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Tea polyphenols can restrict benzo[*a*]pyrene-induced lung carcinogenesis by altered expression of *p53*-associated genes and *H-ras*, *c-myc* and *cyclin D1*

Sugata Manna[a], Sudeshna Mukherjee[a], Anup Roy[b], Sukta Das[a], Chinmay Kr. Panda[a,*]

[a]*Department of Oncogene Regulation, Chittaranjan National Cancer Institute, Kolkata 700026, India*
[b]*Department of Pathology, Medical College and Hospital, Kolkata 700073, India*

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Abstract

The modulatory influence of tea polyphenols (epigallocatechin gallate, epicatechin gallate and theaflavin) on benzo[*a*]pyrene (B[*a*]P)-induced lung carcinogenesis in mice was analyzed using histopathological and molecular parameters. Progression of lung lesions was restricted at the hyperplastic stage by tea polyphenols. A significant reduction in cellular proliferative index and an increase in apoptotic index were noted in the restricted lung lesions. High expression of *H-ras*, *c-myc*, *cyclin D1* and *p53* genes was seen at the inflammatory stage (9th week) and in subsequent premalignant lesions, but down-regulation of *H-ras* at the hyperplastic stage (17th week). Expression of *bcl-2* was high in hyperplastic lesions, whereas the expression of *mdm2* and *bcl-xl* increased only at the moderately dysplastic stage (36th week). The tea polyphenols inhibited inflammatory response in the lung lesions on the 9th week, when decreased expression of *H-ras* and *c-myc* and increased expression of *bax* were noted. Prolonged treatment (>9th week) with tea polyphenols resulted in changes in the expression of some additional genes, such as reduced expression of *cyclin D1* (from the 17th week), *bcl-2* (from the 26th week; mild dysplasia) and *p21* (on the 36th week), and high expression of *p53* (from the 17th week) and *p27* (on the 36th week). These observations indicate that the tea polyphenols can restrict B[*a*]P-induced lung carcinogenesis by differential modulation of the expression of *p53* and its associated genes such as *bax*, *bcl-2*, *mdm2*, *p21* and *p27*, along with *H-ras*, *c-myc* and *cyclin D1*, at different time points.

Keywords: Tea polyphenols; Chemoprevention; Lung cancer; Apoptosis; Proliferation

1. Introduction

Chemopreventive intervention by different phytochemicals, particularly tea polyphenols epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and theaflavin (TF), forms a relatively recent approach to restrict carcinogenesis and prevent cancer [1–3]. Chemical carcinogenesis induced in different susceptible strains provides very useful models for understanding the mechanism of carcinogenesis and its modulation. Better knowledge of genetic alterations taking place during carcinogenesis is important for exploring the mechanism of action of different chemopreventive agents, thereby helping in formulating important intervention strategies.

Lung cancer is a leading cause of cancer deaths worldwide, and it has been estimated that about 90% of lung cancer cases are associated with tobacco use [4]. Chemopreventive strategies may help to reduce lung cancer risk, as implied by the observation that heavy smokers who do not get lung cancer have a higher intake of antioxidants/phytochemicals through vegetables and tea [5]. We have focused attention on the chemopreventive role of tea polyphenols.

Benzo[*a*]pyrene (B[*a*]P)-induced lung carcinogenesis in strain A mice is a commonly used animal model for the study of carcinogenesis and anticarcinogenesis. The present investigation was undertaken in this model to assess the

* Corresponding author. Tel.: +91 33 2474 3922; fax: +91 33 2475 7606.
E-mail address: ckpanda@vsnl.net (C.K. Panda).

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modulatory role of three tea compounds in the expression of some genes associated with carcinogenesis.

Multiple molecular changes, including overexpression of cell-cycle- and apoptosis-regulatory genes *H-ras*, *c-myc*, *cyclin D1* and *p53* [6–9], are known to be associated with the development of spontaneous or induced tumors in susceptible mouse strains. Kang et al. [10] reported altered expression of G1/S-regulatory genes *cyclin D1*, *cyclin-dependent kinase 4* (*cdk4*), *p21*, *p27*, *p15* and *p16* during early lung carcinogenesis in transforming growth factor-β1 heterozygous mice. K-ras mutation was suggested to be associated with the development of hyperplastic lung lesions in mice, whereas mutation of *p53* was found to be associated with the development of adenocarcinoma [11]. Overexpression of antiapoptotic proteins bcl-2 and mdm2 (the negative regulator of p53) is often found in human lung cancer [12–14]. Proapoptotic bax, another bcl-2 family protein, was down-regulated in primary small cell lung cancer that expresses mutant p53 [15]. B[*a*]P is reported to induce the expression of mdm2, bax and p21 in a p53-dependent manner in lung cancer cell lines [16]. However, in Swiss 3T3 fibroblasts, B[*a*]P has been shown to induce nuclear accumulation of p53, with high expression of mdm2 but without any induction of p21 [17]. Alteration of these cell-cycle- and apoptosis-regulatory genes is a common phenomenon in human lung cancer [11]. In spite of all these reports, so far, there has been no comprehensive study characterizing molecular progression simultaneously with histopathological changes in lung carcinogenesis and its intervention by tea compounds in vivo.

Tea polyphenols and tea extract have been shown to prevent chemically induced carcinogenesis at different organ sites in animal models [3,18]. The chemopreventive mechanism of the tea polyphenols during lung carcinogenesis has been suggested to be associated with inhibition of cellular proliferation and induction of cellular apoptosis [19,20]. However, the molecular mechanism associated with these phenomena has not been elucidated in detail. The modulatory effect of tea on precancerous rat liver lesions was suggested to be carried out through alterations in the cell cycle regulators p21, cyclin D1, and cdk4 [21]. EGCG was shown to decrease the expression of c-myc, to affect the cell signaling pathway and to inhibit intestinal tumorigenesis in $Apc^{min/+}$ mice [22]. Inhibition of *c-myc*, *c-raf* and *H-ras* expression in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung carcinogenesis by tea extract and inhibition of *c-myc* expression in 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced skin carcinogenesis by EGCG have been reported [6].

In the present study, we attempted to understand the molecular mechanism of chemoprevention by tea polyphenols EGCG, ECG and TF during the progression of premalignant lung lesions in mice. Analysis of the histopathological changes, in situ cell proliferation, cell death and expression of associated genes *H-ras*, *c-myc*, *cyclin D1*, *p53*, *bax*, *bcl-2*, *bcl-xl*, *mdm2*, *p21* and *p27* was undertaken.

2. Materials and methods

2.1. Animals and chemicals

Inbred strain A mice were obtained from the animal colony of the Chittaranjan National Cancer Institute (Calcutta, India). They were maintained under standard conditions, in an air-conditioned room with 55±10% humidity under a daily cycle of alternating 12-h periods of light and darkness. Drinking water and pellet diet (Lipton India Ltd. India) were provided to the animals ad libitum. All chemicals were of the highest analytical grade available. EGCG, ECG, TF and B[*a*]P were obtained from Sigma Chemical Co. (USA). TRIzol reagent, 5-bromo-2-deoxyuridine (BrdU) labeling and in situ cell death detection kits were purchased from Roche Molecular Biochemicals (Germany). Primary antibodies and the ABC staining system for immunohistochemistry were purchased from Santa Cruz Biotechnology, Inc. (California, USA).

2.2. Experimental design

The induction of histopathological lesions in the lung by B[*a*]P was performed following the method of Yun et al. [23] and was described in detail in our previous report [24].

Newborn mice received a single subcutaneous injection of 0.02 ml of B[*a*]P suspension (0.2 mg in 1% gelatin) at the subscapular region. As newborn mice are more susceptible to carcinogens than adult mice, in this study, male and female newborn strain A mice less than 24 h of age were used. After weaning, male and female mice were caged separately.

Four experimental groups were maintained. One B[*a*]P-administered group was left untreated and served as the carcinogen control. The other three B[*a*]P-administered groups were treated with either EGCG, ECG or TF at a dose of 0.01 mg, 4 μg and 0.02 mg, respectively (minimal effective dose) by daily intraperitoneal injection to facilitate efficient uptake of the compounds. The dose selection was derived from our previous studies using tea infusion, their high-performance liquid chromatography analysis for EGCG, ECG and TF content, and determination of the minimal effective dose.

Mice from each group were sacrificed on the 9th, 17th, 26th and 36th weeks, as our pilot study revealed significant histological changes at these time points. The focal lesions of the lungs were dissected out for histopathological, immunohistological and molecular analyses. A parallel normal control group was maintained for comparison of the different parameters studied in the experimental groups.

2.3. Histological analysis

Formalin-fixed lung tissues were embedded in paraffin in accordance with the standard procedure. Serial sections (5 μm) were cut and mounted on glass slides. Tissue sections were stained with hematoxylin and eosin for histopathological analysis, examined under a light microscope and photographed. Three to five slides per mice were analyzed.

The premalignant lesions described in this article are bronchiolar lesions.

2.4. Determination of in situ cell proliferation

Proliferative cells in lung lesions were detected by BrdU labeling with commercial kits used in accordance with the manufacturer's protocol. Lung tissues were placed in BrdU added prewarmed (37°C) cell culture medium for 30 min and then fixed in 10% formaldehyde. After paraffin embedding and sectioning at 5 μm, four slides of serial sections of each mice were deparaffinized and rehydrated (in xylene and graded series of ethanol; 100% to 50%, respectively). Next, tissue sections were incubated with anti-BrdU, mouse monoclonal antibody and antimouse Ig alkaline phosphatase (AP) (both at 37°C for 30 min in a humid atmosphere), respectively. Color development was achieved by incubation with a substrate solution containing nitroblue tetrazolium (NBT) and X-phosphate for 5 min at room temperature. A negative control was maintained simultaneously. Sections were mounted in glycerin and observed under a light microscope. Proliferative index was determined as the percentage of labeled nuclei with respect to the total number of nuclei counted. Based on lesion size, 5–10 representative microscopic fields were examined. The relative proliferative index of each lesion was determined with respect to the normal lung of the corresponding week.

2.5. In situ cell death detection

Apoptotic cells in lung lesions were detected by the terminal deoxyneucleotidyl transferase (TdT)-mediated dUTP biotin nick end labeling (TUNEL) method with an in situ cell detection kit used in accordance with the manufacturer's protocol. The dissected lung tissues were fixed in 10% formalin. Following paraffin embedding and sectioning at 5 μm, four slides from serial sections of each mice were deparaffinized and rehydrated (in xylene and graded series of ethanol; 100% to 50%, respectively). After permeabilization in proteinase K solution (20 μg/ml in 10 mM Tris–HCl, pH 7.4), sections were washed in phosphate-buffered saline (PBS) and incubated with 50 μl of TUNEL reaction mixture (TdT and fluorescein dUTP) at 37°C for 60 min in a humid atmosphere in the dark. Next, sections were rinsed with PBS, incubated with converter AP (anti-fluorescein antibody, Fab fragment from sheep, conjugated with AP) for 30 min and analyzed under a light microscope after color development, which was achieved by incubation with a substrate solution containing NBT and X-phosphatase for 5 min at room temperature. The sample without primary antibody was used as negative. Apoptotic index was determined as the percentage of labeled nuclei with respect to the total number of nuclei counted. Based on the lesion size, 5–10 representative microscopic fields were examined. The relative apoptotic index of each lesion was determined with respect to the normal lung of the corresponding week.

2.6. Analysis of gene expression by semiquantitative reverse transcription–polymerase chain reaction

Gene expression was analyzed by reverse transcription–polymerase chain reaction (RT-PCR) procedure. Total RNA was extracted from the freshly dissected lung lesions with TRIzol reagent used in accordance with the manufacturer's protocol. cDNA was synthesized from the total RNA with SuperScript III Reverse Transcriptase (Invitrogen, USA) used in accordance with the manufacturer's protocol. After treatment with DNaseI, 1 μg of RNA was taken in a 20-μl reaction volume containing 200 ng of random hexamer, 40 U of RNaseOUT (Invitrogen), 1× reverse transcription buffer [50 mM Tris–HCl (pH 8.3), 75 mM KCl and 3 mM $MgCl_2$], 0.005 M dithiothreitol (DTT), 0.5 mM each of deoxynucleoside triphosphates (dNTPs; Gibco-BRL, USA) and 200 U of SuperScript III. The reaction was performed at 50°C for 60 min, then for 15 min at 75°C.

To analyze the expression of specific genes, 2 μl of the cDNA was taken in a 20-μl PCR mixture containing 1× PCR buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8 at 25°C) and 0.01% Tween-20], 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 3 pmol of each gene-specific primer and 0.3 U of Taq polymerase (Bioline, London, UK) enzyme. *H-ras*, *c-myc*, *cyclin D1*, *p21*, *p27*, *p53*, *bax*, *bcl-2*, *bcl-xl* and *mdm2* genes were coamplified with the hypoxanthine phosphoribosyl transferase (*hprt*) or *β-actin* gene as control. Primers used in this experiment are listed in Table 1. The PCR cycle conditions were as follows: 95°C for 3 min, then 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 60 s, with a final extension step of 7 min at 72°C in a thermal cycler (Applied BioSystem, USA). The PCR products were electrophoresed in 2% agarose gel, stained in ethidium bromide, visualized in UV transilluminator and then photographed. The intensity of the bands was analyzed by scanning with a densitometric scanner (Model CS-9000; SHIMADZU, Japan). In each case, the intensity of the bands was normalized with respect to the control bands of *hprt*/*β-actin*.

2.7. Immunohistochemistry

Excised lung lesions were fixed in 10% formalin and embedded in paraffin in accordance with standard histological procedures. Expression of the proteins in four serial tissue sections per mice was determined by immunostaining following the ABC staining protocol (ABC Staining System; Santa Cruz Biotechnology, Inc.). The endogenous peroxidase of deparaffinized and rehydrated tissue sections was blocked by incubating the tissue sections in 1% hydrogen peroxide in methanol for 15 min at room temperature. Sections were then incubated with a protein block (1.5% normal goat serum in PBS) for 60 min at room temperature. Next, sections were incubated with primary antibody (1:100 dilution/0.5–5 μg/ml in 1.5% blocking serum) for 30 min at room temperature, followed by incubation with a biotinylated anti-mouse secondary antibody (1 μg/ml) for 30 min and incubation with AB enzyme reagent (avidin and biotinylated horseradish

Table 1
Oligonucleotide primer sequences used for RT-PCR analysis of proliferation and apoptosis-associated genes

Gene of interest	Primer sequences	References
H-ras	FP: 5′ACA GAA TAC AAG CTT GTG GTG GTG 3′ RP: 5′CTC TAT AGT GGG ATC ATA CTC GTC 3′	[25]
c-myc	FP: 5′TCC TGT ACC TCG TCC GAT TC 3′ RP: 5′ATT GAT GTT ATT TAC ACT TAA GGG T 3′	[26,27]
cyclin D1	FP: 5′AAC ACC AGC TCC TGT GCT GCG AA 3′ RP: 5′GTC TCC TTC ATC TTA GAG GCC ACG 3′	[28]
p21	FP: 5′AAT CCT GGT GAT GTC CGA CC 3′ RP: 5′AAA GTT CCA CCG TTC TCG G 3′	[29]
p27	FP: 5′GAG GGC AGA TAC GAG TGG CAG 3′ RP: 5′CTG GAC ACT GCT CCG CTA ACC 3′	[29]
p53	FP: 5′ATG ACT GCC ATG GAG GAG TCA CAG T 3′ RP: 5′GTG GGG GCA GCG TCT CAC GAC CTC C 3′	[30]
Bax	FP: 5′AAG CTG AGC GAG TGT CTC CGG CG 3′ RP: 5′GCC ACA AAG ATG GTC ACT GTC TGC C 3′	[31]
bcl-2	FP: 5′CTC GTC GCT ACC GTC GTG ACT TCG 3′ RP: 5′CAG ATG CCG GTT CAG GTA CTC AGT C 3′	[31]
bcl-xl	FP: 5′TGG ATC CTG GAA GAG AAT CG 3′ RP: 5′AGA TCA CTG AAC GCT CTC CG 3′	[26]
mdm2	FP: 5′CGA CTA TTC CCA ACC ATC G 3′ RP: 5′CTA GTT GAA GTA ACT TAG CAC AAT 3′	[32]
β-actin	FP: 5′GTG GGC CGC TCT AGG CAC CAA 3′ RP: 5′CTC TTT GAT GTC ACG CAC GAT TTC 3′	[29]
hprt	FP: 5′GCT GGT GAA AAG GAC CTC T 3′ RP: 5′CAC AGG ACT AGA ACA CCT GC 3′	[27]

FP: forward primer; RP: reverse primer.

peroxidase) for 30 min. Color development was achieved by incubation with 3,3-diaminobenzidine-tetrahydrochloride-containing peroxidase substrate (0.02% hydrogen peroxide) for 10 min at room temperature. The sample without primary antibody was used as negative control in all cases and to see the specificity of the primary antibody. The slides were then counterstained with hematoxylin, dehydrated, mounted in depex polystyrene (DPX) mounting medium and observed under a light microscope. A positive reaction is indicated by light brown to dark brown precipitate in the cytoplasm and/or in the perinuclei of the cells. The percentage of immunoreactive cells was determined in each lesion.

2.8. Statistical analysis

All data were expressed as mean±S.D. Statistical analyses were performed using one-way analysis of variance, followed by paired *t* test with the help of critical difference.

3. Results

3.1. Effect of tea polyphenols on B[a]P-induced lung lesions

It is evident from Fig. 1I that the body weight of mice in different groups did not change significantly. We had earlier reported that B[*a*]P treatment resulted in gradual progression of the lung lesions through inflammation, hyperplasia, mild dysplasia and moderate dysplasia on the 9th, 17th, 26th and 36th weeks, respectively (Fig. 1IIA–D) [24]. The number of inflammatory cells (lymphocyte) on the 9th week was high compared to that of normal lung cells. Hyperplasia was manifested as an increase in cell number with retention of alveolar pattern. During hyperplasia, the single layer of bronchiolar epithelium became multilayered, but the cells maintained their normal appearance (i.e., the cells were well differentiated and lacked nuclear pleomorphism). During mild dysplasia and moderate dysplasia, there was loss of polarity in columnar epithelial cells (<30% and >30% cells, respectively), and the nuclear-to-cytoplasmic ratio of cells increased. Dysplasia was accompanied by occasional metaplasia (normal epithelial cells replaced by flattened squamous epithelium) and inflammation in the peribronchiolar region. Following treatment with black tea polyphenol TF, lung lesions did not progress beyond hyperplasia (Fig. 1IIE–H; Table 2), as seen in our previous study with EGCG and ECG (Table 2). Treatment reduced the size and the number of hyperplastic zones. In addition, infiltration of inflammatory cells in the peribronchiolar region was low in the treatment group compared to the carcinogen controls.

3.2. Inhibition of B[a]P-induced lung hyperproliferation by tea polyphenols

Fig. 2A is a representative photograph that shows the pattern of cellular proliferation in the lungs from different groups on the 36th week. It is evident from Fig. 2B that the relative proliferative index in lung lesions on the 9th week of B[*a*]P exposure was similar to that in the normal lung. However, due to B[*a*]P exposure, a significant increase in the relative proliferative index was seen in the hyperplastic

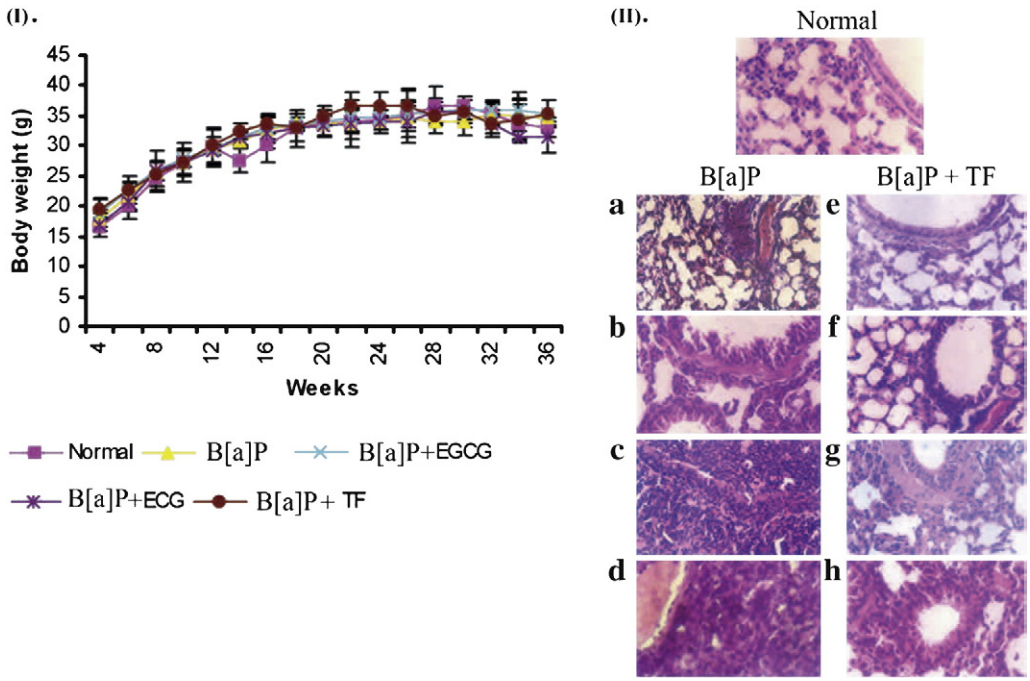

Fig. 1. Influence of TF during the progression of B[*a*]P-induced lung lesions. (I) Effect of tea polyphenols on the body weight of mice treated with B[*a*]P. Body weight was measured biweekly. Values are presented as mean±S.E. (n=5–7 mice). (II) Histopathological analysis of the effect of TF on the progression of lung lesions (n=7–12) stained by hematoxylin and eosin. Normal (original magnification ×500); (a) 9th week (original magnification ×250); (b) 17th week (original magnification ×400); (c) 26th week (original magnification ×400); (d) 36th week (original magnification ×800); (e) 9th week (original magnification ×400); (f) 17th week (original magnification ×400); (g) 26th week (original magnification ×312.5); (h) 36th week (original magnification × 800). TF restricted the progression of carcinogenesis at the hyperplastic stage.

lesions on the 17th week (71%; P<.01), as well as in subsequent weeks (i.e., on the 26th and 36th weeks by 98% and 87%, respectively; P<.01).

Tea polyphenols significantly reduced the relative proliferative index by 24–42% (P<.01) as compared to the B[*a*]P group on the 17th, 26th and 36th weeks.

3.3. Induction of apoptosis in B[a]P-induced lung lesions by tea polyphenols

Fig. 2C is a representative photograph of apoptotic cells in the lung lesions of different groups on the 36th week. The percentage of apoptotic cells on the 9th week in the B[*a*]P-treated group was comparable to that of the normal lung in the corresponding week (Fig. 2D). A gradual and significant decrease in relative apoptotic index was noted during the progression of lung lesions on the 17th, 26th and 36th weeks after B[*a*]P exposure by 19%, 34% and 30% (P<.01), respectively. This was in comparison to that in the lung lesions on the 9th week.

Tea polyphenols did not affect the relative apoptotic index in the lung lesions on the 9th week, which was comparable to that of the corresponding lesions in carcinogen controls. However, a significant increase (31–93%; P<.01) in the relative apoptotic index was observed in the treated groups on the 17th, 26th and 36th weeks.

3.4. Expression of proliferation and apoptosis-associated genes

To analyze the molecular events at the early stages of lung carcinogenesis, the expression of some proliferation- and apoptosis-associated genes was studied by RT-PCR (Fig. 3). There was a differential expression pattern of *H-ras* during early lung carcinogenesis. The expression of *H-ras* was

Table 2
Histopathological pattern of B[*a*]P-induced lung lesions in mice after treatment with tea polyphenols

	9th week	17th week	26th week	36th week
B[*a*]P	Inflammation [100% (10/10)]	Hyperplasia with focal dysplasia [100% (10/10)]	Mild dysplasia [83% (10/12)]	Moderate dysplasia with squamous metaplasia [100% (7/7)]
B[*a*]P+TF	Normal [100% (10/10)]	Hyperplasia with inflammation [100% (10/10)]	Hyperplasia with focal dysplasia [100% (7/7)]	Hyperplasia with focal dysplasia [77.8% (7/9)]
B[*a*]P+EGCG	Normal [100% (10/10)]	Hyperplasia with occasional metaplasia [100% (10/10)]	Hyperplasia with inflammation and occasional metaplasia [87.5 (7/8)]	Hyperplasia with inflammation [100% (6/6)]
B[*a*]P+ECG	Normal [100% (10/10)]	Hyperplasia [100% (10/10)]	Hyperplasia with inflammation [77.8% (7/9)]	Hyperplasia with focal dysplasia [75% (6/8)]

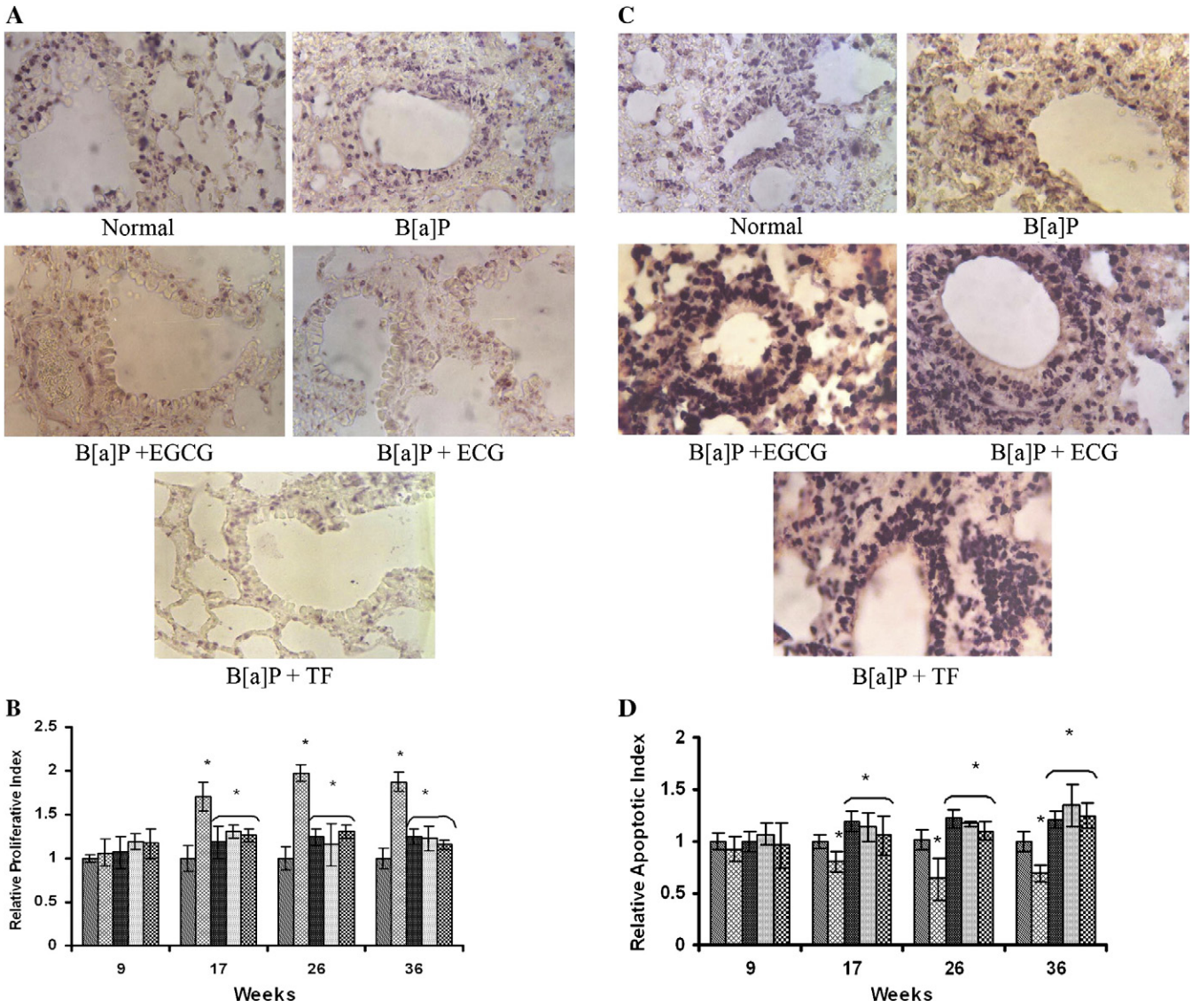

Fig. 2. Inhibition of cell proliferation (detected by BrdU immunohistochemistry) and induction of apoptosis (detected by TUNEL staining) in B[*a*]P-induced lung lesions by tea polyphenols. (A) Representative photograph of BrdU immunohistochemistry of lung lesions on the 36th week (original magnification ×400). (B) Histogram of proliferative cells in lung lesions. (C) Representative photograph of immunohistochemically detected apoptotic cells in lung lesions on the 36th week (original magnification ×400). (D) Histogram of apoptotic cells in lung lesions. Results are presented as mean±S.E. (n=5–7). *P<.01. (▨) Normal; (▩) B[*a*]P; (▩) B[*a*]P+EGCG; (▩) B[*a*]P+ECG; (▩) B[*a*]P+TF.

seen to increase by about 34% (P<.05) on the 9th week compared to that of the respective normal lung. On the 17th week, there was down-regulation, and the level was comparable to that of the corresponding normal lung. On the 26th week, the level of *H-ras* increased by 21%, but the result was not statistically significant. This was followed by an increase of about 41% (P<.01), again in the moderate dysplastic lesions on the 36th week. The expression of *c-myc* was significantly increased by 45% (P<.01) on the 9th week compared to the expression of the corresponding normal lung, which continued to increase further by 59–77% (P<.01) in the subsequent weeks. The expression of *cyclin D1* continued to increase significantly by 27–49% through the 9th week to the 36th week in comparison to the normal lung. Among the different apoptosis-associated genes, the expression of *p53* gene in the lesions was up-regulated by about 33% (P<.05) on the 9th week compared to the normal lung, followed by a gradual increase by 50% (P<.01) on the 17th week, which then gradually decreased to 40% (P<.01) on the 36th week. Expression of the antiapoptotic gene *bcl-2* did not change on the 9th week compared to the normal lung, but increased by 35% and 61% (P<.01) on the 17th and 26th weeks, respectively, and remained more or less constant in the subsequent week. No significant changes in the expression of *p21*, *p27*, *bax*, *bcl-xl* and *mdm2* were observed, except that of an increase (P<.01) in *bcl-xl* and *mdm2* expression on the 36th week by 38% and 70%, respectively.

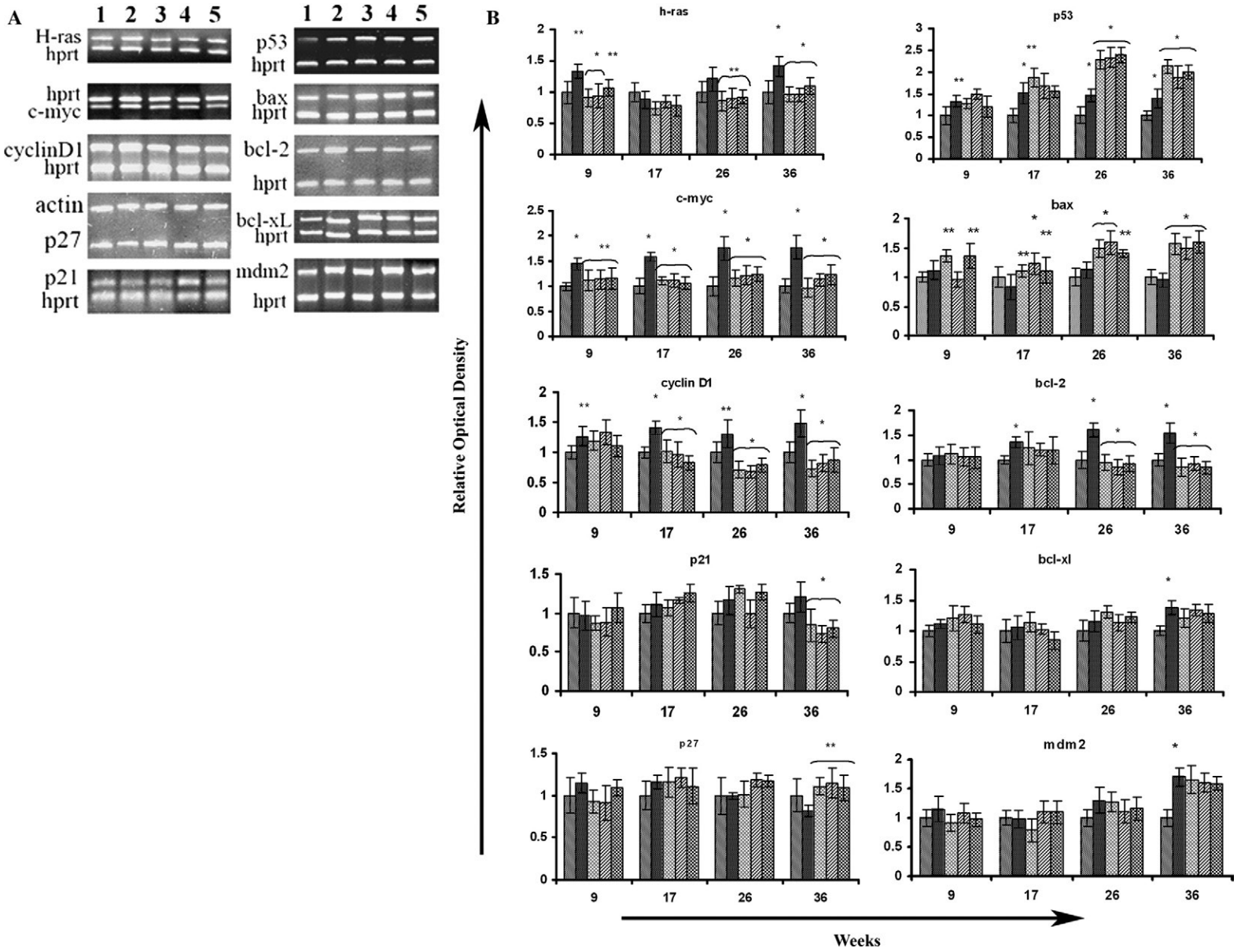

Fig. 3. Effect of EGCG on the mRNA level of apoptotic and cell-proliferation-regulatory genes. The genes were coamplified with either *β-actin* or *hprt*. (A) Representative photographs of ethidium-bromide-stained 2% agarose gels under UV in mice samples on the 36th week. Lane 1: normal lung; lane 2: B[*a*]P-treated lung; lane 3: B[*a*]P+EGCG-treated lung; lane 4: B[*a*]P+ECG-treated lung; lane 5: B[*a*]P+TF-treated lung. (B) Histogram display of the quantitative analysis of different genes at the mRNA level. Results are presented as mean±S.E. (*n*=5 mice). ***P*<.05; **P*<.01. (▨) Normal; (▩) B[*a*]P; (▩) B[*a*]P+EGCG; (▨) B[*a*]P+ECG; (▩) B[*a*]P+TF.

3.5. Expression of proliferation and apoptosis-associated genes in B[a]P-induced lung lesions in the presence of tea polyphenols

It was evident from RT-PCR analysis (Fig. 3) that the tea polyphenols significantly reduced the expression of *H-ras* by 20–32% on the 9th, 26th and 36th weeks compared to the B[*a*]P-treated lung lesions in the corresponding weeks, although its expression remain unaltered on the 17th week after treatment. Unlike *H-ras*, the tea polyphenols significantly reduced the expression of *c-myc* at all time points (i.e., 9th, 17th, 26th and 36th weeks) by 20–45% compared to the corresponding B[*a*]P control. Similar to *c-myc*, a significant reduction (28–51%; $P<.01$) in the expression of *cyclin D1* was also noted, except on the 9th week. The tea polyphenols did not have any effect on the expression of *p53* on the 9th and 17th weeks, except for a 23% increase ($P<.05$) noted in the EGCG-treated group on the 17th week. However, on the 26th and 36th weeks, *p53* expression was found to be significantly increased by 34–64% ($P<.01$) after treatment with the polyphenols. In contrast to *p53*, *bcl-2* expression was significantly reduced (40–47%; $P<.01$) by the tea polyphenols on the 26th and 36th weeks as compared to the B[*a*]P-treated group. The expression of *bax* was significantly increased in all treatment groups (except for the ECG-treated group) by 22–68% on the 9th week. The tea polyphenols did not have any significant effect on the expression of *bcl-xl* and *mdm2* in the lesions. However, there was significant inhibition of *p21* expression (30–40%; $P<.01$) and significant activation of *p27* expression (36–44%; $P<.05$) by the tea polyphenols on the 36th week.

3.6. Immunohistochemical analysis of the B[a]P-induced lung lesions

Immunohistochemical analysis of protein expression was performed in lung lesions on the 26th and 36th weeks, when major changes in histology and mRNA expression pattern were observed (Fig. 4). The expression of H-ras was significantly increased (37% and 54%; $P<.01$) on the 26th and 36th weeks in B[*a*]P-induced lesions compared to the corresponding normal lung, which was then significantly reduced by tea polyphenols. Similar to H-ras, bcl-2 expression was also high (about 2.6-fold; $P<.01$) in the B[*a*]P-treated lung lesions, followed by inhibition of its expression by the tea polyphenols. No significant differences in the expression of bax on the 26th and 36th weeks were noted between the carcinogen control group and the corresponding normal control group. However, the tea polyphenols significantly increased its expression by about 1.6-fold to 2.2-fold ($P<.01$) in the lung lesions.

4. Discussion

An analysis of an experimental B[*a*]P-induced lung carcinogenesis mouse model revealed progressive histopathological changes that could be identified as inflammation, hyperplasia, mild dysplasia and moderate dysplasia on the 9th, 17th, 26th and 36th weeks, respectively, following carcinogenic exposure [24]. It was further observed that chemopreventive intervention with two green tea polyphenols (i.e., EGCG and ECG) could restrict the progression of such lung lesions at the hyperplastic stage [24]. It is evident from the present study that black tea polyphenol TF also restricted the carcinogenic process at the hyperplastic stage, similar to that noted with EGCG and ECG. Budesonide, a corticosteroid, was reported to delay lung carcinogenesis induced by B[*a*]P in adult A/J mice [33]. EGCG is reported to suppress early-stage, but not late-stage, prostate cancer in TRAMP mice [34]. All these studies suggest that it is possible to restrict the progression of lung carcinogenesis by chemopreventive intervention.

While there are some reports to indicate that use of tea compounds could influence body weight, we did not observe any significant change with any of the compounds used. Similarly, Yang et al. [35] did not see any change in the body weight of mice during the progression of NNK-induced premalignant lung lesions, as well following treatment with TF. Mimoto et al.[36] reported that EGCG could prevent cisplastin- and NNK-induced weight loss during lung carcinogenesis in A/J mice. These are in contrast to the report that black tea extract reduced body weight after the 24th week of treatment in NNK-induced lung carcinogenesis in mice, although the animals were healthy and active, with low tumor multiplicity and tumor volume [37].

Cell proliferation and apoptosis detected in situ in lung lesions reveal no significant changes at the inflammatory stage (9th week after B[*a*]P exposure) in comparison to the normal lung. However, the expression of *H-ras*, *c-myc*, *cyclin D1* and *p53* increased significantly at this stage (Fig. 5), suggesting a role of these genes in the inflammatory process. The increased expression of *H-ras* has been reported in rat lung inflammatory cells [38]. Activation of the *myc* oncogene engaged a broad array of inflammatory signals (influx of mast cells), which in turn recruited immune cells to the tumor. Wong et al. [39] reported the important role of *cyclin D1* in ulcerative-colitis-related inflammation. Hudson et al. [40] reported that *p53* expression increases during inflammation. The observation that macrophage migration-inhibitory factor (a proinflammatory cytokine released at sites of inflammation) is capable of functionally inactivating *p53*, which normally functions to prevent proliferation of cells carrying genotoxic damage, may provide a mechanistic link between inflammation and cancer. It seems that, during inflammation, cells are in a critical state due to genotoxic stress, which induces the up-regulation of both positive and negative cell cycle regulators. This may lead to the activation of inflammatory NF-κB and PI3 kinase pathways that functionally interact with proinflammatory signaling pathways and release proinflammatory cytokines to regulate cellular activation and survival in inflammation [41,42]. Deregulation of these genes (i.e., overexpression of *c-myc*,

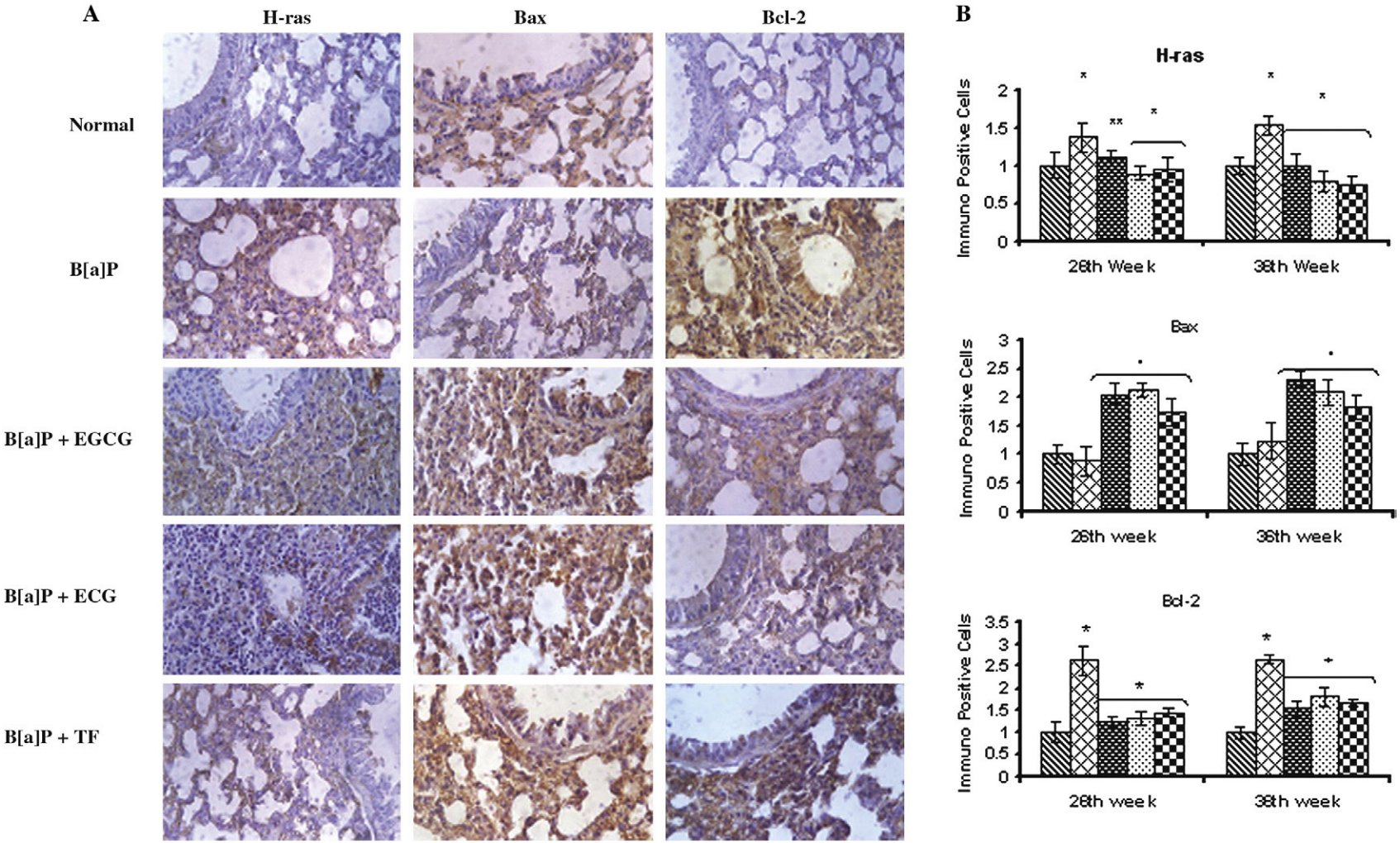

Fig. 4. Effect of tea polyphenols on *H-ras*, Bax and Bcl-2 expression detected by immunostaining. (A) Representative photograph of mouse lung lesions on the 36th week (original magnification ×400). (B) Histogram display of expression level. Results are presented as mean±S.E. (n=5 mice). **P<.05; *P<.01. (▨) Normal; (▩) B[a]P; (■) B[a]P+EGCG; (▩) B[a]P+ECG; (▩) B[a]P+TF.

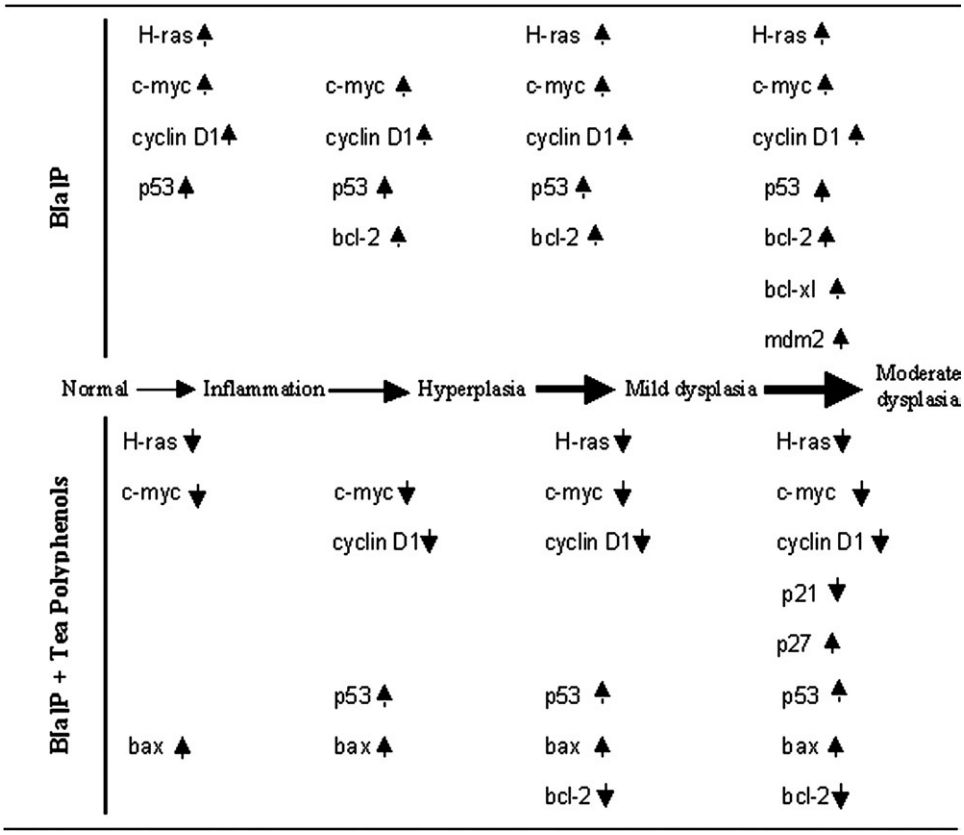

Fig. 5. Expression pattern of proliferation and apoptosis-associated genes during the progression of B[*a*]P-induced lung lesions and their modulation by tea polyphenols.

cyclin D1, *H-ras* and *p53*) has been seen during B[*a*]P, NNK and coal burning smoke-induced mouse lung carcinogenesis [6–10]. B[*a*]P was found to induce the expression of *p53* at both protein and mRNA levels in the A549 and NIH3T3 cell lines [43]. Our study shows that tea polyphenols could reduce the effect of genotoxic stress on early lung lesions by reducing the expression of *H-ras* and *c-myc* and by increasing the expression of *bax*, although cellular proliferation and apoptosis remained almost unaffected. Green tea catechins were found to inhibit chemically induced DNA adduct formation [44,45] and to protect DNA from ·OH-radical-induced strand breaks and base damage [46]. Tea extracts also produced anti-inflammatory activity in chemically induced edema model in rats and mice [47,48]. This anti-inflammatory activity of tea polyphenols may be mediated through modulation of cyclooxygenase-2, inducible nitric oxide synthase and proinflammatory cytokines, and through inhibition of chemokine-induced neutrophil chemotaxis [49–52]. Although the role of tea polyphenols as an anti-inflammatory agent in cancer deserves deeper investigation, all these investigations support the potential of tea polyphenols in reducing chronic or recurrent inflammation, whose role in the development of many types of cancer in humans is well recognized [53,54].

That B[*a*]P-induced genotoxic stress might lead to genetic alterations in subsequent stages of the progression of lung carcinogenesis is evident from this study. Among the up-regulated genes, increased expression of *c-myc*, *cyclin D1* and *p53* genes was seen throughout the progression of early lung lesions up to the premalignant stage of dysplasia. The additional genetic changes noted are likely to afford selective growth advantage to the altered cells. It seems that the increased expression of *c-myc*, *cyclin D1* and *bcl-2* in the hyperplastic lesions could override the high *p53* expression for induction of cellular proliferation and reduction of the apoptotic process at these stages.

Overexpression of c-myc, bcl-2 and mdm2, and mutation in *ras* and *p53* are common phenomena in lung cancer, and mutation in *p53* often occurs late in chemically induced mouse lung tumorigenesis [11]. The increased expression of *p53* noted in this study was not accompanied by any change in the expression of *p21*, *p27* and *bax*, indicating the inability of *p53* to influence *p21*, *p27* and *bax*. However, decreased expression of p21 and p27 was reported in mice lung carcinogenesis [10].

Significant reduction in the presence of apoptotic cells in hyperplastic and dysplastic lung lesions might be due to the increased expression of *bcl-2* at both RNA and protein levels noted in the present study. The increased expression of *H-ras* in mild dysplastic lesions and in moderately dysplastic lesions at both RNA and protein levels indicates that the *H-ras*-mediated signaling pathway was associated with

progression of the premalignant lesions. It was evident that there was cooperation between *myc* and *ras* to induce the S-phase of the cell cycle by activating cyclin/cdk activity and loss of p27 inhibition [55]. It has also been reported that the *ras* gene could positively regulate the synthesis of cyclin D1 [56] and stabilize the myc protein [57]. The increased expression of *bcl-xl* and *mdm2* observed in moderately dysplastic lung lesions might have some additive effect on the reduction of apoptosis at this stage.

Significant reduction in the proliferation and induction of apoptosis by treatment with tea polyphenols beyond the 9th week of B[*a*]P exposure resulted in restriction of lung lesions at the hyperplastic stage. This might be due to significant reduction in the expression of proliferation-associated genes (*H-ras*, *c-myc* and *cyclin D1*) and induction in the expression of apoptosis-associated genes (*p53* and *bax*) noted on the 17th week. Prolonged treatment with tea polyphenols could also significantly induce the expression of the cell cycle inhibitor *p27* in the lung lesion on the 36th week, significantly reducing the apoptotic inhibitor *bcl-2* on the 26th and 36th weeks and the cell cycle inhibitor *p21* on the 36th week. The immunohistochemical observation also supported the RNA data of *bax*, *bcl-2* and *H-ras* in lung lesions on the 26th and 36th weeks.

There are reports showing the inhibition of *c-myc* and *H-ras* by tea and its polyphenols in NNK-induced lung lesions in mice [6], of cyclin D1 and p21 in preneoplastic rat liver cells [21], and of c-myc and cyclin D1 in intestinal tumorigenesis [22]. Several in vitro studies also showed that tea polyphenols could modulate cell-cycle- and apoptosis-regulatory genes in different cancer cell lines [58–60]. EGCG has been shown to induce apoptosis in estrogen-receptor-negative human breast carcinoma cells via inhibition of bcl-2 expression and induction of p53 and bax expression [61]. Tea polyphenols are also able to augment the activity of the apoptosis executor caspase-3 in different cell lines [62]. All these studies have established a well-defined role for tea polyphenols in apoptosis.

The present report is the first of its kind to attempt a characterization of early and precancer lung lesions induced by a chemical carcinogen at the molecular level, which was correlated with histopathological changes, cell proliferation and programmed cell death (apoptosis). The modulation of these pathophysiological changes and associated gene expressions following chemopreventive intervention by tea polyphenols in the same experimental system gives an insight into the understanding of carcinogenesis and anticarcinogenesis. It is evident from the present study that cellular proliferation and apoptosis were found to occur in opposite directions during B[*a*]P-induced lung carcinogenesis and that the interplay of *H-ras*, *c-myc*, *cyclin D1*, *p53* and *bcl-2* genes has an important role in the initiation of lung carcinogenesis. It is of interest to note that the tea polyphenols can restrict lung carcinogenesis at the hyperplastic stage by modulating mainly the expression of these genes and the *bax* gene. For maintenance of restriction at the hyperplastic stage due to prolonged treatment with the tea polyphenols, additional alterations in some cell cycle/apoptosis regulators such as *p21* and *p27* are necessary. This indicates that the tea polyphenols modulate the *p53*-dependent apoptotic pathway, along with the expression of some proliferation-associated genes such as *c-myc*, *cyclin D1* and *H-ras*, to restrict the progression of B[*a*]P-induced lung lesions. These results provide molecular and cellular insights into the claimed beneficial properties of green tea and indicate that tea polyphenols are potent anti-inflammatory and proapoptotic compounds with both preventive and therapeutic potential.

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