RESEARCH COMMUNICATION

Inhibition of Growth, Induction of Apoptosis and Alteration of Gene Expression by Tea Polyphenols in the Highly Metastatic Human Lung Cancer Cell Line NCI-H460

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Abstract

Lung cancer is a complex group of diseases but each lesion is thought to originate from a single mutated progenitor cell. It is evident that multiple genetic changes are involved in the generation of each specific type of lung cancer. Due to the high complexity of these processes and rapid metastasis, treatment of advanced lung cancer, particularly of NSCLCs, is far from satisfactory. Thus, there is a need for innovative strategies for modulation of adverse alteration in protooncogene or tumor suppressor genes so that lung carcinogenesis can be suppressed or delayed. To this end, we have evaluated the effects of tea compounds (theaflavins, epicatechin-gallate and epigallo-catechin-gallate) on proliferation and apoptosis and associated gene expression in a highly metastatic human lung cancer cell line NCI-H460. Significant reduction of cell proliferation, detected in situ by BrdU incorporation, and induction of apoptosis, assessed by the by the TUNEL method, were noted following treatments. Expression of p53, Bcl-2, c-Myc and H-Ras, was localized by immunocytochemistry and analysed by Western blotting. Tea compounds upregulated expression of p53, downregulated expression of Bcl-2 but there was no significant influence on H-ras and c-Myc expressions. It is suggested that tea compounds can influence genetic alteration to disfavour, growth and survival of lung cancer cells.

Key Words: Tea polyphenols - growth - apoptosis - gene expression - lung cancer cell line

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Introduction

Non-small cell lung cancers (NSCLCs) represent approximately 80% of all lung cancers, and no curative treatment is available for advanced stages. Despite considerable advances in the understanding of the molecular pathogenesis of lung cancer, only one in eight patients diagnosed as having lung cancer may respond to treatment (Minna et al, 1989). Current treatment of NSCLC involves surgical resection coupled with the use of cytotoxic drugs. The surgical procedures are highly invasive and are applicable to only a small number of patients. Standard chemotherapy is broadly used but offers only a limited survival benefit at the expense of substantial toxicity, drug resistance, poor target specificity and genetic complexity. Thus, there is a need for innovative strategies that would be effective against the adverse alteration of protooncogene or tumor suppressor genes to delay or suppress lung carcinogenesis and effectively reduce occurrences.

Tea polyphenols have superior antioxidant properties compared to any other antioxidant present in fruits and vegetables, known to mankind (Jhawar, 2000). Chen and Ho extensively investigated the antioxidative properties of various tea polyphenols. (Chen, 1994) Most of the green tea polyphenols are flavonols, commonly known as catechins. (Ahmad and Hassan, 1999). These catechins, especially epigallo-catechin gallate (EGCG) and (-)-epicatechin gallate (ECG), the major polyphenolic constituents of green tea, have been studied as a preventive substance for carcinogenesis (Fujiki et al, 1999). Different green tea catechins are oxidized and dimerized during the manufacture of black tea to form orange-red pigments, theaflavins (TF), a mixture of theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3'-gallate (TF2B) and theaflavin-3, 3'-digallate (TF3) (Roberts, 1962, Davis et al, 1995). According to Halder and Bhaduri (1998) theaflavins and thearubigins, major component of black tea also possess antioxidant properties. We have reported the anticarcinogenic

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and chemopreventive role of black tea in several experimental models including lung (Saha and Das, 2002, 2003, Sengupta et al, 2002,2003, Das et al, 2004, Banerjee and Das, 2004, Banerjee et al 2005)

It has been clearly established that lung cancer is a disease caused by accumulation of multiple genetic alterations in both oncogenes and tumor suppressor genes (Minna et al, 1989). We had reported of the chemopreventive efficacy of tea compounds during progressive lung carcinogenesis in mouse, where we found induction of apoptosis and restriction of cell proliferation resulting in delayed formation of carcinoma in situ (Banerjee and Das, 2005, Banerjee et al, 2005). Altered expression of p53, C-Myc, Bcl-2 and H-Ras was also noted (Saha et al, 2005). The present study was designed to evaluate cell proliferation, apoptosis and expression of these genes following treatment with green tea compounds viz. EGCG, ECG and Black tea extract theaflavins on a highly metastatic human lung cancer cell line (NCI-H640).

Materials and Methods

Tea Compounds: Epicatechin gallate (ECG) and Epigallocatechin gallate (EGCG) extracted from green tea and black tea extract theaflavins (TF) were obtained from Sigma Chemical Co. USA.

Cell culture and treatment: Human non-small cell lung cancer cell line NCI-H640 was obtained from the American Type Culture Collection. Cell line was cultured in RPMI 1840 media supplemented with heat inactivated 10% fetal bovine serum, 0.03% L-glutamine (Gibco, New York, USA), 2000 unit/ml penicillin, 200µg /ml streptomycin and 100 µg/ml genticin in completely humidified air with 5% CO2 at 37^0C. Every third day exponentially growing cells were sub cultured by seeding $2x10^5$ cells/ml in 5 ml tissue culture flasks. Cells counts and viability were determined using hemocytometer and trypan blue dye exclusion.

Cells in fresh media were incubated for 24,48 and 72 hrs. with either ECG, EGCG or TF at a concentration 100µM for $2.5x\ 10^5$ cells. The control group consisted of cells treated with deionized water.

In situ Cell Proliferation: Proliferating cells were localized in situ following incorporation of 5-bromo-2'-deoxy-uridine (BrdU) using BrdU Labeling and Detection Kit II, AP (Roche) as per kit protocol. For this purpose cells attached to cover slips (control & treated) were subjected to BrdU labeling of DNA in proliferating S-phase cells. The cells were harvested, fixed with 4% paraformalin and incubated with an anti-Brdu monoclonal antibody at 37^0C for 30 min, which was detected by an alkaline phosphatase-conjugated-antimouse-immunoglobulin antibody (anti-mouse-Ig-AP). The bound anti-mouse-Ig-AP was visualized using nitroblue tetrazolium (NBT), an AP-substrate. At least 300 proliferating cells were counted for each group with phase contrast microscopy and proliferative index calculated.

In situ cell death detection: 2.5x 105 cells/ml were plated onto cover slips placed in 35mm tissue culture dishes the day before the treatment. Cells were washed with phosphate-buffered saline (PBS) once, then placed into the RPMI 1840 medium containing ECG, EGCG or TF at the concentration indicated. After exposure to tea compounds for the periods of time indicated, the cells were fixed with 4% Paraformalin in PBS. Cells were then incubated with TUNEL reaction mixture provide in the in situ cell detection kit, AP (Roche Molecular Biochemiocals Mannheim, Germany), at 37^0C for 30 min, rinsed in PBS, mounted in glycerol and analyzed under a fluorescence microscope (NIKON-600) using an excitation wave length in the range of 450-500 nm and detection in the range of 515-565 nm. About 300 cells were counted for each group with fluorescence microscopy, and apoptotic index calculated.

Immunocytochemical detection of gene expression: Cells, which were attached to cover slip as described above, were fixed in chilled methanol for 20 min at -20^0C, and incubated in 10% normal blocking serum in PBS to suppress the nonspecific binding and treated with different primary antibodies for p53, Bcl-2, C-Myc and H-ras (Santa Cruz Biotechnology, California, USA), followed by respective FITC conjugated secondary antibodies (Santa Cruz Biotechnology, California, USA). Observation in fluorescence microscope (NIKON ECLITSE E600, Japan) made at Ex- 450-490,Dm-505, Ba –520. Different gene expression were localized in cells, scored and expressed

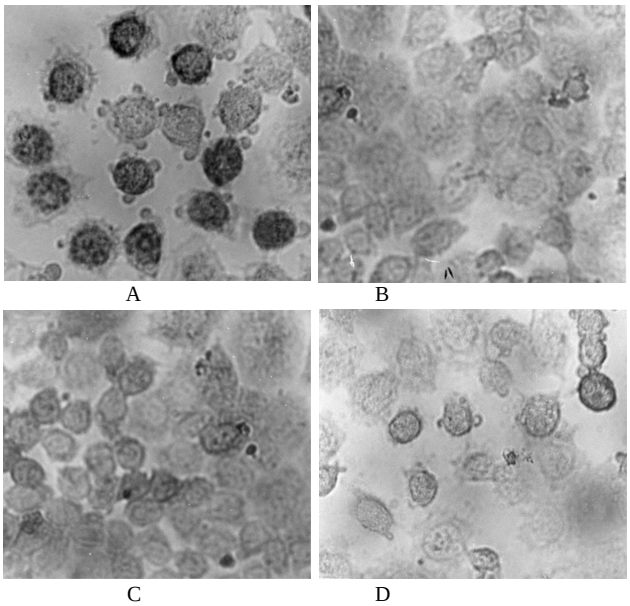

Figure 1. Immunocytochemical Detection of Proliferating Cells.

(a) BrdU labeled proliferating NCI-H460 cells (control)
(b) Reduced number of proliferating cells after 48 hrs. treatment with Theaflavins.
(c) Same as (b) to show inhibitory effect of (-)Epicatechin gallate treatment.
(d) Cell proliferation labeled after 48 hrs, treated with Epigallocatechin gallate.

semi-quantitatively (Carbone et al, 1994) as follows:Negative -, 1-25 % positive cells +, 26-50% positive cells ++, 75% positive cells +++, 76- 100% positive cells ++++.

Western Blot Analysis: 20 µg of total cell lysate solubilized in in ice-cold homogenizing buffer (0.1M NaCl, 0.1M Tris and 0.001M EDTA) containing protease inhibitor (1µg/ml Aprotinin, 0.1mM Leupeptin and 1mM PMSF) was electrophoresed on 12.5 or 15% sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon-P filters (PVDF, Sigma, USA) along with prestained molecular weight markers (Rainbow markers, Amersham, USA). Membranes were subsequently blocked with 5% TBS blotto A (non-fat dried milk, Santa Cruz Biotech, USA) and again incubated with primary antibodies against p53, Bcl-2, H-ras and c-Myc (Santa Cruz Biotechnology, California, USA) and then with a HRP conjugated secondary antibody (Santa Cruz Biotechnology, California, USA). The protein bands were visualized by using Luminol reagent (Santa Cruz Biotech, USA) in the dark room and the bands were exposed to X-ray film and developed by conventional methods.

The data on proliferation and apoptosis assay were analyzed by Students-t-test using MS Excel and a p value of < 0.05 was considered significant.

Results

Effects of Tea Compounds on Proliferation:

BrdU labeled proliferative cells are shown in Fig-1, a-d. Proliferative index in the control group was 24.9, which was reduced to 10.1, 9.2 and 12 by treatment with TF, ECG and EGCG, respectively (Fig-2). The results were highly significant ($p<0.01$) in all cases.

Effect of tea compounds on Apoptosis:

Fluorescent cells representing apoptotic cells seen in control and treated groups are shown in Fig-3, a-d. It may be noted from Fig-4 that the apoptotic index was significantly ($p<0.01$) elevated following treatment. The effect of TF was maximum followed by EGCG and ECG.

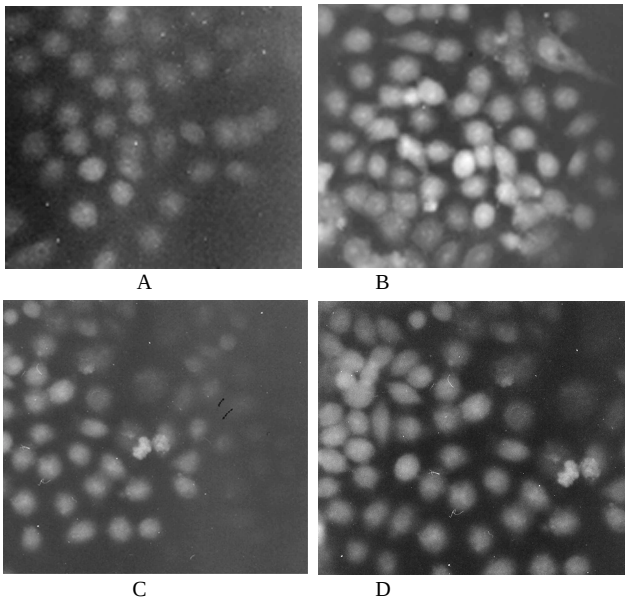

Figure 3. Immunocytochemical Localization of Apoptotic Cells.

(a) Apoptotic NCI-H460 cells (control).
(b) Increased number of apoptotic cells after 48 hrs. treatment with Theaflavins.
(c) Few apoptotic cells after treated with (-)Epicatechin gallate.
(d) Apoptotic cells labeled after 48 hrs, treatment with Epigallocatechin gallate.

Effect of TF, ECG and EGCG on gene expression:

Nature of in situ expression p53, Bcl-2, H-ras and c-Myc in NCI-H460 cells is shown in Fig-5 and the expression of these proteins by western blot is shown in Fig-6. As evident from Table I, both green and black tea compounds

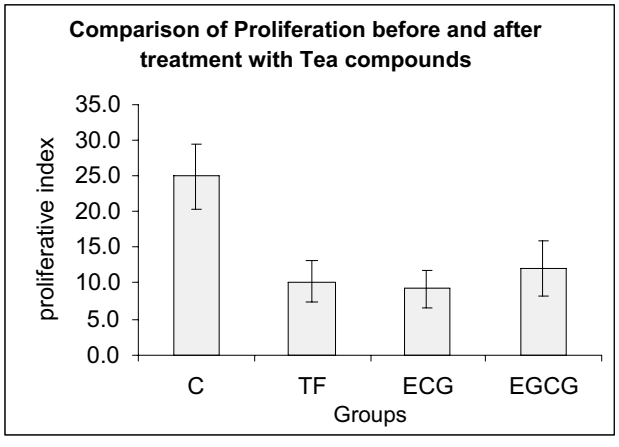

Figure 2. Effect of Theaflavins, Epicatechin Gallate and Epigallocatechin Gallate on NCI-H640 Cell Proliferation Represented Graphically after 48 hrs of Treatment. The influence of treatment may be noted in the form of inhibition on cell proliferation.

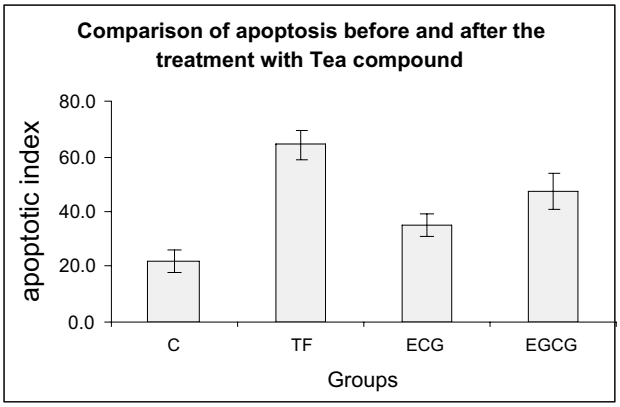

Figure 4. Effect of Tea Compounds (Theaflavins, Epicatechin Gallate and Epigallocatechin gallate) on Cell Death in NCI-H640 Cells. Apoptosis represented graphically after 48 hrs of treatment. Significant induction of apoptosis was noted after every treatment, among them theaflavins showed best action.

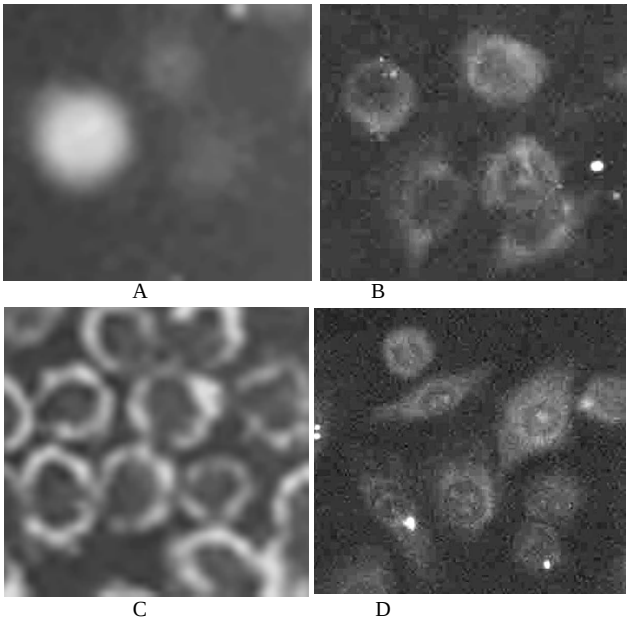

Figure 5. Microphotographs to Show Cellular Localization of p53, Bcl-2, H-Ras and c-Myc Expression in NIH-H640 Cells (X 900).

(a) Distinct nuclear localization of p53 noted in lung cancer cells.
(b) Cytoplasmic localization pattern of Bcl-2 protein expression.
(c) H-ras expression in cell cytoplasm in NIH-H640 cells.
(d) Nuclear localization of c-Myc expression in human lung cancer line.

tested were found to influence p53, Bcl-2, and C-Myc expressions. However no changes were noted in the expression of H-ras. Only 15% of the cell population in the control group was found to be p53 positive during the period of observation (72 hours). While in the TF treatment group p53 expression was initially 20% further increasing to 30%, in case of ECG and EGCG treatment the expression level was around 30% all through. Bcl-2 gene was highly expressed in this cell line. Approximately 60% cells show Bcl-2 positivity throughout (24- 72 hours). TF

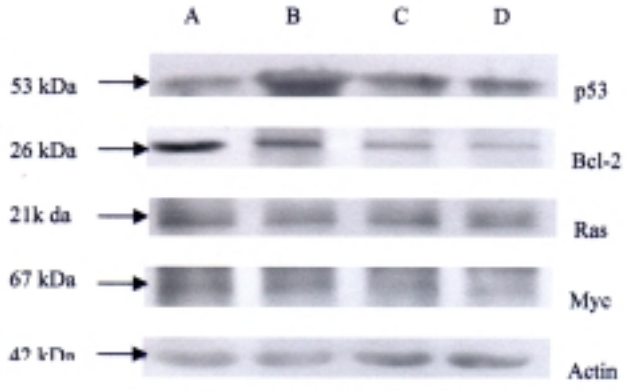

Figure 6. Effects of Tea Compounds on Protein Expression in NIH-H640 Cells Evaluated by Western Blotting. Expression of p53 was upregulated after treatment whereas downregulation of Bcl-2 expression observed after treatment. There was no significant difference in H-ras and c-Myc expression between treated and untreated groups.

Table 1. Effect of Tea Compounds on Gene Expression in NCI-H640 Cells in vitro (C- control, TF- theaflavins, ECG-epicatechin gallate and EGCG-epigallocatechingallate Treatment)

Duration of treatment	C %of+cells	TF %of+cells	ECG %of+cell	EGCG %of+cells
	Expression of p53 gene			
24 hr	+	+	++	++
48 hr	+	++	++	++
72 hr	+	++	++	++
	Expression of Bcl-2 gene			
24 hr	++	+	++	+
48 hr	++	++	++	++
72 hr	+++	++	++	++
	Expression of H-Ras gene			
24 hr	++++	++++	++++	++++
48 hr	++++	++++	++++	++++
72 hr	++++	++++	++++	++++
	Expression of c-Myc gene			
24 hr	++	+	++	+
48 hr	++	+	+	+
72 hr	++	+	+	+

1-25 % positive cells = '+', 26-50% positive cells = '++', 75% positive cells = '+++' and 76- 100% positive cells = '++++'.

downregulated this expression (23% after 24 hours) which was however not sustained. A similar observation was made with respect to ECG and EGCG. H-ras was uniformly expressed with high intensity. Neither TF nor ECG and EGCG had any influence on this gene expression. c-Myc was expressed moderately all through. Both TF and EGCG treatment resulted in decrease of c-Myc expression, which was at 9%, 8% and 6% respectively after 72 hours.

Discussion

In the present study, upregulation of p53 after treatment with tea compounds was observed, suggesting an influence on a major genetic change, presumably related to the induction of apoptosis noted. p53 is a prototype tumor suppressor gene that is the most common genetic lesion in human cancers (Harris and Deichmann,1995) and mutated in over two thirds of lung cancers. (Bennett et al, 1993).

The Bcl-2 oncogene, first identified in follicular lymphoma encoding a membrane associated protein that is present in the endoplasmic reticulum, nuclear and outer mitochondrial membranes, is over expressed in many human neoplasms including lung carcinoma suggesting a role for this oncoprotein in the pathogenesis of neoplasia. Ben-Azra et al,1994, Colombel et al, 1993, Lipponen et al, 1995, Piris,1994.

Increased expression of protein product of Bcl-2 gene appears in the early phase of carcinogenesis leading to apoptosis impairment and in consequence to the progression of neoplastic changes (Jordan et al, 1996, Nakagawa et al, 1994, Reed, 1994). We noted lower expression of this protein in human non-small cell lung cancer cell line NCI-H640 after treatment with tea components in comparison to

untreated group, where the expression was higher (darker band).

It is interesting to note that treatment with TF, ECG and EGCG can upregulate p53 simultaneously with downregulation of Bcl-2, which is reflected in increased apoptotic index. This observation suggests that the tea compounds can modulate these gene expressions in transformed metastatic cells, assuming therapeutic significance.

BP exposed mouse lung revealed G-T transversion in K-ras, (Mass et al, 1993) and a mutated form of the K-ras-2 gene, generally seen in smokers, was suggested to stimulate growth rate in lung cancer cells. Recently we reported of a weak or no expression of this gene in the early stages of BP induced lung carcinogenesis in mouse (hyperplasia and dysplasia), which was however expressed in lesions showing carcinoma in situ (Saha et al, 2005). The present study shows that even in human lung cancer cell line the expression of H-ras was considerably high and not influenced by the treatment.

C- Myc, whose key biological function is related to promotion of cell cycle (Amati.2001, Amati et al, 1998, Dang, 1999, Eilers, 1999) was noted to express increasingly along with progression of carcinogenesis. TF, ECG and EGCG did not seem to influence the expression of these gene associated with cell proliferation, to the extent noted for apoptosis regulatory genes studied here.

It has been estimated that 85% of lung cancer are associated with tobacco use (Shopland, 1995). It may be possible to prevent a substantial portion of lung cancer by cessation of tobacco smoking along with preventive measures including healthy eating and drinking habits. Our studies in vivo and in vitro suggest that tea containing strong anti-carcinogenic compounds like TF, ECG and EGCG should be able to interfere with the expression of p53 and Bcl-2 and alter the process of cell proliferation and apoptosis for prevention and control of cancer.

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