in oral cavity could decrease mice body weights and induce tongue and liver carcinogenesis with similar histological stages (severe dysplasia up to 30th weeks of NDEA administration). Increasing mice body weights were seen in continuous and post EGCG/TF treated groups. EGCG/TF treatment could restrict both the carcinogenesis at similar histological stages showing potential chemopreventive effect in continuous treated groups (mild dysplasia) followed by pre treatment (moderate dysplasia) and therapeutic efficacy in post treated groups (mild dysplasia) up to 30th week. The mechanism of carcinogenesis by NDEA and restriction by the EGCG/TF in both tongue and liver were similar and found to be associated with modulation in cellular proliferation/apoptosis and prevalence of CD44 positive population. The up-regulation of self renewal Wnt/β-catenin, Hh/Gli1 pathways and their associated genes Cyclin D1, cMyc and EGFR along with down regulation of E-cadherin seen during the carcinogenesis processes were found to be modulated during the restriction processes by EGCG/TF.

1. Introduction

Chronic tobacco use is one of the main etiological factors associated with cancers in multiple organs like lung, oral cavity, liver etc due to presence of many carcinogenic compounds including Benzo[a]pyrene, N-nitrosoamines etc (Aguiló et al., 2008; Pfeifer et al., 2002). It was observed that, administration of tobacco associated carcinogen N-nitrosodiethylamine (NDEA) through various routes could induce carcinogenesis in multiple organs that are extensively exposed to the carcinogen, associated with carcinogen metabolism and excretion of the animal (Herrold and Dunham, 1963; Herrold, 1964). It was documented in different experimental animal models, where intra-peritoneal/intra-dermal/intra-tracheal/intra-gastric administration of NDEA either as a single carcinogen or in combination with other carcinogens could induce carcinogenesis initially in lungs/nasal cavity followed by in liver, stomach and kidney (Herrold and Dunham, 1963; Herrold, 1964; Kim et al., 1997; Wang et al., 1992). However, the molecular mechanism of such multi-organ tumorigenesis is not known clearly. Oral cavity is the primary exposure site of tobacco and liver is the main xenobiotic metabolizing organ. But, how oral administration of NDEA induce carcinogenesis in its primary exposure site i.e. in oral cavity as well as in liver of the same set animals is not known.

It was evident from our previous study that intra-peritoneal administration of CCl_4/NDEA could increase CD44 positive cancer stem cell (CSC) population along with activation of self renewal Wnt, Hedgehog (Hh) pathways in the liver lesions during different stages of carcinogenesis (Sur et al., 2015). Activation of Wnt and Hh pathways were also observed in NDEA (intra-peritoneal) induced mouse/rat liver cancer model and Mdr2(−/−) liver cancer transgenic mouse model respectively (Khan et al., 2011; Mercer et al., 2014; Philips et al., 2011). Oral administration of 4-nitroquinoline-1-oxide (4NQO) was shown to activate Wnt and Hh pathways during tongue carcinogenesis (de Oliveira Santos et al., 2014; Osei-Sarfo et al., 2013).

Abbreviations: EGCG, epigallocatechin gallate; TF, theaflavine; NDEA, N-nitrosodiethylamine; 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); EGFR, epidermal growth factor receptor.

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Drinking green tea catechin was found to differentially reduce tumor incidences in lung, fore stomach, small intestine and liver in multi-organ carcinogenesis model of rat, though the mechanism was not clear (Hirose et al., 1993; Wang et al., 1992). Our previous study had documented that oral administration of tea polyphenols Epigallocatechin gallate (EGCG) and Theaflavin (TF) restricted the CCl_4/NDEA (intraperitoneal) induced mouse liver carcinogenesis by reducing the prevalence of CD44 positive CSCs and activity of self renewal Wnt/Hh pathways (Sur et al., 2015). Other reports have also demonstrated that oral administration of tea and its polyphenols mainly EGCG could prevent NDEA induced (intraperitoneal) liver carcinogenesis and 4NQO induced tongue carcinogenesis in rat model separately (Srinivasan et al., 2008; Sumi et al., 2013). But how the oral administration of EGCG and TF modulate the oral and liver carcinogenesis in the same set of animals is unknown.

The present study was undertaken to explore how oral administration of NDEA affect oral cavity particularly tongue as well as liver in the same set of animals and the effect of tea polyphenols EGCG/TF during tongue and liver carcinogenesis. We have therefore, analyzed first the pattern of oral NDEA induced tongue and liver carcinogenesis in mice in absence or presence of EGCG/TF and then tried to understand the underlying mechanism of actions by analyzing: (i) *in-situ* cellular proliferation and apoptosis (ii) determination of prevalence of CD44 positive population and (iii) expression of some key regulatory genes in the Wnt and Hh pathways as well as some of their key regulated genes. Our observation clearly revealed a same pattern of tongue and liver carcinogenesis by NDEA and its restriction by EGCG and TF. The mechanisms of carcinogenesis and restriction were found to be similar too in both the organs.

2. Materials and methods

2.1. Reagents

Epigallocatechin gallate (EGCG; 95%), Theaflavin (TF, >80%) and N-nitrosodiethylamine (NDEA) were obtained from Sigma-Aldrich, St. Louis, MO, USA. 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, Texas, USA.

2.2. Experimental animal

Female Swiss albino mice were obtained from the 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 was 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 the institutional ethical committee.

2.3. Experimental design

Experimental design was adapted from our previous study (Sur et al., 2015). Female Swiss albino mice (5–6 weeks) with average body weight 20–25 g were divided into following experimental groups, having 12 mice in each group:

Group I (normal control group): Mice without any treatment.

Group II (carcinogen control group): Mice of this group received N-Nitrosodiethylamine (NDEA) (10 μl) into the oral cavity at 75 mg/kg body weight weekly for three successive weeks and 100 mg/kg body weight continued till end of the experiment. Before and after NDEA application mice were restrained from drinking for 1 h. The dose selection of NDEA was based on our previous study (Pal et al., 2012).

Group III (pre-treatment group): Mice of this group received oral administration of tea polyphenols (EGCG/TF) daily for 15 days only, prior to carcinogen administration.

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

Group V (post treatment group): Mice in this group received oral administration of EGCG/TF daily from 6th week of carcinogen application and continued till the end of the experiment.

Doses of EGCG (aqueous solution of 8 μg/kg body weight) and TF (aqueous solution of 10 μg/kg body weight) were selected based on our previous mouse serum toxicity analysis for oral administration (Sur et al., 2015). Mice from different experimental groups were sacrificed at 10th, 20th and 30th weeks of first NDEA administration. At each time point three animals were sacrificed from each group. All the experiments were repeated once. After sacrifice, tongue and liver tissues from the same mouse were dissected out and divided into different parts. One part was fixed in formalin and paraffin sections were prepared for histopathological analysis, immunohistochemistry and *in-situ* apoptosis analysis. The other fresh tissue parts were used for RNA/protein isolation and *in-situ* proliferation assay. Three samples from prevalent/advanced histological stages were used for the different analysis.

2.4. Histopathological analysis

Tongue and liver tissue samples from different groups were fixed in 10% phosphate buffered formalin, embedded in paraffin and 4–5 μm thick tongue and liver sections were stained by hematoxylin and eosin (HE) for routine histopathological analysis according to standard protocol (Pal et al., 2012).

2.5. In situ cell proliferation and apoptosis assay

Cellular proliferation and apoptosis in the tongue and liver sections of different groups were determined using BrdU labeling and detection kit II and *in situ* cell death detection kit (TUNEL assay) respectively, according to manufacturers' protocol as described previously (Manna et al., 2009). The percentage of BrdU/TUNEL positive cells were determined from labeled nuclei with respect to the total number of nuclei counted at 5–10 randomly chosen microscopic fields.

2.6. Quantitative RT-PCR analysis

Total tissue RNA was extracted from tongue and liver samples by TRIzol reagent followed by cDNA synthesis from the 5 μg of total RNA with Super Script III Reverse Transcriptase (Invitrogen/Life technology, USA) following the manufacturer's protocol. Gene expression was carried out by Real-time PCR (ABI Prism 7500, Life Technology, Massachusetts) 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 (Pal et al., 2012). Mouse β_2-microglobulin gene (B2M) was used as an endogenous control and for target gene normalization. Each sample was loaded in triplicate. Relative expression was graphically represented.

2.7. Protein extraction and Western blot analysis

Protein extraction and Western blot was performed as described earlier (Pal et al., 2012). Protein was extracted from the freshly operated tongue and liver tissues by sonication with extraction buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% Triton-X 100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF and 1 mM sodium

orthovanadate] followed by quantification according to Lowry's method and aliquots were stored at −80 °C. Equal amount of proteins were separated by 10–12% SDS-PAGE and then transferred to polyvinylidine difluoride membrane (PVDF: Millipore, MA). Membranes were incubated with 3–5% non fat dry milk for 1–2 h at room temperature for blocking followed by overnight incubation at 4 °C with desired primary antibodies (1:200–1:1000; Supplementary Table 2) and then with corresponding HRP-conjugated secondary antibodies (1:1000–1:5000; Supplementary Table 2). The target protein bands were then visualized using luminol reagent and autoradiographed on X-ray film (Kodak, Rochester, NY, USA). The band intensities were quantified using densitometric scanner (Bio Rad GS-800, Hercules, California, USA). Peak densities of the proteins of interest were normalized using peak density of loading control α-Tubulin.

2.8. Immunohistochemical (IHC) analysis

IHC analysis was performed on the tongue and liver sections as described previously (Pal et al., 2012). Paraffin sections of tongue and liver were processed for deparaffinization by xylene 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 followed by incubation with desired primary antibody (1:50–1:100; Supplementary Table 2), over night at 4 °C and with HRP conjugated secondary antibody (1:500–1:1000; Supplementary Table 2) for 2 h at room temperature. For colour substrate reaction diaminobenzidine (DAB) was used after incubation with secondary antibody followed by counterstaining with hematoxylin. Negative control slides (without primary antibody) were prepared in the same way as described above except the use of PBS instead of primary antibody. The expression pattern of a particular protein in tongue and liver lesions of Group II–V was compared with the expression in basal layer of normal tongue and central vein centric region in normal liver respectively (Group I). Expression pattern was scored according to the Perrone et al. (2006) with some modifications as described previously (Pal et al., 2012; Perrone et al., 2006).

2.9. Statistical analysis

Data obtained from Group II was compared with Group I and data obtained from Group III, Group IV and Group V were compared with Group II. Statistical analysis was performed using t-test. P-value < 0.05 was considered as statistically significant. Data were expressed as mean with standard deviation (SD).

3. Results

3.1. Analysis of pathological and histological changes in tongue and liver lesions in different experimental groups

First detectable changes encountered following oral NDEA administration and EGCG/TF treatment were with respect to body weight of the experimental animals. In carcinogen control mice (Group II), gradual decrease in body weights were evident up to 30th weeks compared to increasing body weights in normal mice (Group I) (Supplementary Fig. 1). On the other hand, EGCG/TF pre-treatment group (Group III) initially showed quite stable body weights but from 20th week onwards there was gradual fall. Interestingly, in continuous (Group IV) and post treatment (Group V) groups increasing body weights were evident mainly from 10th week onwards (Supplementary Fig. 1).

Macroscopically, no visible changes were seen in tongues of different groups following chronic NDEA exposure, but prominent macroscopic changes were noted in the livers of Group II mice. Rough liver surface was seen in Group II at 10th–20th weeks and foci formation at 30th week compared to smooth surface in normal liver (Group I) (Fig. 1A). However, no remarkable changes persisted in livers in the EGCG/TF treated groups (Group III–V), although slight rough liver surface was noted at 10th week in Group V and at 30th week in Group III (data not shown).

Histological analysis clearly indicated tongue carcinogenesis with moderate dysplasia (83%) at 10th and 20th weeks followed by severe dysplasia (67%) at 30th week in Group II (Table 1, Fig. 1B). Similar histological stages were observed in the livers too with moderate dysplastic changes (100%) at 10th and 20th weeks followed by severe dysplasia (67%) at 30th week, indicating similar pattern of carcinogenesis in both the organs (Table 1, Fig. 1B).

Pre-treatment with EGCG/TF (Group III) could restrict the tongue carcinogenesis at mild dysplastic stages at 10th week followed by moderate dysplastia at 30th week (Table 1, Fig. 1B). While, continuous treatment (Group IV) with these compounds resulted in better chemopreventive effect with restriction of the carcinogenesis at mild dysplasia at all the time points. In post treated Group V, mild to moderate dysplasia appeared in tongue from 10th week, which was downstaged to mild dysplasia at 30th week (Table 1, Fig. 1B).

Similar histological observations were evident in liver sections of the corresponding mice (Table 1, Fig. 1B). EGCG and TF treatment could restrict the liver carcinogenesis showing better chemopreventive action in the continuous treated Group IV (mild dysplasia) in comparison to the pre-treated Group III (moderate dysplasia) up to 30th week (Table 1, Fig. 1B). On the other hand, in the post treatment protocol (Group V) restriction of carcinogenesis at mild dysplastic stages up to 30th week indicates importance of EGCG/TF in chemotherapy also (Table 1, Fig. 1B).

3.2. Analysis of cellular proliferation and apoptosis during tongue and liver carcinogenesis with/without EGCG/TF

During tongue carcinogenesis, significant increased ($P < 0.05$) proliferation was evident in Group II at 10th week (54%) followed by gradual increase up to 30th week (80%) than Group I (29–30%) (Fig. 1C, Supplementary Fig. 2). The BrdU incorporated cells were found to be located throughout the dysplastic zone in Group II compared to localization in basal layer of normal tongue. Treatment with EGCG and TF could reduce the percentage of proliferating cells in all the treated groups (III–V) at all the time points with significant decrease at 30th week, except in Group V at 10th week (Fig. 1C, Supplementary Fig. 2). However, this reduction was more prominent in Group IV and V than Group III at 30th week.

Similarly, in liver significant increase in ($P < 0.05$) proliferation was evident in Group II from 10th week (55%) to 30th week (82%) compared to normal liver (Group I) (20–22%) (Fig. 1C, Supplementary Fig. 2). The BrdU positive cells were detected throughout the liver lesions of Group II compared to central vein centric localization in normal liver (Group I). Here also, EGCG and TF treatment reduced proliferation in all the treated groups (Group III–V) at all the time points than Group II (with significant decrease at 30th week), except in Group V at 10th week (Fig. 1C, Supplementary Fig. 2). However, this reduction was more prominent in Group IV and V than Group III at 30th week.

Gradual decrease in percentage of apoptotic cells was seen during tongue carcinogenesis in Group II with significant decrease ($P < 0.05$) at 20th and 30th week (3.6%) than Group I (20–24%) (Fig. 1D, Supplementary Fig. 2). EGCG and TF treatment could significantly increase ($P < 0.05$) the percentage of apoptosis in all the treated groups (Group III–V) at different time points than Group II, except in Group V at 10th week (Fig. 1D, Supplementary Fig. 2).

Similar trend in gradual reduction of apoptosis was also seen in the liver lesions in Group II mice from 10th (16%) to 30th week (7.2%) compared to normal liver (10–12%) (Fig. 1D, Supplementary Fig. 2). The restriction of the carcinogenesis by EGCG/TF treatment was accompanied by significant increase in ($P < 0.05$) percentage of apoptotic cells in all the treated groups (Group III–V) at different time points than Group II, except in Group V (EGCG/TF) and Group III (TF) at 10th week

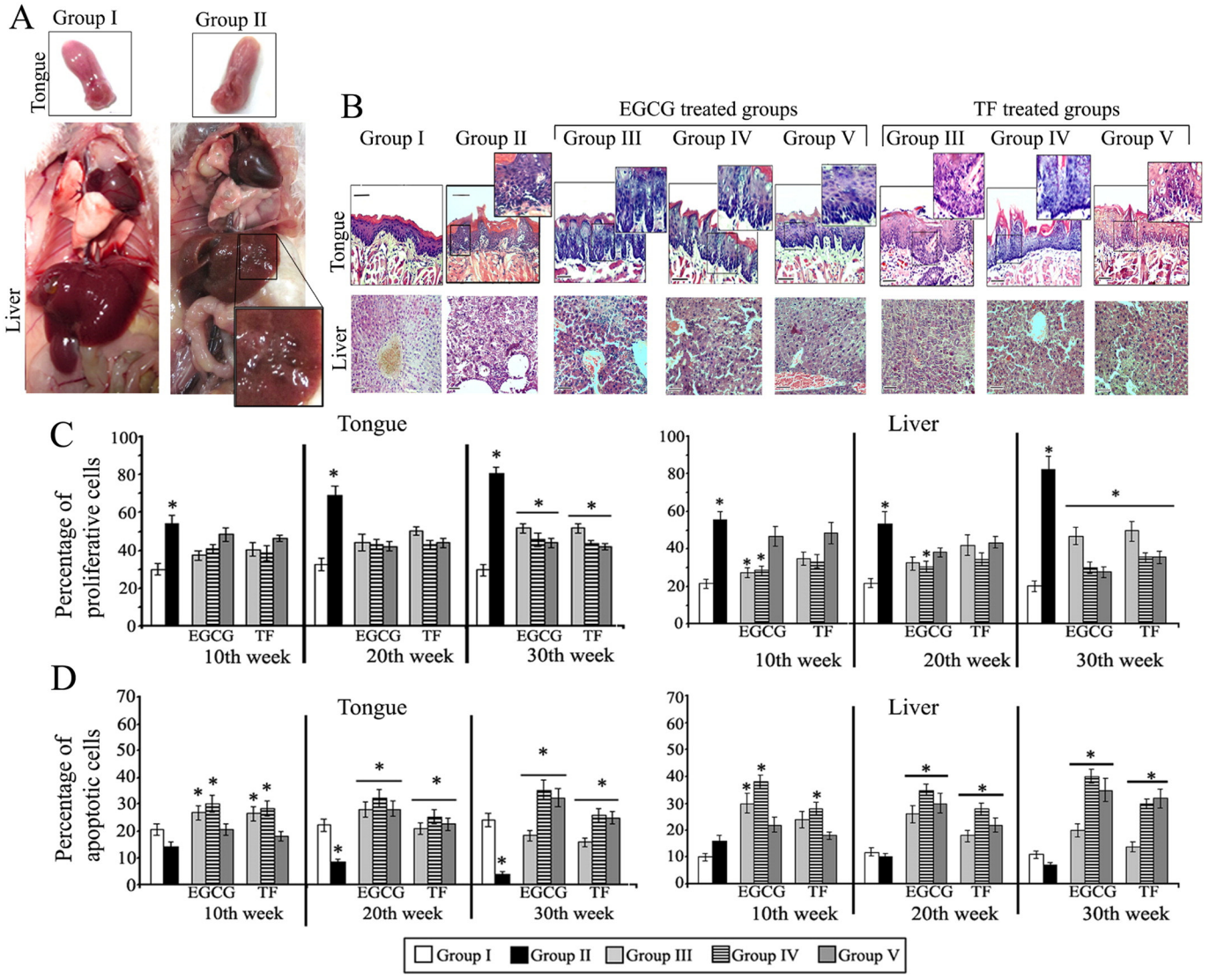

Fig. 1. NDEA induced multi-organ carcinogenesis in tongue and liver in absence or presence of EGCG/TF in same mice. (A) Macroscopic observations of tongue and liver in normal mouse (Group I) and carcinogen control mouse (Group II) at 30th week of oral NDEA administration. (B) Representative histological images of tongue and liver sections stained by hematoxylin–eosin in different groups at 30th week. Magnification 20×. Scale bar represents 50 μm. (C) Graphical representation of percentage of proliferating cells/BrdU incorporated cells and (D) apoptotic cells/TUNEL positive cells in tongue and liver sections of different groups at different time points. Data presented as mean ± SD. *Represents significant *P* value ($P < 0.05$) in Group II with respect to Group I and in Group III–V with respect to Group II.

(Fig. 1D, Supplementary Fig. 2). However, the induction of apoptosis was more prominent in Group IV and V than Group III at 30th week.

3.3. Prevalence of CD44 positive stem cell population during tongue and liver carcinogenesis with/without EGCG/TF

To understand the mechanism of both tongue and liver carcinogenesis in same mouse as well as the action of tea polyphenols during the restriction process, expression and prevalence of CD44 positive population was checked in the tongue and liver lesions. Western blot analysis showed gradual and significant increase in CD44 expression ($P < 0.05$) in the tongue lesions of Group II at different time points than Group I (Fig. 2A). Interestingly, both the tea polyphenols could significantly reduce ($P < 0.05$) its expression in all the treated groups (Group III–V) at different time points, except in Group V at 10th week (Fig. 2A). Immunohistochemical (IHC) analysis revealed ~10–12% of CD44 positive cells in basal layer of normal tongue (Group I) (Fig. 2B, C). In comparison with Group I, significant increase ($P < 0.05$) in CD44 positive population was detected in tongue lesions of Group II at 10th week (60%) followed by gradual increase up to 30th week (80%). EGCG treatment significantly reduced ($P < 0.05$) CD44 positive populations in all the treated groups (Group III–V) at different time points, except in Group V at 10th week (Fig. 2B, C). Similar trend was seen in TF treated groups with significant decrease ($P < 0.05$) in all the groups mainly at 30th week (Fig. 2B, C). However, at 30th week this reduction was more prominent in Group IV and V irrespective of EGCG/TF treatment.

Western blot analysis of liver lesions showed similar trend of increased ($P < 0.05$) CD44 expression during liver carcinogenesis in Group II and reduced expression in the EGCG and TF treated groups (Group III–V) (Fig. 2A). Similarly, IHC analysis showed increased ($P < 0.05$) CD44 positive cells in liver lesions of Group II at 10th week (12%) followed by gradual increase up to 30th week (22%) in comparison to the normal liver (Group I; ~3%) (Fig. 2D, E). Both EGCG and TF significantly reduced ($P < 0.05$) CD44 positive cells in all the treated groups at different time points, except in Group V at 10th week (EGCG/TF) and 20th week (TF) (Fig. 2D, E). However, at 30th week this reduction was more prominent in Group IV and V. Thus, the observation indicates that modulation in CD44 positive population might be associated with NDEA induced tongue and liver carcinogenesis and EGCG/TF mediated restriction.

Table 1
Histopathological observations of tongues and livers in same set mice of different experimental groups at different time points.

Groups	10th week		20th week	30th week
Histological observations of tongues				
Normal control group	Group I	Normal	Normal	Normal
Carcinogen control group	Group II	Mild (1/6; 16.7%), Mod (5/6; 83.3%)	Mild (1/6; 16.7%), Mod (5/6; 83.3%)	Mod (2/6; 33.3%), Sev (4/6; 66.7%)
EGCG treated groups	Group III	Mild (5/6; 83.3%), Mod (1/6; 16.7%)	Mild (4/6; 66.7%), Mod (2/6; 33.3%)	Mild (2/6; 33.3%), Mod (4/6; 66.7%)
	Group IV	Mild (6/6; 100%)	Mild (6/6; 100%)	Mild (6/6; 100%)
	Group V	Mild (4/6; 66.7%), Mod (2/6; 33.3%)	Mild (4/6; 66.7%), Mod (2/6; 33.3%)	Mild (6/6; 100%)
TF treated groups	Group III	Mild (4/6; 66.7%), Mod (2/6; 33.3%)	Mild (2/6; 33.3%), Mod (4/6; 66.7%)	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	Mild (1/6; 16.7%), Mod (5/6; 83.3%)	Mild (4/6; 66.7%), Mod (2/6; 33.3%)	Mild (4/6; 66.7%), Mod (2/6; 33.3%)
Histological observations of livers				
Normal control group	Group I	Normal	Normal	Normal
Carcinogen control group	Group II	Mod (6/6; 100%)	Mod (6/6; 100%)	Sev (4/6; 66.7%), Mod (2/6; 33.3%)
EGCG treated groups	Group III	Mild (6/6; 100%)	Mild (4/6; 66.7%; 66.7%), Mod (2/6; 33.3%)	Mod (6/6; 100%)
	Group IV	Mild (6/6; 100%)	Mild (6/6; 100%)	Mild (6/6; 100%)
	Group V	Mild (2/6; 33.3%), Mod (4/6; 66.7%; 66.7%)	Mild (4/6; 66.7%; 66.7%), Mod (2/6; 33.3%)	Mild (6/6; 100%)
TF treated groups	Group III	Mild (6/6; 100%)	Mild (4/6; 66.7%; 66.7%), Mod (2/6; 33.3%)	Mod (6/6; 100%)
	Group IV	Mild (6/6; 100%)	Mild (6/6; 100%)	Mild (4/6; 66.7%), Mod (2/6; 33.3%)
	Group V	Mod (6/6; 100%)	Mild (4/6; 66.7%; 66.7%), Mod (2/6; 33.3%)	Mild (5/6; 83.3%), Mod (1/6; 16.7%)

Mild: mild dysplasia; Mod: moderate dysplasia; Sev: severe dysplasia.

3.4. Analysis of self renewal Wnt pathway during tongue and liver carcinogenesis with/without EGCG/TF

To understand the molecular mechanism of tongue and liver carcinogenesis and restriction by EGCG/TF on self renewal Wnt pathway, expressions (mRNA/protein) of effector gene β-catenin along with pathway antagonists' sFRP1 and APC were analyzed in the tongue and liver lesions of different groups.

During tongue carcinogenesis, significant increase ($P < 0.05$) in mRNA expression of the β-catenin was seen in Group II at 10th week followed by gradual increase up to 30th week as compared to normal tongue (Group I) (Fig. 3A). EGCG treatment significantly reduced ($P < 0.05$) its expression in all the treated groups (Group III–V) at different time points, except in Group V at 10th week. TF treatment reduced its expression in all the treated groups only at 20th and 30th weeks, except in Group III at 20th week (Fig. 3A). This reduction was more prominent at 30th week in Group IV and V. Western blot analysis of the β-catenin showed concordance with respective mRNA expression in different groups during the carcinogenesis and following EGCG/TF treatment (Fig. 3B).

Similarly, IHC analysis showed high nuclear and membrane/cytoplasmic expression ($P < 0.05$) of β-catenin in the tongue lesions of Group II during carcinogenesis compared to low expression in basal layer of normal tongue (Group I) (Fig. 3C, Supplementary Table 3, Supplementary Fig. 3). In case of Group III and IV, low nuclear and membrane/cytoplasmic ($P < 0.05$ than Group II) expression was evident in the tongue lesions at 10th week, whereas high expression was noted in Group V at 10th week (Fig. 3C, Supplementary Table 3, Supplementary Fig. 3). However, during the restriction, its expression remained low in Group IV, but gradually increased in Group III and decreased gradually in Group V (Fig. 3C, Supplementary Table 3, Supplementary Fig. 3).

Gradual decrease in mRNA expressions of sFRP1 and APC were seen in the tongue lesions of Group II up to 30th week (Fig. 3A).Interestingly, EGCG and TF treatment could increase the mRNA expressions in all the treated groups (Group III–V) up to 30th week (Fig. 3A). However, the increased expression of sFRP1 and APC was more prominent in Group IV and V at 30th week. Similarly, IHC analysis showed low cytoplasmic expressions of sFRP1 and APC in the tongue lesions of Group II compared to high expression in normal tongue (Group I) up to 30th week (Fig. 3C, Supplementary Table 3). High cytoplasmic expressions of sFRP1 and APC were seen in Group III–IV along with low–moderate expression in Group V at 10th week (Fig. 3C, Supplementary Table 3). However, during the restriction by EGCG/TF, their expressions remained high in Group IV but gradually reduced in Group III and increased in Group V up to 30th week (Fig. 3C, Supplementary Table 3).

Similarly, in case of liver lesions significant increase in ($P < 0.05$) mRNA expression of β-catenin was evident in Group II during carcinogenesis and reduced expression was seen in Group III–V due to EGCG and TF treatment (Fig. 4A). Western blot analysis in the liver lesions also showed concordance with mRNA expression in respective groups (Fig. 4B). Likewise, IHC analysis showed high nuclear and membrane/cytoplasmic expression ($P < 0.05$) of the protein in the liver lesions of Group II during carcinogenesis compared to low expression of normal liver (Group I) (Fig. 4C, Supplementary Table 4, Supplementary Fig. 3). EGCG and TF treatment could reduce its nuclear as well as membrane/cytoplasmic expression in different treated groups compared to Group II (Fig. 4C, Supplementary Table 4, Supplementary Fig. 3). However, during the restriction this reduction was more prominent ($P < 0.05$) in Group IV and V at 30th week (Fig. 4C, Supplementary Table 4, Supplementary Fig. 3).

In expression analysis of sFRP1 and APC in the liver lesions, similar trend of gradual reduction in their mRNA expressions were evident during liver carcinogenesis in Group II and significant up-regulation ($P < 0.05$) were seen in different EGCG/TF treated groups (Group III–V) up to 30th weeks (Fig. 4A). IHC analysis showed high cytoplasmic expression of sFRP1 and APC in normal liver (Group I) (Fig. 4C, Supplementary Table 4). In Group II, low sFRP1 expression was evident during the carcinogenesis, whereas moderate (10th week) to low (30th week)

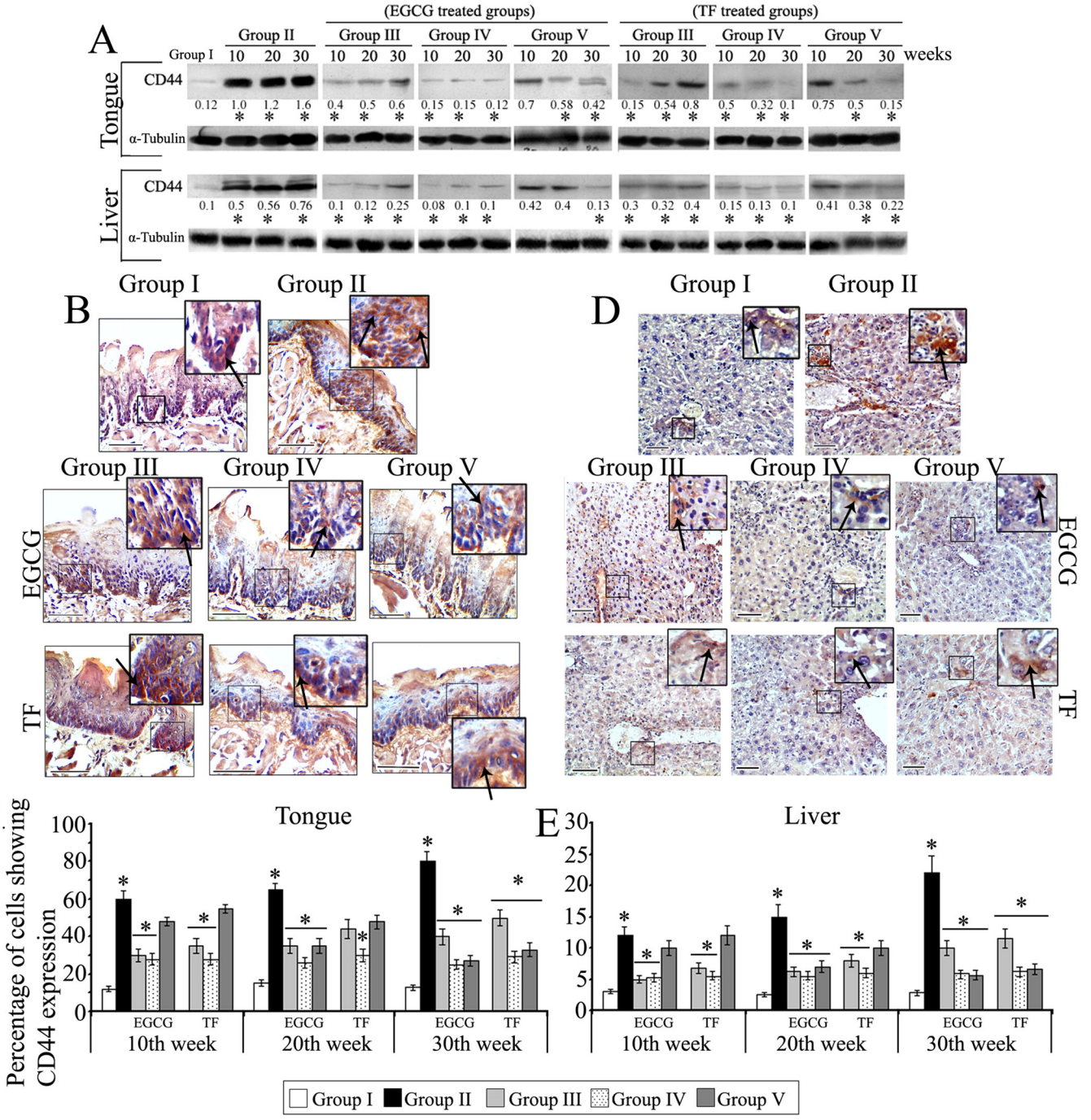

Fig. 2. Prevalence of CD44 positive population during tongue and liver carcinogenesis with/without EGCG/TF. (A) Western blot analysis of CD44 in tongue and liver tissues of different groups at 10th, 20th and 30th weeks. α-Tubulin used as loading control. Relative peak density was normalized with loading control. (B) Representative photographs of immunohistochemical expressions of CD44 in tongue sections of different groups at 30th week. Magnifications 40×. Scale bar represents 50 μm. Arrows indicate CD44 positive cells. (C) Graphical representation of percentage of CD44 positive cells observed by IHC analysis in different groups at different time points. (D) Representative photographs of immunohistochemical expressions of CD44 in liver sections of different groups in the corresponding mice at 30th week. Magnifications 20×. Scale bar represents 50 μm. Arrows indicate CD44 positive cells. (E) Graphical representation of percentage of CD44 positive cells in the liver sections of different groups at different weeks. Data presented as mean ± SD. *Represents significant *P* value ($P < 0.05$) in Group II with respect to Group I and in Group III–V with respect to Group II.

expression of APC was noted in the liver lesions (Fig. 4C, Supplementary Table 4). High cytoplasmic expressions of sFRP1 and APC were seen in the liver lesions of Group III–IV and low to moderate expressions in Group V at 10th week. However, during EGCG/TF mediated restriction, their expressions remained high in Group IV, but decreased gradually (moderate to low level) in Group III and increased gradually in Group V (Fig. 4C, Supplementary Table 4). Thus, similar modulation of the Wnt pathway might be associated with NDEA induced tongue and liver carcinogenesis as well as restriction of the processes by EGCG/TF in same set of mouse model.

3.5. Analysis self renewal hedgehog (Hh) pathway during tongue and liver carcinogenesis with/without EGCG/TF

To study the self renewal Hh pathway during simultaneous carcinogenesis of tongue and liver as well as during the restriction by EGCG/TF,

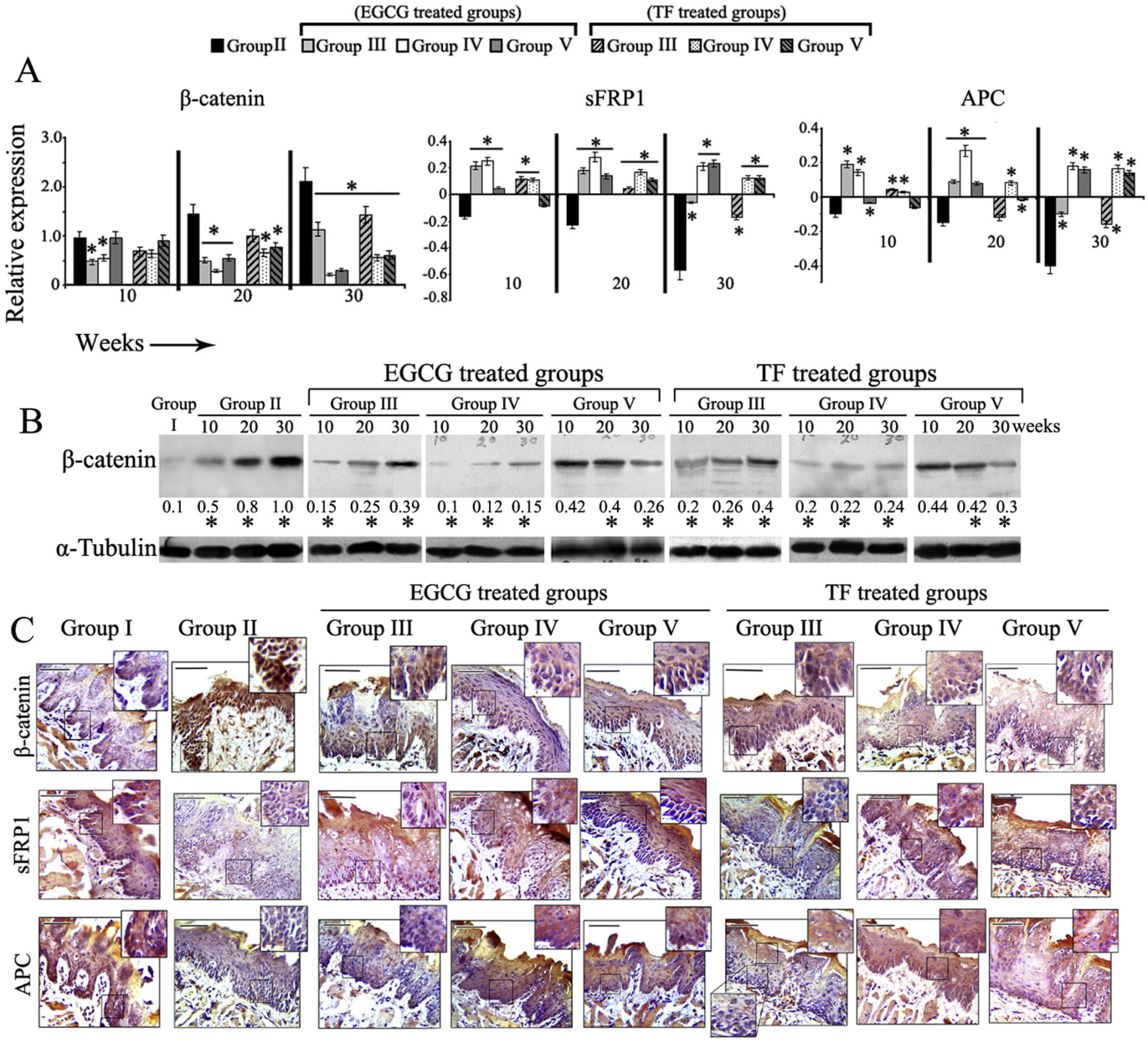

Fig. 3. Expressions of key regulatory genes of Wnt pathway during tongue carcinogenesis with/without EGCG/TF. (A) Relative mRNA expression of β-catenin, sFRP1 and APC in different groups at different weeks analyzed by quantitative RT-PCR. Mouse β_2-microglobulin gene (B2M) was used as endogeneous control and for target gene normalization. Data presented as mean ± SD. (B) Western blot analysis of β-catenin in different groups at 10th, 20th and 30th weeks. α-Tubulin used as loading control. Relative peak density was normalized with loading control. (C) Representative photographs of immunohistochemical expressions of β-catenin, sFRP1 and APC in the tongue sections of different groups with/without EGCG and TF at 30th week. Magnifications 40×. Scale bar represents 50 μm. *Represents significant *P* value ($P < 0.05$) in Group II with respect to Group I and in Group III–V with respect to Group II.

expressions (mRNA/protein) of effector gene Gli1 along with its upstream regulator SMO were analyzed in the tongue and liver lesions of different groups. Significantly increased mRNA expression of the effector gene Gli1 was evident from the 10th week in Group II (Fig. 5A). EGCG treatment could significantly ($P < 0.05$) reduce the Gli1 expression in all the treated groups (Group III–V) at different time points than Group II, except in Group V at 10th week. Similarly, TF treatment could reduce its expression significantly ($P < 0.05$) in all the treated groups mainly at 20th and 30th weeks than Group II, except in Group III at 20th week (Fig. 5A). In western blot analysis, similar trend of increased ($P < 0.05$) Gli1 expression was seen during tongue carcinogenesis in Group II and reduced expression was seen in Group III–V following EGCG/TF treatment up to 30th week (Fig. 5B). However, the reduced RNA and protein expression of Gli1 was more prominent ($P < 0.05$) in Group IV and V at 30th week. Similarly, IHC analysis showed moderate ($P < 0.05$) nuclear and cytoplasmic expression of the protein in the tongue lesions of Group II at 10th week followed by high expression in the subsequent weeks up to 30th weeks compared to low expression in basal layer of normal tongue (Fig. 5C, Supplementary Table 3, Supplementary Fig. 3). EGCG and TF treatment could reduce its nuclear and cytoplasmic expression in all the treated groups (Group III–V) than Group II (Fig. 5C, Supplementary Table 3, Supplementary Fig. 3). However, the reduced expression was more prominent in Group IV–V (low expression) than Group III (moderate) at 30th week.

Up-regulation in mRNA expression of SMO was evident during tongue carcinogenesis in Group II and down regulation was seen following EGCG/TF treatment in Group III–V (Fig. 5A). IHC analysis showed moderate cytoplasmic expression of SMO in the tongue lesions of Group II at 10th week followed by high expression up to 30th week

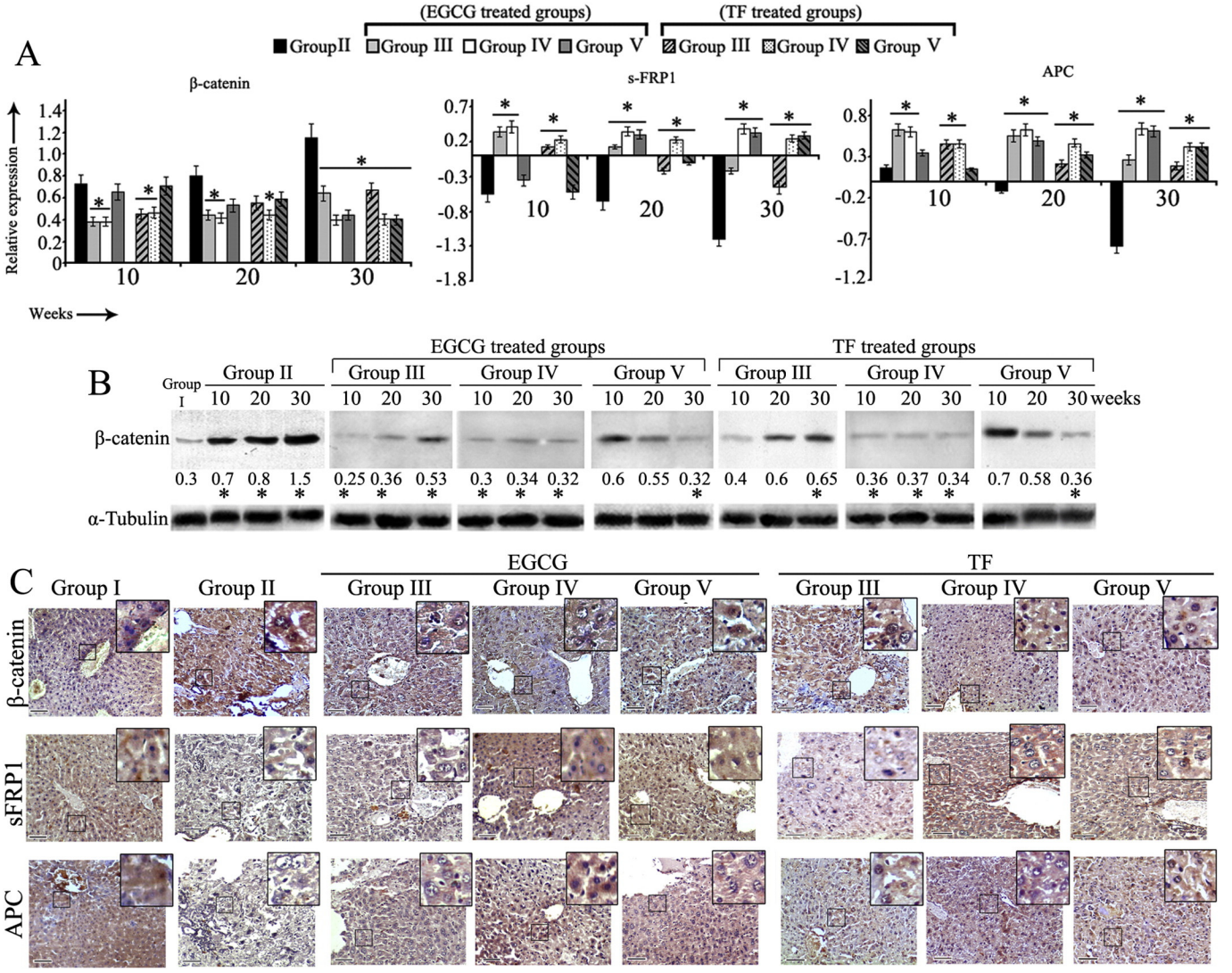

Fig. 4. Expressions of some key regulatory genes of Wnt pathway during liver carcinogenesis of same set of mice with/without EGCG/TF. (A) Relative mRNA expression of β-catenin, sFRP1 and APC in different groups at different weeks analyzed by quantitative RT-PCR. Mouse β_2-microglobulin gene (B2M) was used as endogeneous control and for target gene normalization. Data presented as mean ± SD. (B) Western blot analysis of β-catenin in different groups at 10th, 20th and 30th weeks. α-Tubulin used as loading control. Relative peak density was normalized with loading control. (C) Representative photographs of immunohistochemical expressions of β-catenin, sFRP1 and APC in the liver sections of different groups with/without EGCG and TF at 30th week. Magnifications 20×, Inset magnification 40×. Scale bar represents 50 μm. *Represents significant *P* value ($P < 0.05$) in Group II with respect to Group I and in Group III–V with respect to Group II.

compared to low expression in normal tongue (Group I) (Fig. 5C, Supplementary Table 3). Low cytoplasmic expression of SMO was seen in Group III–IV and moderate expression was seen in Group V at 10th week. However, during the restriction by EGCG/TF, its expression remained low in Group IV, but increased at moderate level in Group III and decreased at low level in Group V up to 30th week (Fig. 5C, Supplementary Table 3).

Similar trend in mRNA expressions of Gli1 and SMO were evident in liver lesions during carcinogenesis (Group II) and during EGCG/TF treatment (Group III–V) (Fig. 6A). Western blot analysis of Gli1 in the liver lesions of different groups showed concordance with their respective mRNA expression (Fig. 6B). IHC analysis revealed high nuclear and cytoplasmic expression of Gli1 along with high cytoplasmic expression of SMO in the liver lesions of Group II during carcinogenesis compared to low expression in normal liver (Group I) (Fig. 6C, Supplementary Table 4, Supplementary Fig. 3). EGCG and TF treatment could reduce both nuclear/cytoplsamic expression of Gli1 along with cytoplasmic expression of SMO in different treated groups (Group III–V) during the restriction (Fig. 6C, Supplementary Table 4, Supplementary Fig. 3). However, the reduction in Gli1 and SMO expressions was more prominent in Group IV–V (low) than Group III (high–moderate) at 30th week (Fig. 6C). Thus, the observation suggests similar modulation of the Hh pathway in both tongue and liver by the NDEA as well as EGCG and TF.

3.6. Analysis of some Wnt and hedgehog regulatory genes during tongue and liver carcinogenesis with/without EGCG/TF

To study some Wnt/Hh regulatory cellular pathways during carcinogenesis and restriction, expressions (mRNA) of some β-catenin and Gli1 regulatory genes like, Cyclin D1 and cMyc (trans-activated by both β-catenin and Gli1), EGFR (trans-activated by β-catenin) and E-cadherin (downregulated by Gli1), were analyzed in different groups. Similar to the β-catenin and Gli1 expression during tongue carcinogenesis, significant up-regulation ($P < 0.05$) of Cyclin D1, cMyc and EGFR was evident in Group II at 10th week followed by gradual increase in the subsequent weeks than Group I (Fig. 7A). Interestingly, during the restriction, both

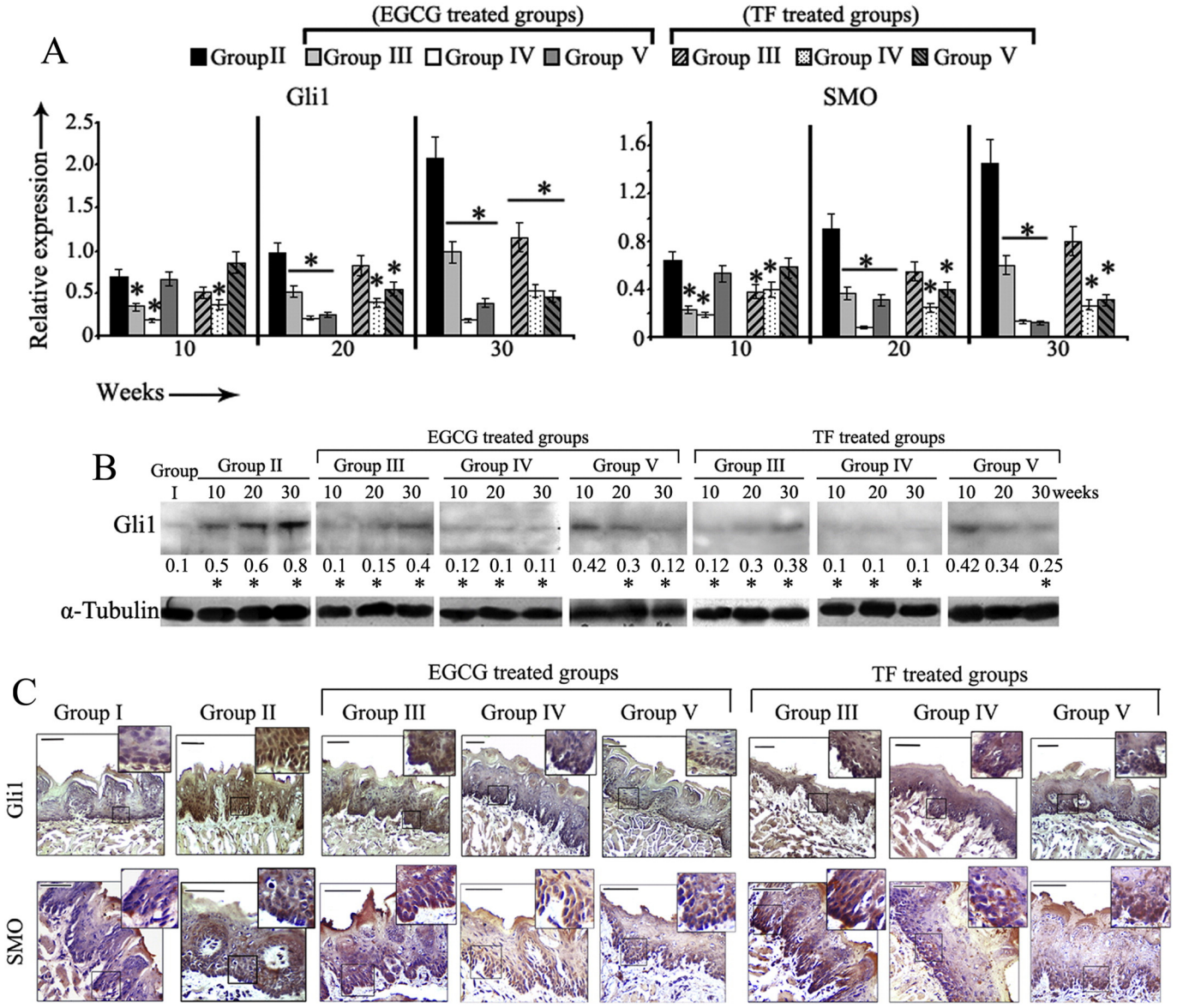

Fig. 5. Expressions of some key regulatory genes of Hedgehog pathway during tongue carcinogenesis with/without EGCG/TF. (A) Relative mRNA expression of Gli1 and SMO in different groups at different weeks analyzed by quantitative RT-PCR. Mouse β_2-microglobulin gene (B2M) was used as endogeneous control and for target gene normalization. Data presented as mean ± SD. (B) Western blot analysis of Gli1 in different groups at 10th, 20th and 30th weeks. α-Tubulin used as loading control. Relative peak density was normalized with loading control. (C) Representative photographs of immunohistochemical expressions of Gli1 and SMO in the tongue sections of different groups with/without EGCG and TF at 30th week. Magnifications 40×. Scale bar represents 50 μm. *Represents significant *P* value ($P < 0.05$) in Group II with respect to Group I and in Group III–V with respect to Group II.

EGCG and TF could reduce their expressions in different treated groups (Group III–V) up to 30th weeks (Fig. 7A). However, significant differences ($P < 0.05$) in their expressions were seen in Group IV at all the time points and in Group V from 20th week irrespective of polyphenols treatment than Group II. On the other hand, significant decrease ($P < 0.05$) in mRNA expression of E-cadherin was seen in the tongue lesions of Group II at 10th week and in the subsequent weeks up to 30th week (Fig. 7A). Both the tea polyphenols (EGCG/TF) could significantly increase ($P < 0.05$) its expression in all the treated groups (Group III–V) than Group II, except in Group V at 10th week (Fig. 7A).

Similar trend in mRNA expressions of Cyclin D1, cMyc, EGFR and E-cadherin were seen in the liver lesions also during carcinogenesis (Group II) and due to EGCG/TF treatment (Group III–V) up to 30th weeks (Fig. 7B). Thus, in concordance with Wnt/Hh pathway, modulation in their regulatory genes might associate with tongue and liver carcinogenesis as well as EGCG and TF mediated restriction.

4. Discussion

The aim of this study is to understand the molecular mechanism of NDEA induced carcinogenesis simultaneously in tongue and liver in same set of mice as well as mechanism of restriction of carcinogenesis by tea polyphenols EGCG/TF, if any. It was evident that chronic NDEA exposure in oral cavity resulted in decrease in body weights and simultaneous initiation of carcinogenesis in both tongue and liver with similar histological stages i.e. severe dysplasia up to 30th weeks of NDEA application (Table 1, Fig. 1A–B, Supplementary Fig. 1). There are reports to show that single or multiple doses of NDEA could induce carcinomas in multiple organs including liver following different routes of administration like intra-peritoneal/intra-gastric/oral gavage in mice, rat and hamster (Herrold and Dunham, 1963; Herrold, 1964; Kim et al., 1997; Wang et al., 1992). Oral administration of EGCG and TF continuously during the experimental period (Group IV) produced better chemo-

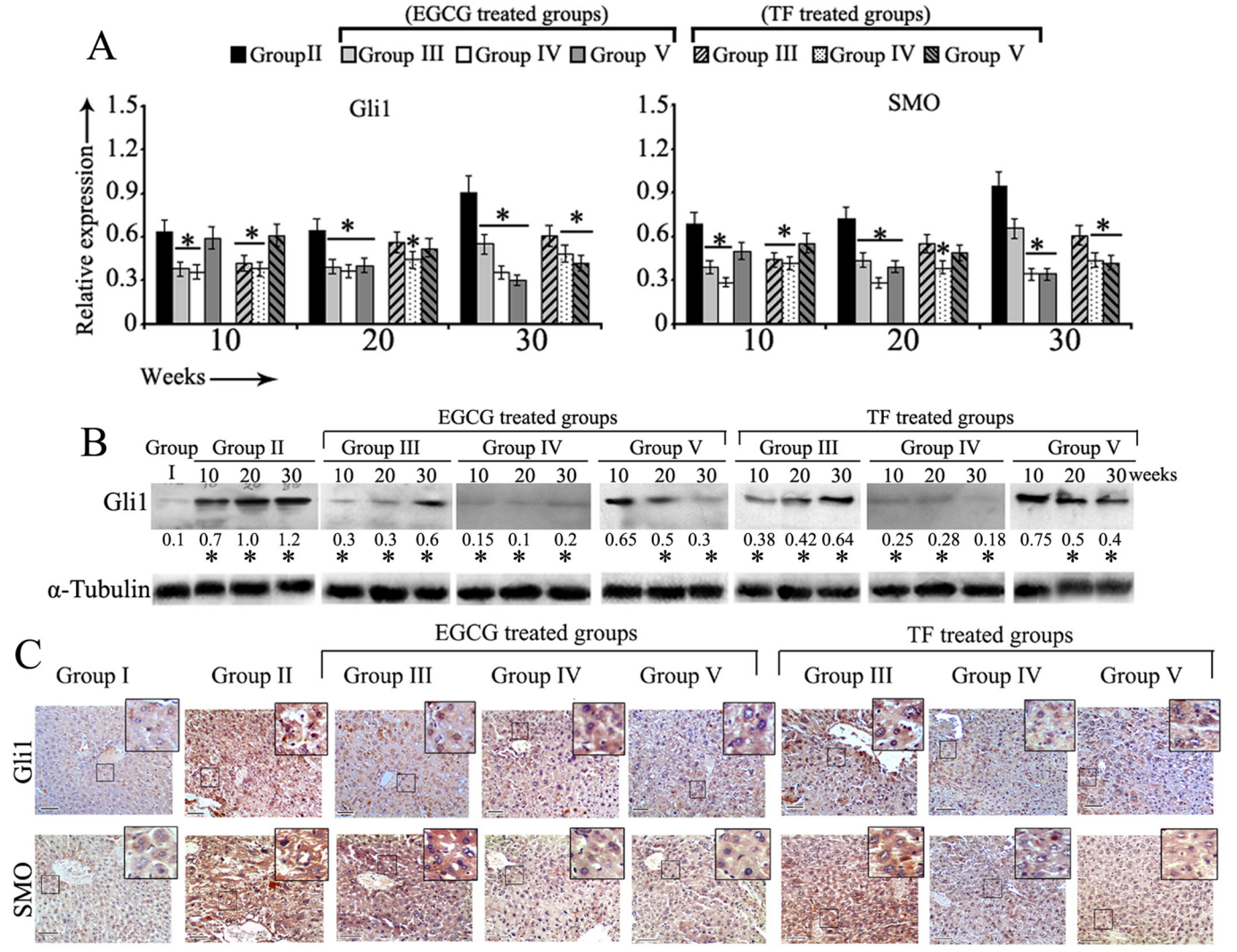

Fig. 6. Expressions of some key regulatory genes of the Hedgehog pathway during liver carcinogenesis with/without EGCG/TF (A) Relative mRNA expression of Gli1 and SMO in different groups at different weeks analyzed by quantitative RT-PCR. Mouse β_2-microglobulin gene (B2M) was used as endogeneous control and for target gene normalization. Data presented as mean ± SD. (B) Western blot analysis of Gli1 in different groups at 10th, 20th and 30th weeks. α-Tubulin used as loading control. Relative peak density was normalized with loading control. (C) Representative photographs of immunohistochemical expressions of Gli1 and SMO in the liver sections of different groups with/without EGCG and TF at 30th week. Magnifications 20×, Inset magnification 40×. Scale bar represents 50 μm. *Represents significant *P* value ($P < 0.05$) in Group II with respect to Group I and in Group III–V with respect to Group II.

preventive effect by downstaging the histopathological lesions (mild dysplasia) in both tongue and liver than treatment at the pre-initiation stages only (Group III; moderate dysplasia) up to 30th weeks (Table 1, Fig. 1A–B, Supplementary Fig. 1). This indicates that EGCG/TF application only before carcinogen administration in Group III failed to provide effective protection against carcinogenesis. The result of post treatment protocol of EGCG/TF (Group V) suggests potential therapeutic effect of the polyphenols against both types of the carcinogenesis (mild dysplasia up to 30th week) (Table 1, Fig. 1A–B, Supplementary Fig. 1). This observation is indicative of tea polyphenols EGCG and TF (at slightly higher dose than EGCG) being able to act similarly to modulate the tumor initiating clones in tongue and liver by similar mechanism to be in the early pre-malignant lesions and thereby to restrict both types of carcinogenesis in similar histological stages. In another mouse multi-organ carcinogenesis study, continuous administration of green tea infusion with drinking water followed by administration at post initiation stages showed better effect than treatment only at pre-initiation stage for reduction of NDEA induced (oral gavage) lung and fore-stomach cancer incidences in mice (Wang et al., 1992). Our study further revealed that the development of tongue and liver tumors was associated with increase in cellular proliferation and decrease in apoptosis, while tea polyphenols (EGCG/TF) could restrict the carcinogenesis processes at both sites through modulation of cellular proliferation and apoptosis (Fig. 1C–D, Supplementary Fig. 2). Similar effect of the tea polyphenols was also seen in our previous study during restriction of mouse hepatocellularcarcinoma induced by intra-peritoneal administration of CCl_4/NDEA as well as in other *in-vivo* and *in-vitro* studies (Sur et al., 2015; Manna et al., 2009; Thakur et al., 2012).

The gradual increase in cellular proliferation during tongue/liver carcinogenesis might be due to increased proliferation of tumor initiating clones/stem cells. We noted high prevalence of CD44 positive population in the present study during both types of the carcinogenesis processes (Fig. 2). Increased prevalence of CD44 positive stem cell population was also reported in primary oral and liver cancer samples and in respective cell lines (Perez et al., 2013; Ji and Wang, 2012). Over expression of CD44 in oral cancer cell line SCC25 was found to induce cellular proliferation, cisplatin resistance and reduce apoptosis (Perez et al., 2013). It is interesting to note reduced prevalence of CD44 population in tongue and liver lesions following EGCG and TF treatment in all the treated groups (Fig. 2). Similar inhibitory effect of

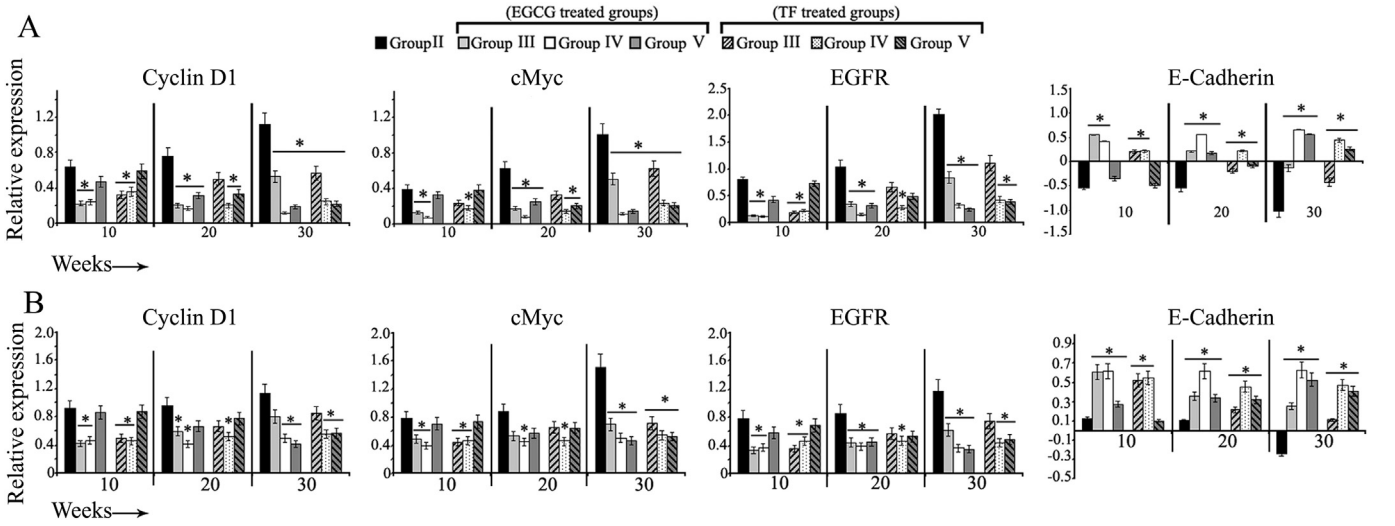

Fig. 7. Expressions of some Wnt and Hedgehog pathways regulated genes during tongue and liver carcinogenesis with/without EGCG/TF. Relative mRNA expression of Cyclin D1, cMyc, EGFR and E-cadherin in (A) tongue and (B) in liver tissues of different groups in same set of mice at different weeks analyzed by quantitative RT-PCR. Mouse β2-microglobulin gene (B2M) was used as an endogeneous control and for target gene normalization. Data presented as mean ± SD. *Represents significant *P* value ($P < 0.05$) in Group II with respect to Group I and in Group III–V with respect to Group II.

the EGCG was reported against CD44 positive population in oral cancer cell line resulting in reduction of cellular proliferation, induction in apoptosis and increased cisplatin sensitivity (Lee et al., 2013). Thus, modulation in CD44 positive population can be considered as an important key event during NDEA induced tongue and liver carcinogenesis and EGCG/TF mediated restriction processes.

It was reported that, CD44 is trans-activated by the Wnt pathway effector molecule β-catenin (Zeilstra et al., 2008). Increased expression (RNA/protein) of the β-catenin seen during the tongue and liver carcinogenesis in the present study indicates the activation of Wnt pathway during the processes (Figs. 3–4). Similar, increased expression of β-catenin was also evident in other models of liver and tongue carcinogenesis (Sur et al., 2015; Mercer et al., 2014; Khan et al., 2011; Osei-Sarfo et al., 2013). The high mRNA expression of β-catenin during carcinogenesis is possibly due to transcriptional modulation. The high protein expression of β-catenin in cytoplasm and nucleus might be due to its increased mRNA expression and/or stabilization of the protein due to down–regulation of Wnt pathway antagonists' sFRP1/APC as seen in our study during carcinogenesis (Figs. 3–4). In primary oral and liver cancer samples and cell lines inactivation of sFRP1 and APC mainly by promoter hyper-methylation were reported (Sogabe et al., 2008; Pérez-Sayáns et al., 2012; Takagi et al., 2008; Pez et al., 2013; Jain et al., 2011).Studies indicated that ectopic over expression of sFRP1/APC or restoration of their expressions from hypermethylated states by 5-aza-2′-deoxycytidine or other DNA methylation inhibitory drugs could reduce β-catenin protein expression in liver cancers (Takagi et al., 2008; Pez et al., 2013; Jain et al., 2011). Moreover, high nuclear expression of the β-catenin seen in our study might be due to its C-terminal phosphorylation (Y-654) by increased expression of EGFR, downstream effector of β-catenin (Fig. 7) (van Veelen et al., 2011; Tan et al., 2005; Guturi et al., 2012). Thus, during the carcinogenesis process there was activation of β-catenin and EGFR mediated signaling processes. On the other hand, tea polyphenols EGCG/TF could reduce β-catenin mRNA expression as well as cytoplasmic/nuclear protein expression during restriction of both tongue and liver carcinogenesis (Figs. 3–4, Supplementary Fig. 3). Different *in-vitro* studies also indicated that tea polyphenols EGCG and TF could reduce β-catenin expression and its nuclear activation in different cancers (Sarkar et al., 2010; Halder et al., 2012). The reduced β-catenin expression (mRNA/protein) during the restriction of the carcinogenesis by EGCG/TF might be due to β-catenin transcriptional inactivation or inhibition of mRNA stability, and/or increased protein destabilization due to up-regulation of sFRP1 and APC (Figs. 3–4) (Annabi et al., 2007). It was reported that tea polyphenol EGCG could restore sFRP1 expression from its hypermethylated state in hepatoblastoma cells, resulting in inactivation of Wnt signaling (Godeke et al., 2013). The reduced β-catenin expression might affect EGFR expression thereby down-regulating its signaling (Fig. 7). It is therefore suggested that modulation of the Wnt/β-catenin signaling is the key event in initiating NDEA induced carcinogenesis in tongue and liver as well as during restriction by EGCG/TF.

It seems that modulation in Wnt pathway might affect the Hedgehog pathway (Hh) due to their co-operative signaling as evident in development and cancers (Sarkar et al., 2010; Noubissi et al., 2009; Singh et al., 2012; Li et al., 2007; Maeda et al., 2006). Increased mRNA expression of Gli1 along with its high nuclear and cytoplasmic protein expression during tongue and liver carcinogenesis indicates activation of the Hh pathway (Figs. 5–6, Supplementary Fig. 3). Increased Gli1 expression was also observed in our previous mouse liver carcinogenesis study along with in other cancer studies separately (Sur et al., 2015; Dimitrova et al., 2013; Carpenter and Lo, 2012; Chen et al., 2014). Down-regulation of Gli1 expression by siRNA could inhibit liver cancer cell proliferation and migration/invasion (Chen et al., 2014). The increased Gli1 expression might be due to up-regulation of its up-stream regulatory molecule SMO as seen in our study during tongue and liver carcinogenesis (Figs. 5–6). Over expression of SMO was also evident in different cancers (McCabe and Leahy, 2015). Inhibition of SMO by cyclopamine could down-regulate Gli1 expression resulting in inactivation of Hh pathway in cholangiocarcinoma (El Khatib et al., 2013). In addition, increased Gli1 expression might be due to its mRNA stabilization associated with β-catenin expression or by EGFR mediated activation in SMO independent pathway (Noubissi et al., 2009; Maeda et al., 2006; Eberl et al., 2012). On the other hand, reduced Gli1 expression (mRNA/protein) during restriction by EGCG/TF could be due to down regulation of SMO (Figs. 5–6). Earlier reports have shown that reduced Gli1 and SMO expression by EGCG or TF was associated with prevention of mouse liver carcinogenesis and regulation of characteristics of pancreatic cancer stem cells (Sur et al., 2015; Tang et al., 2012). In addition, reduced Gli1 expression might also be due to down-regulation of β-catenin and EGFR expressions (Figs. 3–4, Fig. 7). Thus, modulation in Gli1 expression is an important key event during NDEA induced tongue and liver carcinogenesis as well as EGCG/TF mediated restriction.

The up-regulation of β-catenin and Gli1 during the liver and tongue carcinogenesis processes might have induced the expressions of two cell cycle regulators cMyc and Cyclin D1 (Fig. 7). In addition, during

the carcinogenesis process down-regulation of E-cadhrein seen in our study might be due to increased expression of its repressor Snail by Gli1 (Fig. 7) (Li et al., 2007; Carpenter and Lo, 2012). Similar expressions of these genes were also evident in different oral and liver cancers studies separately resulting in alterations of their associated pathways like cell cycle, cell signaling, epithelial to mesenchymal transition etc (Williams, 2000; Chin et al., 2004; Llovet and Bruix, 2008). Decreased expressions of Cyclin D1 and cMyc, along with up-regulation of E-cadherin as seen during the restriction might be due to EGCG/TF mediated down-regulation of β-catenin and Gli1 (Fig. 7). Different studies indicated the effect of tea polyphenols mainly EGCG on expressions of these genes in different cancers, resulting in modulation of their respective signaling pathways (Thakur et al., 2012). Thus, the modulation of Wnt and Hh pathways might be important event not only in regulation of CD44 positive stem cell population, but also in regulation of different cellular pathways during NDEA induced tongue and liver carcinogenesis as well as during EGCG/TF mediated restriction.

Overall, data presented in this report do indicate that chronic NDEA exposure in the oral cavity simultaneously induce tongue and liver carcinogenesis and oral administration of EGCG and TF can restrict both types of carcinogenesis in same mouse model. Interestingly, the mechanism of tongue and liver carcinogenesis as well as the action of EGCG and TF were similar in both tongue and liver. However, further investigations are needed to clarify the detail molecular mechanism of multi-organ carcinogenesis and anti-carcinogenic action of the tea polyphenols.

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Conflicts of interest

The authors declared that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can found, in online version.

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