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Original Article

Tea polyphenols prevent lung from preneoplastic lesions and effect p53 and bcl-2 gene expression in rat lung tissues

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Abstract: Lung cancer is one of the cancers that have the highest incidence and the highest mortality rate, and it is of great interest to identify ways to prevent its occurrence. We had established an animal model by using 3,4-benzopyrene intra-pulmonary injection in our previous study, and had observed that the rats lung carcinoma incidence and multiplicity were significantly reduced by green tea administration. This study further investigated the effect of tea polyphenols on rat lung preneoplastic lesions using the lung carcinoma model established by 3,4-benzopyrene intra-pulmonary injection. Sprague–Dawley rats of the same age were randomly divided into 10 groups and treated with 3,4-benzopyrene by intra-pulmonary injection. Five groups were given 0.3% solution of tea polyphenols (equivalent to 1.2% of green tea) in drinking water, while the other 5 groups were given pure drinking water. The rats were sacrificed at 0, 1, 4, 8 and 16 weeks after carcinogen treatment. In the control groups of rats, local bronchial inflammation were observed at 1 week after 3,4-benzopyrene treatment. From 4 weeks to 16 weeks after carcinogen treatment, hyperplasia, cell hyperproliferation, heterogeneity were observed in the bronchial epithelium. Meanwhile, the expression of p53 mRNA and protein, as well as the level of bcl-2, increased in the bronchial epithelial lesion. Tea polyphenols treatment significantly alleviated the bronchial epithelial lesions. At the same time, tea polyphenols treatment enhanced p53 expression, but reduced bcl-2 expression. These results indicated that tea polyphenols may have preventive effect against lung preneoplasm lesions, possibly through regulating the expression of some critical genes such as p53 and bcl-2.

Keywords: Lung cancer, cancer prevention, tea polyphenols, p53 gene, bcl-2 gene

Introduction

Since lung cancer is one of the cancers that have the highest incidence and the highest mortality rate, it is of great importance to reduce its impact on human life [1]. To this end, continuous research efforts against lung cancer have been made during the past decades, and many successes were achieved in fields such as operation, radiation and chemotherapy. However, lung cancer remains a major health threat to human health [2]. It is widely accepted that lung cancer prevention is an appropriate strategy to reduce the lung-cancer burden, and it is of great interest to discover effective agents for lung cancer prevention.

Green tea is among the most popular beverages in the world, and its benefits to human health are well-known. Extracts of green tea and green tea polyphenols exhibit inhibitory effects against the formation and development of tumors at different organ sites in animal models [3, 4]. It has been reported that when given after the NNK-treatment period until the end of the experiment, 0.6% green tea extract decreased the tumor incidence and multiplicity in A/J mice by 30% and 85%, respectively [5]. Our previous study also showed that green tea significantly reduced the incidence of rat lung carcinoma induced by 3,4-benzopyrene intra-pulmonary injection [6].

In vitro experiment have indicated that polyphenols from green tea had obvious anticancer activity in human lung cancer cell lines [7-12]. Green tea extract could alter the levels of many

proteins involved in the growth, motility and apoptosis in A549 cells [7]. Some studies demonstrated that green tea catechins (-)-epigallocatechin-3-gallate (EGCG) and (-)-epigallocatechin (EGC) had strong growth inhibitory effects on lung cancer cell lines H661 and H1299 [8], as well as A549 cells [9]. EGCG, a green tea polyphenol, is a potent apoptosis inducer that functions through a p53-dependent pathway in A549 cells [9]. In drug-resistant lung cancer cells, EGCG plays a role in inhibiting telomerase and inducing cell apoptosis [10]. EGCG is also able to inhibit the migration of bronchial tumor cells and could therefore be an attractive candidate to treat tumor invasion and cell migration [13].

These in vitro and in vivo experiments suggest that tea polyphenols might be a promising cancer chemopreventive agent against lung cancer. However, an important question remains to be answered: Do green tea and tea polyphenols prevent lung cancer from the beginning of carcinogen attack, or is its role limited to existing lung cancer cells? In our previous study, we established a rat lung carcinoma model using 3,4-benzopyrene intra-pulmonary injection, and reported that green tea regulated p53 and bcl-2 gene expression in rat lung carcinoma tissues [6]. The purpose of this study was to find out whether tea polyphenols can alleviate preneoplasm lesion induced by carcinogen treatment through regulating p53 and bcl-2 gene expression in lung tissues.

Materials and methods

Animals and treatment

One hundred of female Sprague–Dawley (SD) rats, with weights ranging from 180 g to 220 g, were purchased from the Experimental Animal Department of Central South University, and were randomly divided into ten groups. All the rats were kept in the same condition with 5 animals per cage (cage size: 50 cm × 34 cm × 21 cm) at room temperature and natural light intensity. Tea polyphenols were added to distilled water. The tea polyphenols solution prepared with each 100 mL of tea polyphenols solution contained 200 mg of EGCG, 27 mg of epicatechin (EC), 16 mg of EGC and 19 mg of (-)-epicatechin-gallate (ECG). The Catechins EGCG content in the tea polyphenols is equivalent to 1.2% of green tea. Five groups of animals were given 0.3% tea polyphenols as the sole source of drinking fluid during the experiment. The solution was prepared freshly and placed in the water bottle every day. The other 5 groups of animals were given pure water. All of the animals were treated humanely and comply with Animal Welfare Act of American. This experiment was carried out in accordance with the Animal Research: Reporting In Vivo Experiments—The ARRIVE Guidelines [14], and approved by the Medical Ethics Board of Xiangya Hospital, Affiliated to Central South University.

Carcinogen treatment and lung tissue analysis

The animals were treated with the carcinogen 3,4-benzopyrene after 2 weeks of adaption. In brief, the rats were anesthetized by intraperitoneal injection of pentobarbitone sodium (30 mg/kg body weight). The rats were then punctured in the second spatium intercostale for injection of 3,4-benzopyrene into the lung. The carcinogen 3,4-benzopyrene (from Sigma, American) was dissolved in corn oil and injected into the middle lobe of right lung at a 2-week interval, for a total of four injections. During the injection, animals were punctured at the central-point of the second spatium intercostale. After 2 mm of the needle tip-end entered into the lung tissue, the needle was stopped by a block catheter. All the animals were intramuscularly injected with penicillin (40,000 units/day for 2 days) to prevent infection following intra-pulmonary injection. The rats were closely monitored after treatment. Body weights and food consumption were measured weekly. The rats were sacrificed for tissue collection at 0, 1, 4, 8 and 16 weeks after the first 3,4-benzopyrene treatment. Animal lungs were carefully dissected and collected for pathological examination. The lung tissues were fixed in 10% formalin for 24 hour at room temperature, and were embedded in paraffin and cut into 3-mm serial sections. The lung tissues were examined blindly by pathologists.

In situ hybridization

The levels of p53 and bcl-2 mRNA were analyzed by in situ hybridization on 3 mm sections of lung tissues using digoxigenin-labeled oligonucleotide probes (1:100 dilutions). The experiment was carried out according to the instruction of Digoxigenin Labeled Probes Detection

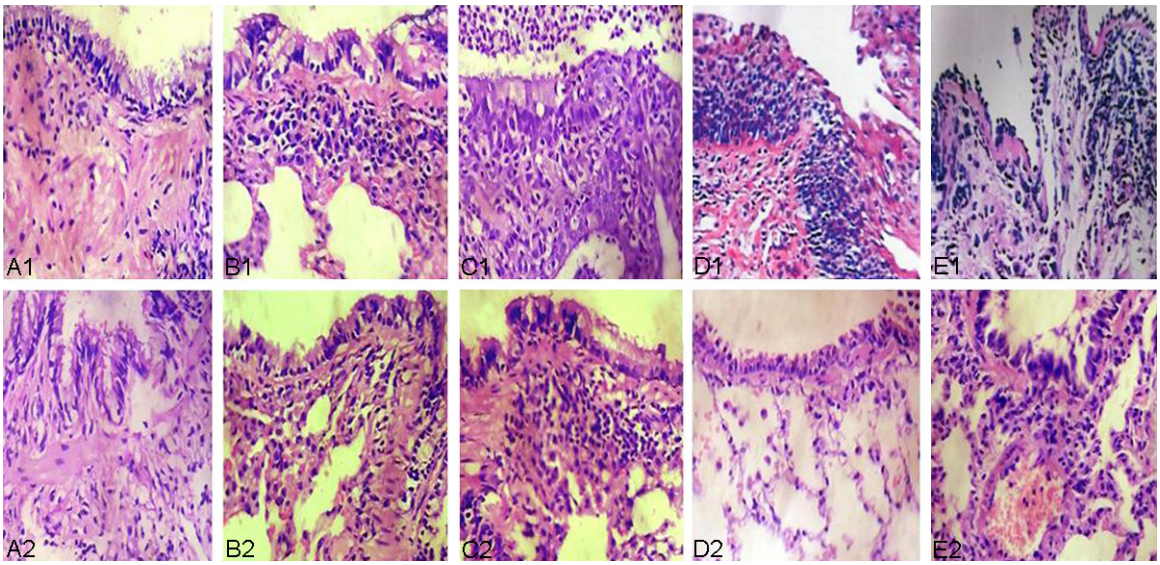

Figure 1. Pathological examination of rat lung tissue sections (×300, H&E staining). A1-E1: displayed lung tissue sections of rat lungs in different groups. A1: lung tissues of rats without 3,4-benzopyrene treatment. No abnormality was found. B1: One week after carcinogen treatment. Local bronchial mucosal inflammatory infiltration of a large amount of neutrophils and lymphocytes were observed. C1: Four weeks after carcinogen treatment. There were abnormal macrophage infiltration, local epithelial cell proliferation and cellular metaplasia in the bronchial mucosa, as well as hyperplasia of bronchial submucous gland. D1: Eight weeks after carcinogen treatment. There were cell disarrangement, epithelial cell proliferation in the bronchial mucosa and submucosa tissues. E1: Sixteen weeks after carcinogen treatment. There were severe atypical hyperplasia and heterogeneity of bronchial epithelial cells. A2-E2: showed lung tissue sections of rats treated with tea polyphenols. A2: No abnormality was found in the bronchial mucosa and submucosa tissues. B2: One week after carcinogen treatment. Slight bronchial mucosal inflammatory infiltration of fewer neutrophils and lymphocytes were observed compared to the control group. C2: Four weeks after carcinogen treatment. Relatively fewer local proliferative epithelial cells and cellular metaplasia were found compared to the control group. D2: Eight weeks after carcinogen treatment. The epithelial cells in the bronchial mucosa comparatively integrated. E2: Sixteen weeks after carcinogen treatment. No severe atypical hyperplasia and heterogeneity of bronchial epithelial cells were observed in tea polyphenols treated groups.

Kit (from Haoyang Corp of Tianjin). The sequences of gene-specific oligonucleotide probes were 5'-TTTTCTTCCTCTGTCCGACGGTCTCTCCCA-3' for p53, and 5'-CTTCAGAGACAGCCAGGAGAAATCAAACAGAGG-3' for bcl-2. The expression of β-actin mRNA in known positive samples was used as the positive control for probe sensitivity. Probe specificities for p53 and bcl-2 mRNA were confirmed by in situ hybridization on known positive sections in the absence of the probes. The scores of hybridization signals were evaluated by two researchers in a blinded fashion, according to the guideline of Pathology Techniques [15].

Immunohistochemical staining

Lung tissue sections were immunostained with antibodies specific to p53 or bcl-2 (NeoMarkers: 1:100 dilutions). The specificity of the immunostaining was confirmed by control experiments eliminating the primary antibody from the staining. Expression levels of p53 and bcl-2 proteins were estimated by counting immunostained cells and measuring staining intensity according to methods previously reported [16]. Briefly, score 0 indicates a percentage of positive cell less than 5%; scored 1 indicates 5–25% positive cells; score 2 indicates 25%–50% positive cells; score 3 indicates 50%–75% positive cells; and score 4 indicates more than 75% positive cells. The intensity of DAB staining was also defined as: score 1 for light yellow, score 2 for dark yellow or yellow–brown, and score 3 for brown staining. On each slide, five vision fields were evaluated and the average score was used for statistical analysis. All slides were scored by two researchers in a blinded fashion.

Statistical analysis

Data were analyzed with SPSS 13.0 software. The differences in p53 and bcl-2 mRNA expres-

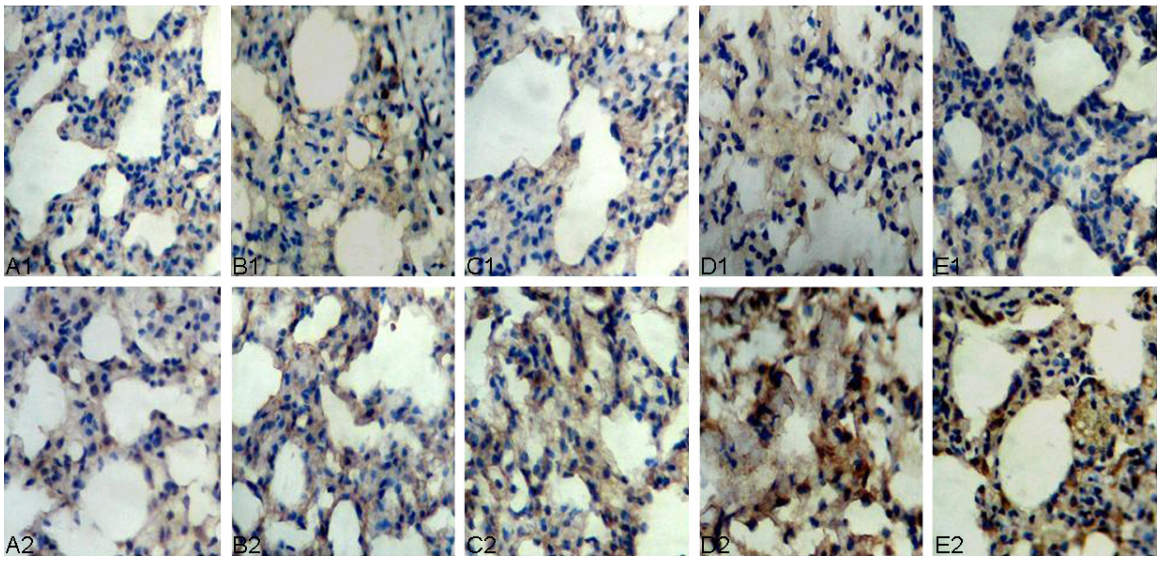

Figure 2. In situ hybridization illustrating p53 mRNA expression in rat lung tissues (×300, DAB staining). A1: Expression of p53 mRNA was negative in lung tissues of rats without carcinogen intra-pulmonary injection. B1-E1: showed p53 mRNA expression in lung tissues of rats in the control groups at 1, 4, 8 and 16 weeks after 3,4-benzopyrene treatment. The expression of p53 gradually increased in epithelial cells after 1 week of 3,4-benzopyrene intra-pulmonary injection, and p53 expression reached the highest level at 16 weeks after carcinogen treatment. A2-E2: illustrated p53 mRNA expression in lung tissues of rats in tea polyphenols treated groups. Tea polyphenols treatment further enhanced p53 expression and increased the percentage of p53-positive cells compare to the control groups.

sion were compared between tea polyphenols-treated rats and untreated rats using rank sum test. Scores of immunohistochemical staining were expressed as means±SD, and were compared by t-test. In all analyses, the null hypothesis was rejected at a level of 0.05.

Results

Histopathology

All animals recovered from intra-pulmonary injection at 12 h after treatment. Although food intake decreased slightly in the injection day and the next two days, no significant changes in overall health were observed in the rats. At 1 week after the first intra-pulmonary injection, local bronchial inflammatory infiltration of a large amount of neutrophils and lymphocytes were observed in the control group of rats. From 4 weeks to 16 weeks after intra-pulmonary injection, the rats treated with carcinogen exhibited abnormal macrophage infiltration, cell disarrangement, epithelial cell proliferation and cellular metaplasia in the bronchial mucosa and submucosa tissues, as well as hyperplasia in bronchial submucous glands. Compared with the control groups, these pathological changes induced by carcinogen were significantly alleviated in the rats with tea polyphenols administration (**Figure 1**).

Expression of p53 mRNA

The sections of lung tissues were analyzed for p53 mRNA expression by in situ hybridization with p53 specific oligonucleotide probe. No p53 mRNA was detected in lung tissues of rats without carcinogen intra-pulmonary injection. At 1 week after 3,4-benzopyrene intra-pulmonary injection, the expression of p53 increased in epithelial cells, and p53 expression reached the highest level at 16 weeks after carcinogen injection. Moreover tea polyphenols treatment further enhanced p53 expression and increased the percentage of p53-positive cells (**Figure 2** and **Table 1**).

Expression of bcl-2 mRNA

In situ hybridization revealed that the rat lung tissue had high level of bcl-2 mRNA expression at 1 week after 3,4-benzopyrene intra-pulmonary injection. Its expression reached the highest level at 16 weeks after carcinogen injection. Tea polyphenols treatment significantly reduced the percentage of bcl-2 expressing cells and

Table 1. Expression of p53 mRNA in lung tissues of rats induced by 3,4-benzopyrene treatment

Group	Total	Negative	Positive	Strongly positive	*P* value
1 week:					
Tea Polyphenols	10	3	7	0	=0.189
Model	10	6	4	0	
4 weeks:					
Tea Polyphenols	10	1	7	2	=0.111
Model	10	3	7	0	
8 weeks:					
Tea Polyphenols	10	0	7	3	=0.039
Model	10	4	5	1	
16 weeks:					
Tea Polyphenols	10	0	8	2	=0.111
Model	10	3	6	1	

reduced the level of bcl-2 mRNA in the bcl-2 positive cells (**Figure 3** and **Table 2**).

Immunohistological analysis of p53 and bcl-2

Lung tissue sections were immunostained with to p53 or bcl-2 antibodies, and the percentage of positive cells and the intensity of immunoreactivity were quantified. Epithelial cells of the lung tissue were partially stained by p53 antibody in the nuclei. In contrast the bcl-2 staining signal was mainly in the cytoplasm. Consistent with in situ hybridization results for p53 and bcl-2 mRNA, immunohistochemistry revealed a similar trend for the changes of p53 and bcl-2 proteins in the lung tissues after treatment. Tea polyphenols treatment enhanced the signal intensity of p53 immunostaining compared to control rats. In contrast tea polyphenols treatment significantly reduced the percentage of bcl-2 positive cells, as well as its signal intensity (**Figures 4**, **5** and **Table 3**).

Discussion

Protection of tea polyphenols against lung lesions induced by 3,4-benzopyrene

Due to the lack of effective screening methods and poor prognosis, the outcome of lung cancer treatment is far from ideal. Therefore, it may be a good strategy to minimize the impact of lung cancers by efficient preventive measures [17]. It is widely recognized that many chronic diseases, including lung cancer, are preventable. The development of lung cancer is consisted of several stages which include epithelial cell hyperplasia, hyperproliferation, heterogeneity and carcinoma in situ. The blocking and reversing of any point during that process by natural or synthetic agent may delay lung cancer development and reduce its morbidity. In this study, the bronchial epithelium of carcinogen-treated rats began to develop lesions of local hyperplasia, squamous metaplasia, hyperproliferation, heterogeneity at 8 weeks after the first 3,4-benzopyrene intra-pulmonary injection. However, the pathological changes of hyperplasia, hyperproliferation, and heterogeneity were significantly alleviated in tea polyphenols treated groups, which indicated that tea polyphenols might play a role in lung cancer prevention.

Extracts of green tea and green tea polyphenols have exhibited inhibitory effects against the formation and development of tumors at different organ sites in animal models [3, 4]. Green tea contains the active ingredient polyphenol. Many studies suggest that green tea polyphenol may inhibit lung cancer cell proliferation and enhance lung cancer cell apoptosis [8-10, 18]. Furthermore, green tea is a popular beverage and its health benefits are well known [19], which suggests that the risk of preventing lung cancer by daily consumption of green tea is minimal. Therefore, green tea may be a very promising beverage for lung cancer prevention. However it remains not clear whether green tea, which contains abundant tea polyphenols, prevents lung carcinogenesis through the alleviation of precancerous lesions. In this study, the rat bronchial epithelial lesions (include hyperplasia, cell hyperproliferation and heterogeneity) in tea polyphenols treated groups appeared later and were smaller than those in the control groups. The observation that tea polyphenols alleviated bronchial epithelial precancerous lesions suggests that green tea might play a preventive role against lung carci-

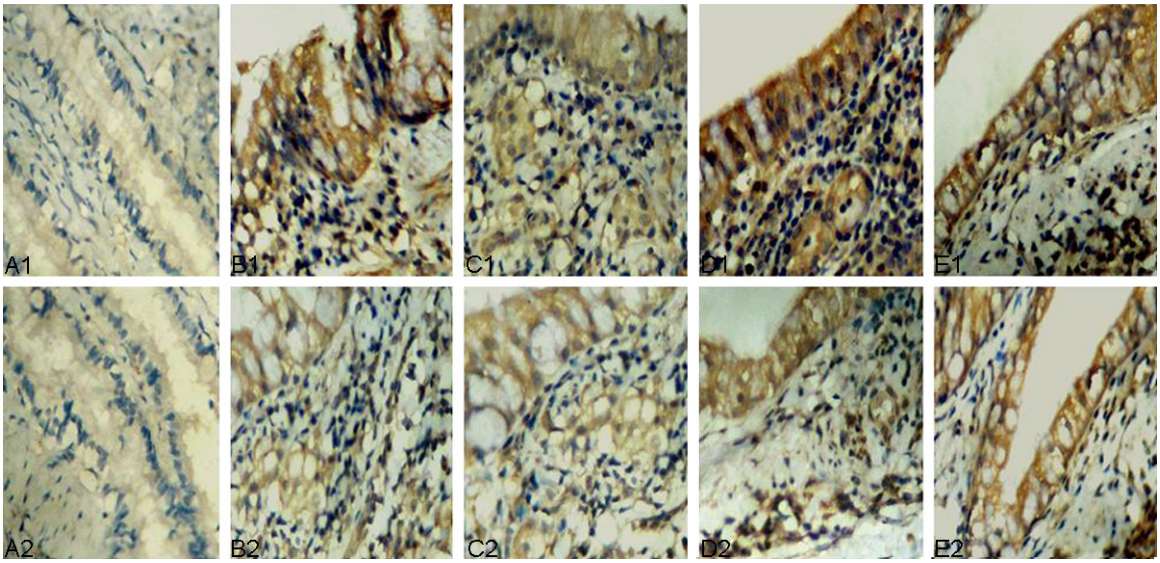

Figure 3. In situ hybridization illustrating bcl-2 mRNA expression in rat lung tissues (×300, DAB staining). A1: Expression of bcl-2 mRNA was negative in lung tissues of rats without carcinogen intra-pulmonary injection. B1-E1: showed bcl-2 mRNA expression in lung tissues of rats in the control groups at 1, 4, 8 and 16 weeks after 3,4-benzopyrene treatment. The expression of bcl-2 gradually increased in epithelial cells after 1 week of 3,4-benzopyrene intra-pulmonary injection, and bcl-2 expression reached the highest level at 16 weeks after carcinogen treatment. A2-E2: illustrated bcl-2 mRNA expression in lung tissues of rats in tea polyphenols treated groups. Tea polyphenols treatment significantly reduced bcl-2 expressing cells, as well as the levels of bcl-2 mRNA in bcl-2 positive cells compared to the control groups.

Table 2. Expression of bcl-2 mRNA in lung tissues of rats induced by 3,4-benzopyrene treatment

Group	Total	Negative	Positive	Strongly positive	*P* value
1 week:					
Tea Polyphenols	10	7	3	0	=0.028
Model	10	2	8	0	
4 weeks:					
Tea Polyphenols	10	6	4	0	=0.018
Model	10	1	8	1	
8 weeks:					
Tea Polyphenols	10	4	5	1	=0.039
Model	10	0	7	3	
16 weeks:					
Tea Polyphenols	10	5	4	1	=0.021
Model	10	0	7	3	

nogenesis in the early stages when carcinogenic attack initiates.

Effect of tea polyphenols on p53 expression in lung tissues

Genetic mutation or epigenetic modification of p53 is observed in more than 50% of human cancers [20]. The mechanism by which p53 inhibits carcinogenesis is through its function on cell cycle bock in the G1/G2 phase [21, 22]. Anything (natural or synthetic agent) regulating p53 expression may have the potential to prevent cancer. It has been reported that tea polyphenols upregulated p53 expression in lung cancer cells [9, 23]. In our previous studies, upregulation of p53 expression by green tea was also noted in cancer tissues of rat lung induced by 3,4-benzopyrene intra-pulmonary injection [6]. In the current study, lesions of hyperplasia, squamous metaplasia, hyperproliferation and heterogeneity were observed in rat lungs after 3,4-benzopyrene injection. But in tea polyphenols treated rats, the premalignant lesions were less severe. The p53 expression level was also higher in the lung tissues of these rats. Regulation of p53 expression by tea polyphenols at least partly accounted for lung

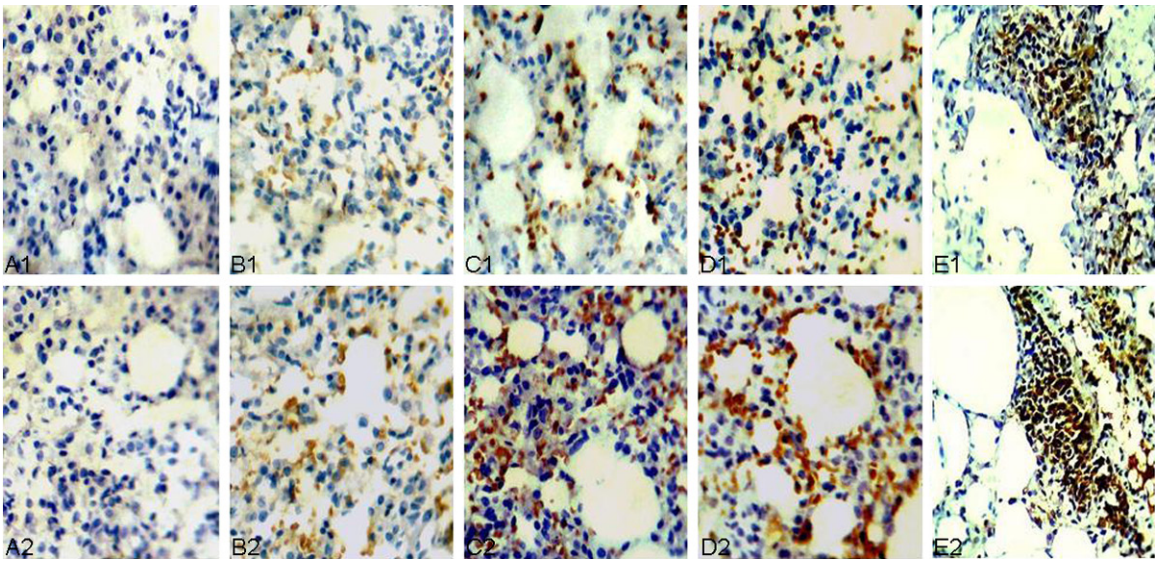

Figure 4. Immunohistochemical staining of p53 in rat lung tissues (×300, DAB staining). A1: P53 immunostaining of rat lung tissues without carcinogen intra-pulmonary injection. B1-E1: showed p53 immunostaining of lung tissues of rats in the control groups at 1, 4, 8 and 16 weeks after 3,4-benzopyrene intra-pulmonary injection. Immunohistochemistry detected p53 protein in rat lung tissues after 1 week of 3,4-benzopyrene intra-pulmonary injection, and p53 protein reached the highest level at 16 weeks after carcinogen treatment. A2-E2: showed p53 protein in lung tissues of tea polyphenols treated groups. Tea polyphenols treatment further enhanced p53 protein expression.

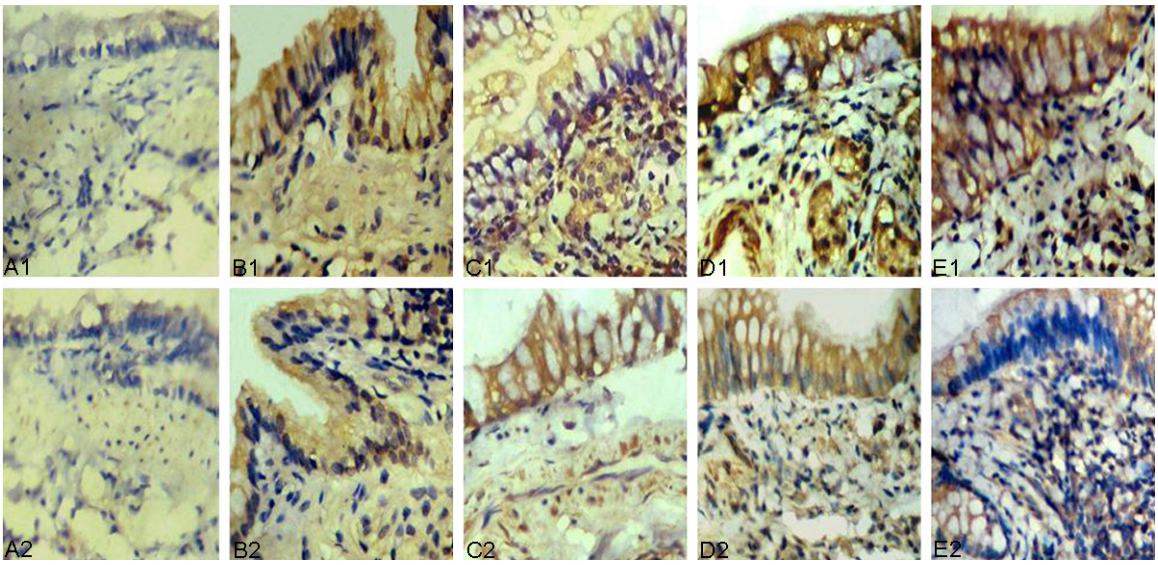

Figure 5. Immunohistochemical staining of bcl-2 in rat lung tissues (×300, DAB staining). A1: Bcl-2 immunostaining in rat lung tissues without carcinogen intra-pulmonary injection. B1-E1: showed bcl-2 immunostaining in lung tissues of rats in the control groups at 1, 4, 8 and 16 weeks after 3,4-benzopyrene intra-pulmonary injection. Immunohistochemistry detected bcl-2 protein in rat lung tissues after 1 week of 3,4-benzopyrene intra-pulmonary injection, and bcl-2 protein reached the highest level at 16 weeks after carcinogen treatment. A2-E2: showed immunohistochemical staining of bcl-2 protein in lung tissues of tea polyphenols treated groups. Tea polyphenols treatment significantly reduced the percentage of bcl-2 positive cells, as well as the intensity of bcl-2 immunostaining.

cancer prevention. When bronchial epithelia injury occurred due to the carcinogen treatment, the up-regulation of p53 by tea polyphenols caused the cells with DNA damage to arrest in G1/G2 phase, resulting in the delay of lung carcinogenesis.

Table 3. Expressions of p53 and bcl-2 proteins in lung tissues of rats induced by 3,4-benzopyrene treatment (Means±SD)

group	p53	*P* value	Bcl-2	*P* value
Before treatment:				
Tea Polyphenols	0.34±0.31	=0.599	0.76±0.47	=0.401
Model	0.26±0.35		0.92±0.34	
1 week:				
Tea Polyphenols	3.20±1.30	=0.214	2.00±1.44	=0.013
Model	2.40±1.47		3.58±1.13	
4 weeks:				
Tea Polyphenols	3.96±1.31	=0.136	2.38±1.40	=0.019
Model	2.98±1.49		3.86±1.15	
8 weeks:				
Tea Polyphenols	4.66±0.91	=0.008	3.34±1.64	=0.022
Model	2.96±1.54		4.84±0.93	
16 weeks:				
Tea Polyphenols	4.20±0.97	=0.129	3.02±1.61	=0.038
Model	3.26±1.60		4.40±1.10	

Effect of tea polyphenols on bcl-2 expression in lung tissues

As a suppressor gene of cell apoptosis, bcl-2 inhibits cell apoptosis by regulating apoptosis-related proteins. Cell apoptosis can be regulated by activation or inactivation of an inner mitochondrial permeability transition pore, which is involved in the regulation of matrix Ca^{2+}, pH and voltage. Several reports suggest that there are PT pores on the inner mitochondrial membrane which indirectly mediate the release of cytochrome c [24], and an earlier implication of MAC pore on the outer membrane [25, 26]. In the process of apoptosis, apoptosis-related proteins mediate the release of cytochrome c into the cytosol through regulating pro-apoptotic and anti-apoptotic proteins. The cytochrome c, once in the cytosol, activates caspase-9 and caspase-3, leading to apoptosis. Bcl-2 is an essential component in this process because it suppresses the initiation of apoptosis by inhibiting mitochondrial permeability transition pore. Overexpression of bcl-2 could inhibit cell apoptosis and increase the risk of cell malignant transformation. In this study, we showed that tea polyphenols treatment significantly reduced the levels of bcl-2 mRNA and protein, which reversed the effect of carcinogen 3,4-benzopyrene. Therefore, the inhibition of bcl-2 gene expression by tea polyphenols may play a role in early prevention of epithelial cell lesions.

In conclusion, at the basis of the lung tumor-bearing model, rats lung preneoplastic lesion had generated in the bronchial epithelial before carcinoma formed. Meanwhile, the expression of p53 mRNA and protein, as well as the level of bcl-2, increased in the bronchial epithelial lesion. As proposed, tea polyphenols treatment significantly alleviated the bronchial epithelial lesions. At the same time, tea polyphenols treatment enhanced p53 expression, but reduced bcl-2 expression. Our preliminary data suggests that tea polyphenols may exert its preventive effect against lung cancer by regulating the expression of p53 and bcl-2 genes. However, the mystery is far from solved, and other mechanisms could exist. Further study is warranted to unravel the mechanisms by which tea polyphenols suppresses lung cancer formation.

Acknowledgement

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

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