Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry xx (2015) xxx–xxx

Journal of Nutritional Biochemistry

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

Subhayan Sur[a], Debolina Pal[a], Syamsundar Mandal[b], Anup Roy[c], Chinmay Kumar Panda[a,*]

[a]Dept. of Oncogene Regulation, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata 700 026, West Bengal, India
[b]Department of Epidemiology and Biostatistics, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata 700 026, India
[c]North Bengal Medical College and Hospital, West Bengal, India

Received 29 April 2015; received in revised form 9 August 2015; accepted 10 August 2015

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.

* Corresponding author. Department of Oncogene Regulation, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata 700026, West Bengal, India. Tel.: +91 33 2474 3922; fax: +91 33 2475 7606.

E-mail addresses: subhayansur18@gmail.com (S. Sur), dbln.pl@gmail.com (D. Pal), ckpanda.cnci@gmail.com (C.K. Panda).

http://dx.doi.org/10.1016/j.jnutbio.2015.08.016
0955-2863/

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.