Role of Ku70 and Bax in epigallocatechin-3-gallate-induced apoptosis of A549 cells *in vivo*

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Abstract. EGCG (epigallocatechin-3-gallate), the major catechin found in green tea, has been demonstrated to inhibit proliferation and induce apoptosis in a number of types of tumors. Recent studies reveal that EGCG has various anticancer effects. This study investigated a further possible molecular mechanism of the anticancer effects of EGCG in murine lung cancer xenografts. In the study, A549 human lung cancer cells were injected into nude mice. Tumor volume was used to measure cancer cell growth. The weight of the animals was used to assess the toxicity of the drugs. The expression of protein and mRNA was assayed by western blot analysis and RT-PCR, respectively. The interaction between Bax and Ku70 was determined by immunoprecipitation. Our results suggest that EGCG induced A549 lung cancer cell apoptosis *in vivo*, and had less toxic effects compared to classical anticancer drugs. EGCG may inhibit the surrogate markers of proliferation and apoptosis (caspase 3) in A549 tumor xenografts *in vivo*. In addition, EGCG downregulated the expression of Bcl-xl and upregulated the expression of Bax mRNA and protein. Further experiments indicated that EGCG downregulated the protein expression of Ku70 and interrupted the binding of Ku70 and Bax. This is the first study demonstrating that the induction of apoptosis by EGCG may be caused by the downregulation of Ku70 and that EGCG disrupts the interaction between Ku70 and Bax in lung cancer.

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Abbreviations: EGCG, epigallocatechin-3-gallate; NHEJ, nonhomologous end-joining; BIP, Bax-inhibition peptide; FBS, fetal bovine serum; IP, immunoprecipitation; RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, one-way analysis of variance; DSB, double-strand break; HDAC, histone deacetylase

Key words: EGCG, lung cancer, apoptosis, Ku70, Bax

Introduction

Green tea, the second most popular beverage worldwide, has potential chemotherapeutic effects against a wide range of malignancies. Epidemiological and rodent carcinogenesis studies have provided evidence that green tea, particularly its major constituent, epigallocatechin-3-gallate (EGCG) has various anticancer effects, including inhibition of carcinogen-induced mutagenesis (1,2), induction of cell cycle arrest (3), induction of apoptosis (4), inhibition of growth factor-mediated proliferation (5), inhibition of transformation (6), inhibition of angiogenesis (7) and inhibition of telomerase activity (8). To date, there have been no studies examining Ku70 in lung cancer; however, such studies may lead to new approaches in the treatment of lung cancer.

Ku70 was first characterized as part of the Ku70/Ku80 heterodimer that is essential for the repair of DNA double-strand breaks (DSBs) by the nonhomologous end-joining (NHEJ) pathway and rearrangement of antibody and T cell receptor genes via V(D) J recombination (9). It has been associated with numerous diseases, including *Rickettsia conorii* infection (10), sterility (11), Fanconi anemia (12) and cancer (13). It is a highly versatile regulatory protein that has been implicated in several nuclear processes, including DNA repair, telomere maintenance and apoptosis. Accordingly, Ku70 is considered to play a vital role in the maintenance of chromosomal integrity and cell survival. A study has suggested that there is a positive correlation between Ku70 and the development of cancer (14), indicating that Ku70 is an important candidate target for anticancer drug development. Specifically, further studies have suggested that a delicate balance exists in Ku70 expression, with overexperssion of Ku70 leading to genomic instability and tumorigenesis (15,16).

Another study demonstrated that Ku70 is able to suppress apoptosis by sequestering Bax from the mitochondria in cancer tissues (17). In contrast, when Ku70 is released from Bax, it allows Bax to translocate to the mitochondria and trigger cytochrome C release, leading to caspase-dependent apoptosis. These studies suggest that Ku70 degradation is a necessary step in the activatation of Bax-mediated apoptosis. Further analysis revealed that Bax inhibition peptide (BIP), which is comprised of five amino acids designed from the Bax-binding domain of Ku70, is able to inhibit Bax-mediated apoptosis and suppress the mitochondrial translocation of Bax (18). Pretreatment of Hela cells with BIP peptides for 1 h

was sufficient to provide protection from staurosporine (STS)- and ultraviolet C (UVC)-induced apoptosis (18).

However, the exact role of Ku70 in the anticancer mechanism of EGCG remains unknown. *In vivo* animals are considered a gold standard in chemotherapy studies, as they provide clear indications on the pharmacological and therapeutic effects of chemotherapy agents, which may then be extrapolated to humans, as examined in this study.

Materials and methods

Chemicals and antibodies. Purified EGCG (>95% pure) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies against various proteins were obtained; Ku70 mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA), Bcl-xl (Proteintech Group, Chicago, IL, USA), cleaved caspase 3 (Assay Technology Inc., Livermore, CA, USA), pan-acetylated-lysine (Santa Cruz Biotechnology) and β-actin (Sigma-Aldrich). Goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugates and goat anti-mouse IgG-HRP conjugates were purchased from Sigma-Aldrich.

Cell lines and cell culture. The human lung cancer A549 cell line was purchased from the Cell Center of Central South University, Hunan, China. The cells were cultured as a monolayer in 10% fetal bovine serum (FBS)-supplemented RPMI-1640 containing 100 units/ml penicillin and 100 Ag/ml streptomycin and were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C.

Animal experiments. A total of 24, 4- to 6-week-old, female BALB/c mice were kept in groups of six per cage and provided with food and water *ad libitum*. The animals were acclimatized for 1 week before use and maintained throughout at standard conditions: 24±2˚C temperature, 50±10% relative humidity and 12-h light/12-h dark cycle.

A549 cells were detached from the culture dishes by trypsinization and then collected, washed and resuspended in RPMI-1640. To establish A549 tumor xenografts, mice were injected s.c. with 1-2x10^6 A549 cells in the right flanks. The mice were then randomly divided into three groups, each consisting of 8 animals. Animals in group I (control) received normal saline at 10 ml/kg, i.p. daily for 2 weeks; group II received cisplatin at 4 mg/kg, i.p. Q4dX3; and group III received EGCG at 50 mg/kg, i.p. daily for 2 weeks. Body weight was recorded each day throughout the study. Once xenografts started growing, their sizes were measured each day using Vernier calipers. The tumor volume was calculated using the formula: Volume = π/6 x length x $width^2$. When the tumors reached a volume of 100 mm^3, the animals were administered different drugs. At the termination of the experiment, animals were sacrificed, and the tumors were surgically removed, weighed and then stored at -80˚C for further biochemical analysis.

All animals were housed and handled according to Central South University Institutional Animal Care and Use Committee guidelines and animal work was approved by the appropriate committee. All experiments were performed according to institutional guidelines and approved by the Ethics Committees of our hospital and conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

Western blot analysis and immunoprecipitation. Western blot analysis was conducted to determine the expression of different proteins. Tumor tissues were collected at the termination of the experiment, minced, homogenized with homogenizer in ice-cold lysis buffer and lysed with ice-cold NP-40 lysis buffer for 30 min, then centrifuged at 14,000 x g for 20 min at 4˚C. The supernatant was collected and either used immediately or stored at -80˚C to examine the expression of different proteins using western blot analysis and immunoprecipitation. Protein concentration was determined using a BCA protein assay kit according to the manufacturer's instructions.

Western blot analysis was conducted to analyze the expression of various proteins following the manufacturer's instructions. Briefly, aliquots of equal amounts of protein (40-80 μg) from the tumor lysates were subjected to 12% SDS-PAGE electrophoresis and transferred to PVDF membranes. The membranes were blocked with blocking buffer by incubating for 1 h at room temperature, then probed overnight with the desired primary antibody at 4˚C. Following washing, the membranes were incubated with the respective HRP-conjugated secondary antibody for 1 h at room temperature. After further washing, protein expression was detected by enhanced chemiluminescence detection systems (Thermo Scientific, Waltham, MA, USA).

For immunoprecipitation of Ku70, 1 mg protein was precleared by incubation with protein A/G Sepharose beads (Santa Cruz Biotechnology). The supernatant was incubated with agarose-conjugated anti-Ku70 antibody, followed by three washes in 1% Triton in PBS. The immunocomplex was separated by SDS-PAGE and proteins were detected with anti-pan-acetyl-lysine (panAc-K) antibody or anti-Bax antibody.

Reverse transcription (RT)-PCR. RT-PCR analysis was conducted to determine the expression of different mRNA. Tumor mRNA from different groups was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed with primer pairs for Bax: forward, 5'-AGCTCT GAGCAGATCATGAAG-3' and reverse, 5'-GGTGGACGC ATCCTGAG-3'; for Bcl-xl: forward, 5'-ACTGTGCGT GGAAAGCGTAG-3' and reverse, 5'-AAAAGTATCCCA GCCGCC-3'; for Ku70: forward, 5'-TCTTGGCTGTGG TGTTCTATGGT-3' and reverse, 5'-GAGTGAGTAGTC AGATCCGTGGC-3'; for GADPH: forward, 5'-AGAAGG CTGGGGCTCATTTG-3' and reverse, 5'-AGGGGCCAT CCACAGTCTTC-3'. The standard PCR conditions were: 95˚C for 15 min and then 35 cycles at 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec.

Statistical analysis. Data are presented as the means and standard deviations (SDs). Groups were compared by a one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

EGCG inhibits in vivo growth of A549 lung cancer cells. The rate of tumor growth of A549 cells, evaluated by measuring

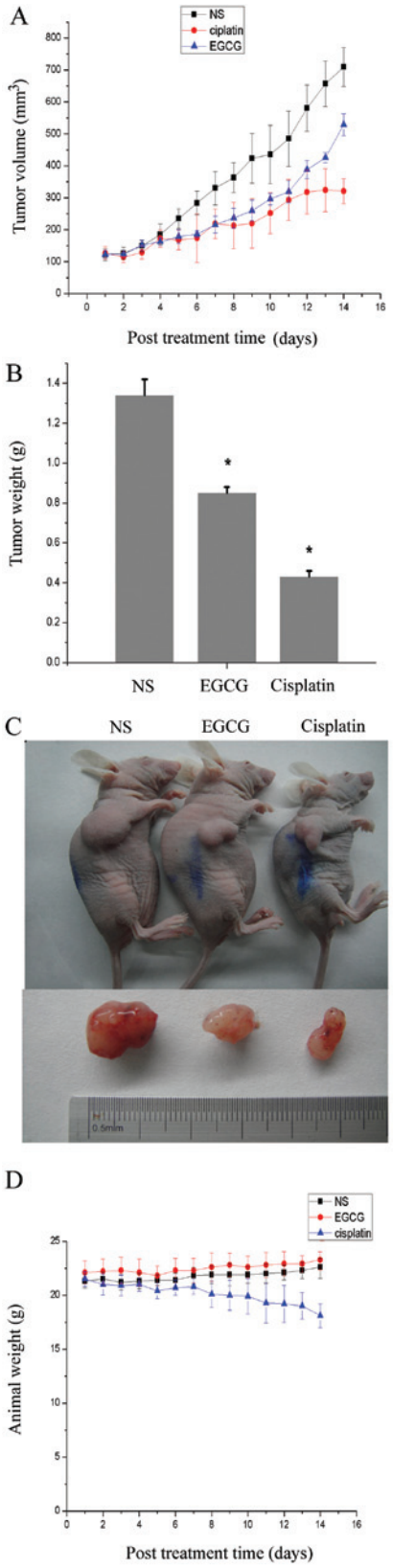

Figure 1. *In vivo* antitumor effect of EGCG on A549 cell-induced tumor xenografts in nude mice and the toxic effect of EGCG. (A) Treatment of A549 tumors with NS, EGCG and cisplatin. Points, mean tumor volume versus time; bars, SD. (B) Tumor weight recorded at the termination of the experiment in the three groups. (C) Mice with A549 xenografts and representative tumors. (D) Treatment for A549 tumors with NS, EGCG and cisplatin. Points, mean animals weight versus time; bars, SD. ANOVA test: *P<0.05 vs. control. EGCG, epigallocatechin-3-gallate; NS, normal saline; SD, standard deviation.

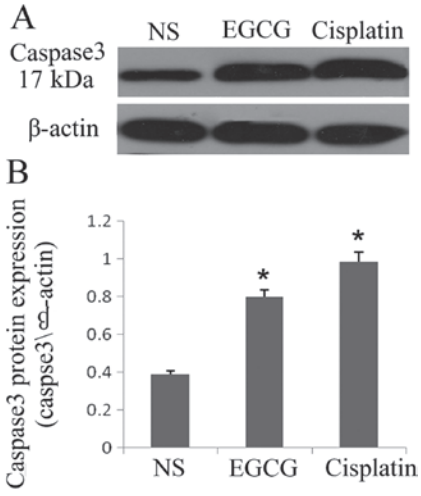

Figure 2. Treatment of A549-induced tumors in mice with EGCG increases caspase 3 activity and increases the protein expression level of cleaved caspase 3. (A) The level of cleaved caspase 3 in tumor tissue lysates was determined using western blot analysis after treating the mice with NS, EGCG or cisplatin for 14 days. (B) The bands were quantified by densitometry and are represented graphically. The results are the mean of three independent experiments. Bars, SD. ANOVA test: *P<0.05 vs. control. EGCG, epigallocatechin-3-gallate; NS, normal saline; SD, standard deviation.

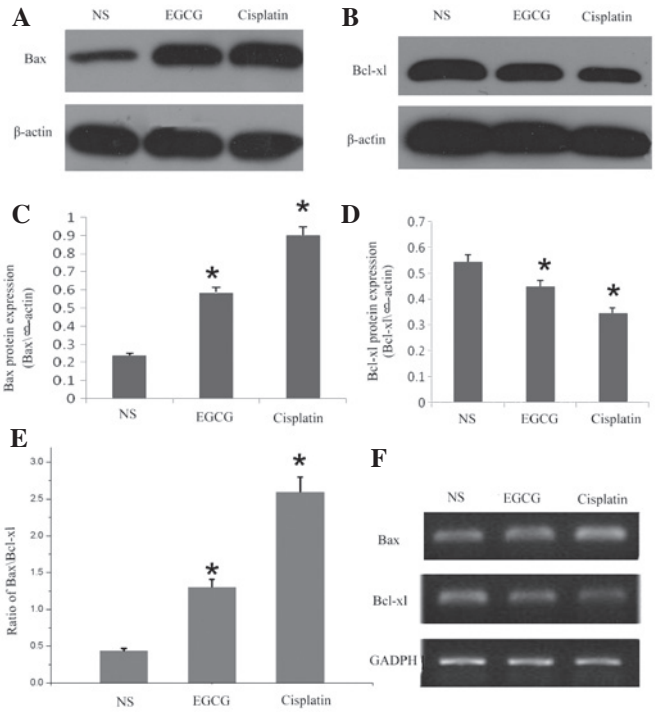

Figure 3. Treatment of A549-induced tumors in mice with EGCG increases the levels of protein and mRNA expression of Bax and decreases the levels of protein and mRNA expression of Bcl-xl. The levels of Bax (A) and Bcl-xl (B) protein in tumor tissue lysates were determined using western blot analysis after treating the mice with NS, EGCG or cisplatin for 14 days. (B) After determining the levels of Bax (C) and Bcl-xl (D) protein by western blot analysis, the bands were quantified by densitometry and are represented graphically. (E) The ratio of Bax and Bcl-xl protein expression was determined from three separate experiments by comparing the relative intensities of the protein bands. (F) The levels of Bax and Bcl-xl mRNA in tumor tissue were determined using RT-PCR after treating the mice with NS, EGCG or cisplatin for 14 days. The results are the mean of three independent experiments. Bars, SD. ANOVA test: *P<0.05 vs. control. EGCG, epigallocatechin-3-gallate; NS, normal saline; SD, standard deviation.

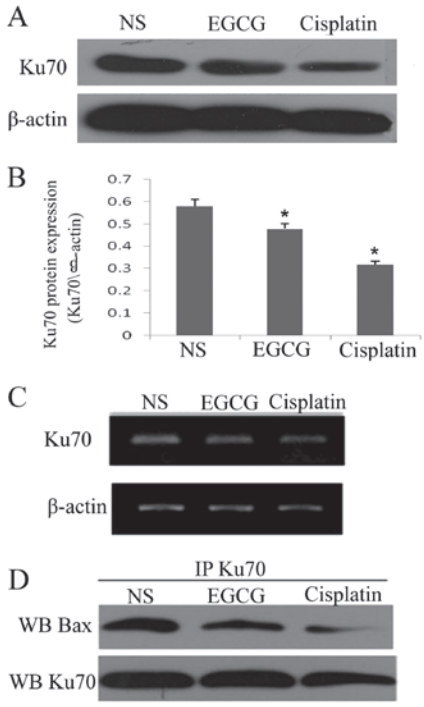

Figure 4. Treatment of A549-induced tumor in mice with EGCG decreases the levels of protein and mRNA expression of Ku70, and interrupts the binding of Ku70 and Bax. (A) The level of Ku70 protein in tumor tissue lysates were determined using western blot analysis after treating the mice with NS, EGCG or cisplatin for 14 days. (B) The bands were quantified by densitometry and are represented graphically. Bars, SD. ANOVA test: *P<0.05 vs. control. (C) The level of Ku70 mRNA in tumor tissue was determined using RT-PCR after treating the mice with NS, EGCG or cisplatin for 14 days. (D) Total cell lysates after treating the mice with NS, EGCG or cisplatin for 14 days were immunoprecipitated with an anti-Ku70 antibody and the presence of Bax in the immunoprecipitates was detected with anti-Ku70 antibody. The results are the mean of three independent experiments. EGCG, epigallocatechin-3-gallate; NS, normal saline; SD, standard deviation.

tumor volume at regular intervals, was decreased in animals administered EGCG and cisplatin, compared with NS control animals (Fig. 1A). Administration of EGCG resulted in a 37% inhibition in tumor weight when recorded at the termination of the experiment, while the inhibition in the cisplatin group was 50% (Fig. 1B). As observed in Fig. 1C, EGCG inhibited the tumor growth; however, the efficiency was lower than cisplatin, the classic chemotherapy agent of major cancers. However, due to the toxicity of drugs, the weight of the animals was decreased, and the average weight of the EGCG treatment animals was 23.3 g, equal to the NS control group, while the cisplatin treatment animals had a lower weight of only 18.1 g (Fig. 1D). It was also demonstrated that the toxicity of EGCG was milder than cisplatin.

EGCG inhibits the surrogate markers of proliferation and apoptosis (caspase 3) in A549-induced tumors in BALB/c mice. As treatment of EGCG inhibited the cell proliferation and viability in the *in vivo* system, we examined the effect of EGCG on markers of cell proliferation by assessing the protein expression of activated caspase 3 in the tumors. Cleaved caspase 3 is considered a hallmark of apoptosis. As determined by western blot analysis, the level of cleaved caspase 3 in the tumors was markedly increased in EGCG- and cisplatin-treated animals compared with the NS-treated animals (Fig. 2A and B). The expression of the basal level of caspase 3 was not detectable in the tumors as the antibodies that were used only recognized cleaved caspase 3. These observations support the evidence that administration of EGCG inhibited tumor growth possibly through the induction of apoptosis in A549 tumor cells. The administration of EGCG may affect the expression of activated caspase 3 in tumor-bearing mice.

EGCG downregulates the expression of Bcl-xl and upregulates the expression of Bax mRNA and protein in tumors in vivo. Furthermore, we examined the effect of EGCG on the apoptotic protein involved in mitochondrial disruption pathway in *in vivo* tumor development. As shown in Fig. 3A, B, C and D, western blot analysis revealed that treatment with EGCG downregulated the expression of antiapoptotic protein Bcl-xl, but increased the expression of proapoptotic protein Bax. As shown in Fig. 3E, the increase in the ratio of Bax/Bcl-xl in *in vivo* tumors suggested that the susceptibility of tumor growth was blocked or inhibited in EGCG-treated BALB/c mice. In addition, using RT-PCR, we also assessed the effect of EGCG on the mRNA expression of Bax and Bcl-xl during apoptosis. As indicated by the western blot result, EGCG induced a decrease in the expression of mRNA coding for Bcl-xl and increased Bax mRNA (Fig. 3F).

EGCG downregulates the expression of Ku70 mRNA and protein and interrupts the binding of Ku70 and Bax. Ku70 is a multifunctional protein playing roles in DNA repair and cell survival and it has been demonstrated to inhibit Bax-mediated cell death by binding Bax. As mentioned above, EGCG was able to upregulate the expression of Bax protein and mRNA, therefore, we examined the effect of EGCG on Ku70. Western blot (Fig. 4A and B) and RT-PCR (Fig. 4C) analysis revealed that EGCG treatment is able to decrease the expression of Ku70 mRNA and protein, similar to cisplatin. To determine whether the Bax-Ku70 interaction was affected by EGCG treatment, the protein was co-immunoprecipitated with Bax antibody. Notably, Ku70-Bax complexes decreased in the EGCG and cisplatin groups compared with the NS group (Fig. 4D). These results suggest that EGCG is able to decrease the expression of Ku70 and interrupt the binding between Bax and Ku70.

Discussion

In this study, we identified that EGCG, the major polyphenolic agent present in green tea, inhibited the growth of lung cancer A549 cells *in vivo*, which was consistent with previous studies indicating that EGCG inhibited growth and apoptosis in human lung, colon, gastric, prostate and mammary carcinoma (19-23). At present, a number of agents are used to clinically treat carcinoma, including cisplatin, gemcitabine and paclitaxel. However, most of these agents have numerous toxicities and side-effects, including bone marrow depression, loss of weight, gastrointestinal reaction and hepatic function failure, which may cause great agony to patients and have a negative impact on their daily life. In animals, loss of weight is the best marker to evaluate the toxicities and side-effects of an agent. In our study, mice treated with cisplatin had lower weight due to the toxicities. Inversely, in the EGCG-treated

group, the weight of the animals was equal to the control group. The present study also revealed that EGCG had less toxicities and side-effects than the existing anticancer agents in human normal cells, while it was able to induce apoptosis and arrest the cell cycle in human carcinoma cells (24). Due to its wide range of pharmacological properties and reduced toxicities and side-effects, EGCG is one of the most promising chemotherapy agents for cancer.

Many of the molecular alterations that accompany carcinogenesis lead to uncontrolled proliferation and the ability of transformed cells to evade apoptosis. Apoptosis is a genetically controlled mechanism of cell death involved in the regulation of tissue homeostasis. The Bcl-2 protein family plays an important role in the control of apoptosis (25,26). Bcl-2 and Bcl-xl are the prototypes of this family and inhibit the induction of apoptosis, while Bax and Bad are proapoptotic. High concentrations of Bcl-2 or Bcl-xl affect cell susceptibility to the induction of apoptosis by altering the ratio of death promoters to suppressors, providing tumor cells with a survival advantage and permitting expansion of transformed cells harboring mutations within their genome. The Bcl-xl protein and other family members locate at the intracellular organelles, including the endoplasmic reticulum and the outer mitochondrial and nuclear membranes, where they modulate responses to diverse death stimuli (27). In Ewing family tumor (EFT) cells, apoptosis by EGCG correlated with altered expression of Bcl-2 family proteins, including increased expression of proapoptotic Bax and decreased expression of prosurvival Bcl-2, Bcl-xl and Mcl-1 proteins (28). In addition, EGCG enhanced apoptosis as demonstrated by an increase in the Bax:Bcl-xl ratio, cleavage of procaspases 3, 8 and 9 and poly (adenosine diphosphate ribose) polymerase and accumulation of subG1 cells in skin cancer cells (29). In our study, we detected the protein and mRNA expression of the Bcl family. Treatment with EGCG resulted in downregulation of the expression of the antiapoptotic protein Bcl-xl, while it increased the expression of proapoptotic protein Bax. The ratio of Bax/Bcl-xl in lung cancer increased compared with the control group, and EGCG increased the expression of activated caspase 3 in tumor-bearing mice. In the human head and neck squamous cell carcinoma cell line, EGCG also induced apoptosis via a mitochondrial pathway, revealing that EGCG caused a decrease in the Bcl-2 and Bcl-xl proteins, as well as an increase in Bax protein and activation of caspase 9 (3). Together, these results suggest that EGCG induces carcinoma cell apoptosis via the mitochondrial pathway.

As mentioned above, EGCG is able to regulate a number of molecules involved in cell apoptosis and cell cycle arrest, including Bax-mediated apoptosis. The mechanism of how Bax is kept inactive remained unclear until 2003, when yeast-based functional screening of Bax inhibitors from mammalian cDNA libraries identified Ku70 as a new Bax suppressor. Bax-mediated apoptosis was suppressed by overexpression of Ku70 in mammalian cells, but enhanced by downregulation of Ku70 (30). Ku70 forms a heterodimeric Ku protein complex with Ku80, which represents a crucial component of the NHEJ DNA double-strand break (DSB) repair machinery (31). In cooperation with Ku80, Ku70 binds and bridges two proximal broken DNA ends, which facilitates DNA end-joining through a cascade of reactions that involve DNA-dependent protein kinase and DNA ligase IV. Ku70 contains two DNA-binding domains at NH2 and COOH termini, both of which are required for the high affinity to DNA (32-35). In early breast cancer patients, low expression of Ku70/Ku80 predicts a good response to radiotherapy (36). Wortmannin pretreatment of A549 cells causes increased apoptosis induced by docetaxel, due to the inhibition of the DNA repair process by wortmannin, and downregulation of DNA repair proteins, including Ku70 (37).

In the present study, for the first time, we reveal that the induction of apoptosis by EGCG may be caused by the downregulation of Ku70. Knockdown of Ku70 has been previously found to enhance gefitinib-induced death in NSCLC cells (38). In addition, recent evidence indicates that Ku70 interacts with Bax, and that the carboxyl terminus of Ku70 and the amino terminus of Bax are required for this interaction (30), and the binding of Ku70 and Bax suppresses its apoptotic translocation to the mitochondria (39). In tumorigenic neuroblastic cell models of NB, the disruption of Ku70 binding to Bax caused activated Bax to translocate from the cytosol to the mitochondria and trigger cell death (40). In order to explore the effect of EGCG on the interaction between Ku70 and Bax, co-immunoprecipitation experiments of Ku70 and Bax were performed. Our data also demonstrated that EGCG is able to disrupt the interaction between Ku70 and Bax.

However, the molecular mechanisms of how EGCG regulates the expression of Ku70 was not explored in this study. Recent studies demonstrate that the activity of Ku70 might be regulated at both the transcriptional and post-translational levels in response to apoptotic stimuli. Particularly, it is noteworthy that Ku70 is targeted for acetylation and deacetylation by histone acetyltransferases and histone deacetylase (HDAC), respectively, *in vivo* (41-43). Evidence indicates that increased acetylation levels of Ku70, as a result of HDAC inhibition, abolishes its ability to bind Bax and suppress Bax-mediated apoptosis (41-44). The question of whether EGCG regulates the expression of Ku70 via regulating the balance between acetylation and deacetylation of Ku70 requires investigation in future studies.

In conclusion, in the present study, we have demonstrated that EGCG is able to inhibit the growth of lung cancer A549 cells *in vivo*. The possible molecular mechanism indicated is that EGCG treatment may interrupt the binding of Ku70 and Bax, resulting in the upregulation of Bax and a parallel downregulation of Bcl-xl, which might ultimately initiate the activation of the caspase cascade leading to apoptosis. These results provide new insights into the anticancer effects of EGCG. The mechanism for the regulation of Ku70 is currently being investigated in our laboratory.

Acknowledgements

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