

Epigallocatechin gallate promotes p53 accumulation and activity via the inhibition of MDM2-mediated p53 ubiquitination in human lung cancer cells

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Abstract. Epigallocatechin gallate (EGCG), which is derived from green tea, is well known for its chemopreventive activity. Several studies have shown that p53 plays an important role in the activity of EGCG; however, the mechanism by which EGCG regulates p53 requires further investigation. In the present study, we showed that EGCG inhibits anchorage-independent growth of human lung cancer cells by upregulating p53 expression. EGCG treatment can substantially increase p53 stability, promote nuclear localization of p53 and decrease nuclear accumulation of MDM2. We also found that EGCG increases the phosphorylation of p53 at Ser15 and Ser20 and enhances its transcriptional activity. Although EGCG promotes MDM2 expression in a p53-dependent manner, the interaction between MDM2 and p53 was significantly inhibited following EGCG treatment, which resulted in the inhibition of MDM2-mediated p53 ubiquitination. Thus, our results suggest that the stabilization and activation of p53 may partly contribute to the anticancer activity of EGCG.

Introduction

According to the results of epidemiologic studies, green tea consumption has a preventive effect on carcinogenesis (1-6). It is thought that polyphenols, also known as catechins, play an important role in the chemopreventive effects mediated by

green tea. (-)-Epigallocatechin gallate (EGCG), a type of polyphenol, which is the most well known, abundant and active compound found in green tea, exerts its anticancer effects in a wide range of malignancies (2,7). Previous studies have suggested that multiple signaling pathways and mechanisms are involved in the antitumor activity of EGCG (1,8-10), including suppression of various protein kinases (11-13); disruption of the activation of transcription factors such as EGFR, NF- κ B, AP-1 and STATs (14-16); induction of cell cycle arrest or apoptosis (17,18); and inhibition of cell migration and metastasis (19-24).

p53, commonly referred to as the 'cellular gatekeeper' or 'the guardian of the genome', is a crucial tumor suppressor gene that is mutated in more than half of all types of human cancer. As a transcription factor, p53 functions to regulate cell fate following various types and levels of cellular stress through its downstream target genes. In addition to its canonical functions of inducing DNA repair, cell cycle arrest and apoptosis (25,26), recent studies have also revealed that p53 is involved in the regulation of various other cellular functions, such as senescence, metabolism and autophagy. Due to the importance of p53, its activation is regulated by complicated post-translational modifications, such as phosphorylation, acetylation, ubiquitination and sumoylation (8,27-29). Previous studies have shown that the phosphorylation and acetylation of p53 promotes the expression of p53 target genes (28,30,31), whereas other modifications, such as ubiquitination and sumoylation, are considered to be associated with the suppression of p53-mediated transcription and nuclear export of p53 (32-34). MDM2, a Ring finger domain-containing protein, is the first identified E3 ligase that can induce p53 ubiquitination and proteasomal degradation (33). However, as a transcriptional target of p53, MDM2 expression is also regulated in a p53-dependent manner. As p53 increases, the expression of MDM2 is strengthened, which induces degradation of p53 and achieves balance.

Previous studies have demonstrated that EGCG treatment increases the expression levels of p53 in various human cancer

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Abbreviations: EGCG, epigallocatechin gallate, CHX, cycloheximide;

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cells (35-38). Previous reports found that cells expressing wild-type p53 are more sensitive than p53-null or p53 knock down cells to EGCG-induced growth inhibition and apoptosis (36,39,40). However, the molecular mechanisms underlying p53 regulation by EGCG and the anticancer effects of green tea via targeting of the p53 tumor suppressor gene are poorly understood. In the present study, we demonstrated that EGCG treatment can induce p53 accumulation and enhance the stability of this protein by disturbing the interaction between p53 and MDM2. Furthermore, our data showed that EGCG inhibits p53 ubiquitination in a dose-dependent manner. These results indicate a novel mechanism for the preventive effects of EGCG on cancer.

Materials and methods

Cell culture and transfection. All cell lines were obtained from the American Type Culture Collection and were grown in a 37°C incubator with 5% CO₂ according to the American Type Culture Collection protocols. For transfection experiments, the Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions.

Reagents and antibodies. EGCG, cycloheximide (CHX) and MG132 were obtained from Sigma (St. Louis, MO, USA). The GFP-p53, His-Ub and HA-MDM2 plasmids were gifts from Dr Bo Liu and were previously described (41). Anti-p53, anti-MDM2, anti-β-actin, anti-GFP, anti-HA, anti-His, anti-rabbit IgG-HRP, anti-mouse IgG-HRP, anti-goat IgG-HRP, and normal mouse/rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-p53 (Ser15), anti-phospho-p53 (Ser20), anti-p21, anti-lamin B, anti-tubulin and anti-ubiquitin antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Immunoprecipitation. 293T and H1299 cells were first co-transfected with 2 μg of GFP-p53 and 2 μg of HA-MDM2. Twenty-four hours after the transfection, these cells were treated with 40 μM EGCG for 24 h. Cells were harvested and washed twice with ice-cold PBS and lysed in NP40 lysis buffer (50 mmol/l Tris-HCl, pH 8.0; 150 mmol/l NaCl; 0.5% NP40) with protease cocktail (Roche Diagnostics GmbH, Mannheim, Germany). All immunoprecipitation procedures were carried out at 4°C. The lysates were then incubated with the appropriate antibodies, followed by incubation with protein A/G agarose beads. The protein-antibody complexes were recovered and subjected to western blot analysis after separation by SDS-PAGE.

Western blotting. Cells were harvested by trypsinization and pelleted by centrifugation. Cell pellets were lysed in NP40 lysis buffer supplemented with protease inhibitors. Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories Philadelphia, PA, USA). Proteins were separated by SDS-PAGE and electrically transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking in 5% non-fat dry milk in TBS, the membranes were hybridized to specific primary antibodies overnight at 4°C, washed three times with TBS Tween-20, and then incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Next,

the membranes were washed three times in TBS Tween-20 at room temperature. The protein bands were visualized using ECL chemiluminescence reagents (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's protocol.

Measurement of p53 and MDM2 half-lives. For p53 and MDM2 protein stability experiments, after the treatment of A549 cells with 40 μmol of EGCG for 24 h, 30 μg/ml of CHX was added to inhibit protein synthesis. Subsequent time points for incubation in medium containing EGCG and CHX were 0, 20, 40, 60 or 90 min as indicated. Cells were then processed as previously described for western blotting and quantified by densitometry.

Luciferase reporter assay. The pGL3-p53 firefly luciferase reporter plasmid and the internal control pRL-SV40 (Renilla luciferase) plasmid were purchased from Promega. A549 cells were cultured in 24-well plates and co-transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Each transfection contained 800 ng of firefly luciferase reporter plasmid and 80 ng of pRL-SV40 plasmid. Twenty-four hours after the transfection, the cells were treated with different concentrations of EGCG for 24 h. Cell lysates were analyzed for firefly and Renilla luciferase activities according to the Dual Luciferase Reporter Assay kit (Promega) protocol. The firefly luciferase activity was normalized to the value of pRL-SV40 activity for transfection efficiency.

Soft agar colony assay. To examine the anchorage-independent growth, lung cancer cells were suspended (10,000 cells/ml) in 1 ml of 0.3% agar with Eagle's basal medium containing 10% FBS, 1% antibiotics, and different concentrations of EGCG (0, 1, 5, 10, 20 and 40 μmol/l) overlaid into 6-well plates containing a 0.6% agar base. The cultures were maintained in a 37°C, 5% CO₂ incubator for 1-2 weeks, and then colonies were counted under a microscope using the Image-Pro Plus software program (Media Cybernetics, Silver Spring, MD, USA).

Ubiquitination assay. H1299 and 293T cells were co-transfected with 2 μg of GFP-p53, 1 μg of HA-MDM2 and 0.5 μg His-Ub plasmids. Twenty-four hours after the transfection, the cells were treated with different concentrations of EGCG for a further 24 h. Subsequently, 20 μM of MG132 was added to the culture medium for 6 h prior to harvesting. Cells were split into two aliquots, one for immunoblotting and the other for a ubiquitination assay. For the ubiquitination assay, we first used the anti-GFP antibody to immunoprecipitate p53 and then detected the ubiquitination using a His-tag antibody. A549 cells were pre-treated with EGCG for 24 h and MG132 for 6 h. Endogenous p53 was immunoprecipitated from 1 mg of protein lysate using the p53 antibody and then immunoblotted with the anti-ubiquitin antibody to capture polyubiquitinated p53.

Statistical analysis. All the statistical analyses were performed using the SPSS software (version 13.0). The experiments were performed in triplicate. The quantitative data are expressed as mean values ± standard deviation. The significant differences between two groups were assessed by a two-tailed Student's t-test. P<0.05 was considered to represent a statistically significant difference.

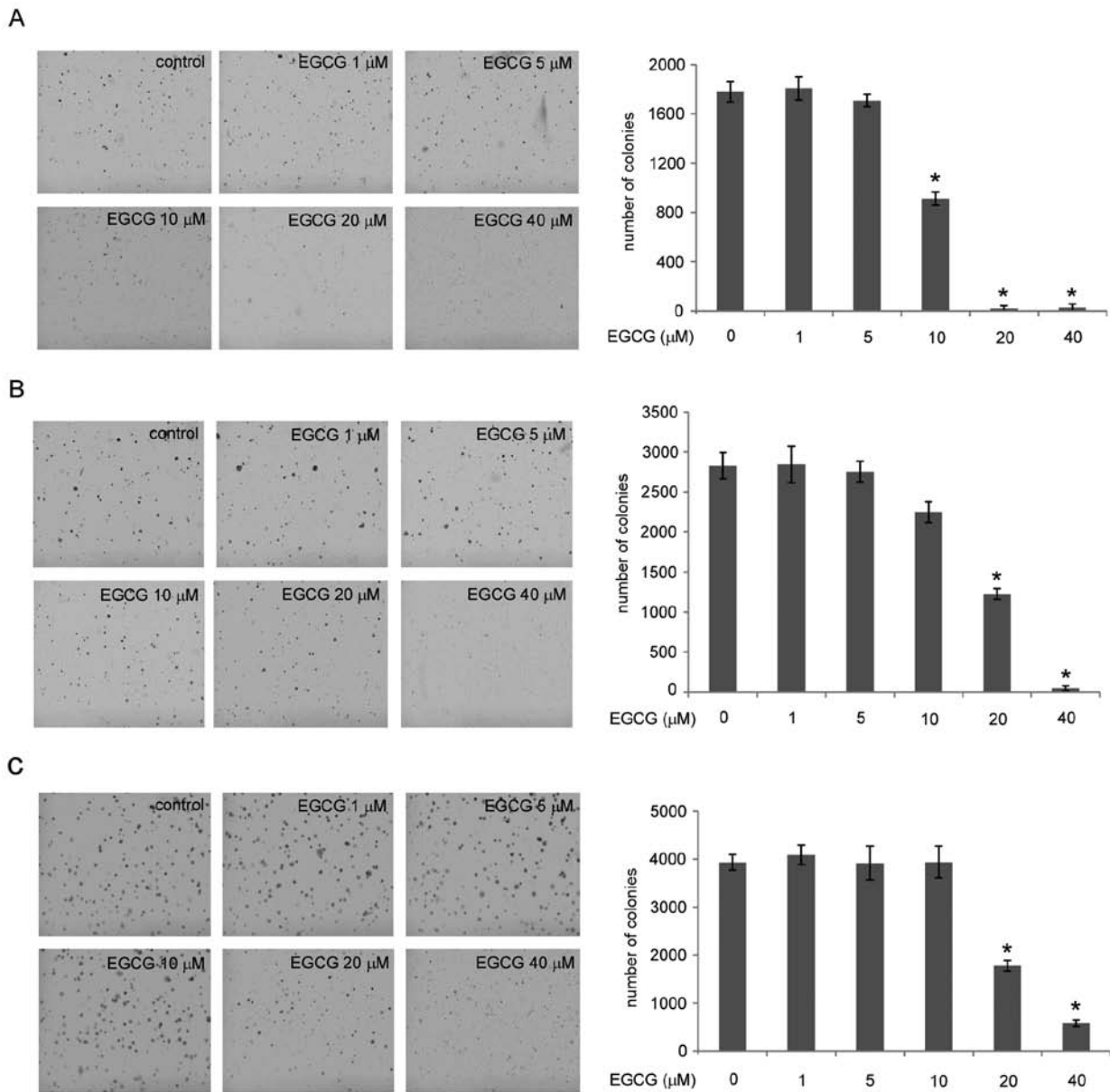


Figure 1. Inhibitory effects of EGCG on anchorage-independent growth of human lung cancer cells. EGCG inhibited anchorage-independent growth in a panel of human lung cancer cells, including (A) H1650, (B) A549 and (C) H460 cells. A colony formation assay was performed as described in Materials and methods. Data shown are the colony formation ability of human lung cancer cells treated with different concentrations of EGCG compared with the dimethyl sulfoxide-treated group. The average colony number was calculated from three separate experiments. Columns, means of the number of colonies as determined from three independent experiments; bars, standard deviation. *P<0.01 significant suppression of colony formation by EGCG.

Results

EGCG inhibits the anchorage-independent growth of human lung cancer cells. Initially, we investigated the effects of EGCG on the anchorage-independent growth of three different types of human lung cancer cell lines: A549, H1650 and H460. The results showed that these three cell lines have different sensitivity to EGCG. At low concentrations (1-5 μM), EGCG does not substantially inhibit the anchorage-independent growth of these lung cancer cells. In H1650 cells, EGCG potentially inhibits the anchorage-independent growth at the concentration of 10 μM, and almost no colonies were formed at 20 μM (Fig. 1A). In A549 and H460 cells, EGCG showed clear inhibitory effects against anchorage-independent growth at the concentration of 20 μM (Fig. 1B and C,

respectively). These results indicate that EGCG inhibits the anchorage-independent growth of human lung cancer cells in a dose-dependent manner.

EGCG induces p53 accumulation and upregulates its target genes. Based on the results of the soft agar experiments, we detected the effects of EGCG on p53 expression in three p53-wild type human lung cancer cells: A549 (Fig. 2A), H1650 (Fig. 2B) and H460 (Fig. 2C). Our results showed that EGCG dose-dependently increased the endogenous p53 expression. At the concentration of 5 μM, the expression of p53 was substantially increased following the treatment of EGCG for 24 h. In addition, we investigated the effects of EGCG on its downstream target genes, p21 and MDM2. Similar to the effect on p53, EGCG enhanced the expression levels of p21

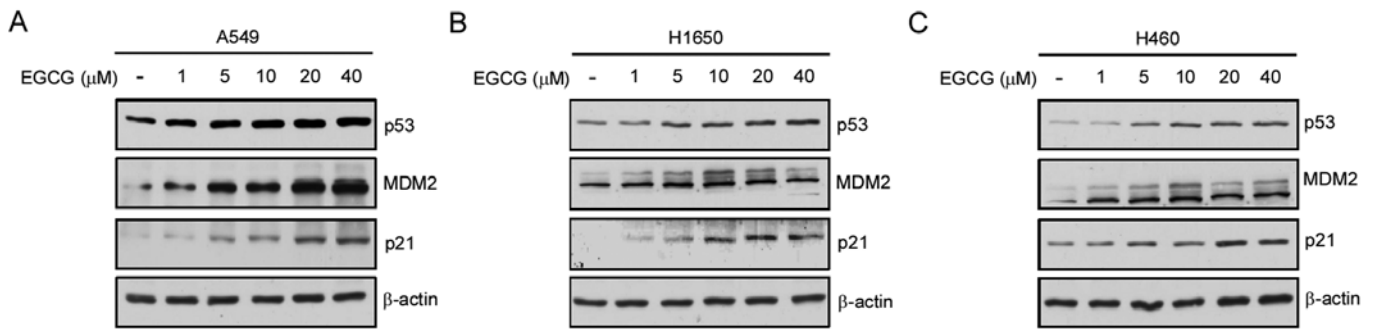


Figure 2. EGCG induces p53 accumulation and upregulates its target genes. EGCG upregulates endogenous p53, p21 and MDM2 expression in a dose-dependent manner in human lung cancer (A) H1650, (B) A549 and (C) H460 cells. The cells were seeded in 10-cm dishes and treated with varying concentrations of EGCG for 24 h. The cells were then harvested, and protein levels were determined by western blot analysis.

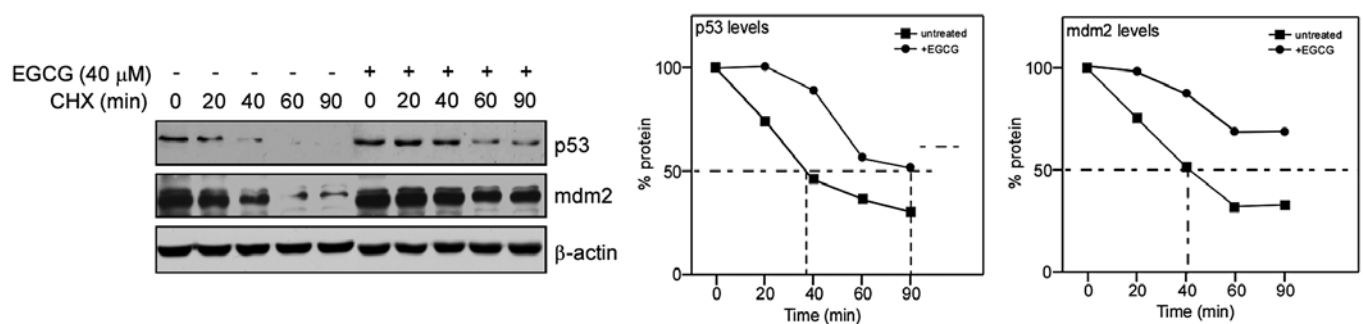


Figure 3. EGCG promotes p53 and MDM2 protein stability. A549 cells were seeded in 6-cm dishes, and following culture with 40 μM of EGCG for 24 h, 20 μg/ml CHX was added to the medium. The cells were harvested at 0, 20, 40, 60 and 90 min. p53 and MDM2 expression levels were determined by western blot analysis and were quantified by densitometry.

and MDM2. These data indicate that EGCG increases the expression of p53 and its target genes.

EGCG promotes the stability of p53 and MDM2. As a short-lived protein, p53 is degraded quickly by ubiquitination under normal physiologic status. Enhancement of protein stability is the first step for p53 to perform its functions. To expose the mechanism of p53 upregulation by EGCG, we first set up a half-life assay to investigate the effects of EGCG on p53 stability. We adopted CHX treatment to block the protein synthesis in A549 cells, and western blotting to detect the expression levels of p53 following treatment with EGCG for 24 h. We found that EGCG significantly upregulated the half-life of p53 from ~40 min to >90 min. Meanwhile, as its target gene and major negative regulatory factor, the half-life of MDM2 also clearly increased after EGCG treatment (Fig. 3). These results demonstrate that EGCG promotes the stability of p53 and MDM2.

EGCG promotes nuclear localization and activity of p53. Ubiquitination of p53 results in targeting either for proteasomal degradation or nuclear export. As an important transcriptional factor, p53 stability and nuclear localization are essential for its tumor suppressor function (34). In the nucleus, as a result of MDM2-mediated ubiquitination, p53 is transported into the cytoplasm or is degraded by the 26S proteasome. Both in the nucleus and in the cytoplasm, the phosphorylation of p53 is considered to be a counteractive post-translational modifica-

tion that promotes p53 stability and transactivation. Previous studies have reported that the phosphorylation of p53 at Ser15 or Ser20 increases p53 stability by disrupting the interaction between p53 and MDM2 (42,43). In the present study, we found that EGCG increased Ser15 and Ser20 phosphorylation of p53 in a dose-dependent manner (Fig. 4A). We also confirmed the localization of p53 and MDM2 by cellular fractionation. As shown in Fig. 4B, EGCG treatment only slightly promoted the accumulation of p53 in the cytoplasm. However, in the nucleus, EGCG dose-dependently increased the accumulation of p53, with a significant increase at the concentration of 20 μM. By contrast, MDM2 in the nucleus was mildly decreased as the concentration of EGCG increased. Furthermore, we tested whether EGCG treatment affects the transactivation ability of p53 using a Dual Luciferase Reporter Assay system. As shown in Fig. 4C, the transactivation ability of p53 was significantly elevated following EGCG treatment. At the concentration of 20 μM, the luciferase activity increased nearly 3 times. These results suggest that EGCG potentiates p53 function in human lung cancer cells.

EGCG inhibits proteasomal degradation-dependent p53 ubiquitination. Based on the observation that EGCG promotes the stability of p53, we further investigated the effects of EGCG on p53 ubiquitination. Plasmids GFP-p53 (3 μg), HA-MDM2 (0.5 μg) and His-Ub (0.5 μg) were co-transfected into 293T cells, which were used as an overexpression system.

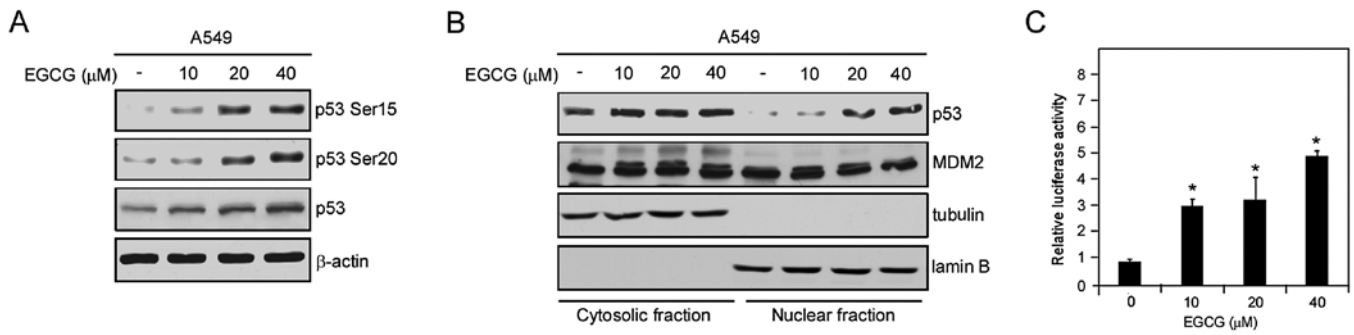


Figure 4. EGCG promotes nuclear localization and activity of p53. (A) EGCG increases p53 Ser15 and Ser20 phosphorylation. A549 cells were treated with EGCG for 24 h and the whole cell lysates were subjected to western blot analysis using the indicated antibodies. (B) p53 was upregulated and translocated to the nucleus upon EGCG treatment. Endogenous p53 and MDM2 in both cytoplasm (non-NE) and nucleus (NE) of A549 cells, which were treated with EGCG for 24 h, were detected using anti-p53, MDM2, tubulin and lamin B antibodies for western blotting. (C) EGCG upregulated p53 transcriptional activity. A549 cells were transfected with the indicated plasmids. EGCG treatment and luciferase reporter assays were performed as described. The relative luciferase activity was normalized to the value of Renilla activity.

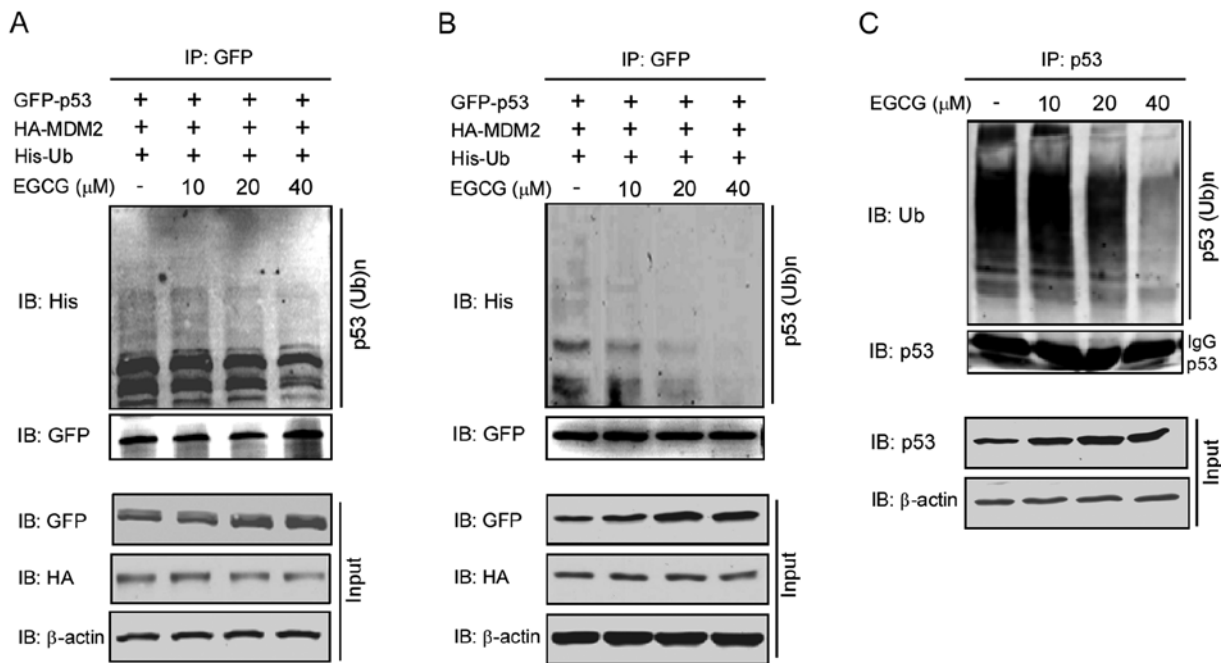


Figure 5. EGCG inhibits p53 ubiquitination. (A) 293T and (B) H1299 cells were co-transfected with different expression vectors and treated with EGCG as described. Cell lysates were immunoprecipitated with GFP antibody and analyzed by immunoblotting with anti-His-tag antibody to capture polyubiquitinated p53. The same cell lysates were subjected to western blotting to determine the expression levels of p53 and MDM2 protein. (C) EGCG inhibited endogenous p53 ubiquitination. A549 cells were treated with or without EGCG as indicated. p53 was immunoprecipitated from 1 mg of cell lysate and immunoblotted with anti-ubiquitin antibody to detect ubiquitinated p53.

As shown in Fig. 5A, EGCG treatment substantially decreased the ubiquitination of p53 with significant inhibition observed at the concentration of 20 μ M. We also tested this phenomenon by transfection of these plasmids in p53-deficient H1299 cells. From the results shown in Fig. 5B, EGCG also dose-dependently inhibited the ubiquitination of p53. In addition to the overexpression system, we also studied the effects of EGCG on the regulation of endogenous p53 ubiquitination (Fig. 5C). In the p53-wild type A549 cell line treated with EGCG at the concentration of 20 μ M for 24 h, the ubiquitination of p53 was markedly decreased. These results suggest that EGCG inhibits p53 ubiquitination.

EGCG inhibits the interaction of p53 and MDM2. The results of the above experiments demonstrated that EGCG inhibits the ubiquitination of p53, promotes its stability and increases its expression. As a downstream target gene of p53, the expression levels of MDM2 also increased with the accumulation of p53 in the nucleus. However, as an important negative regulator of p53, the increase in MDM2 expression may cause the ubiquitination and subsequent degradation of p53. The previous results confirmed that EGCG markedly inhibits the ubiquitination of p53 even in the case of MDM2 overexpression. Therefore, studies were performed to further investigate the effects of EGCG on the interaction between MDM2 and

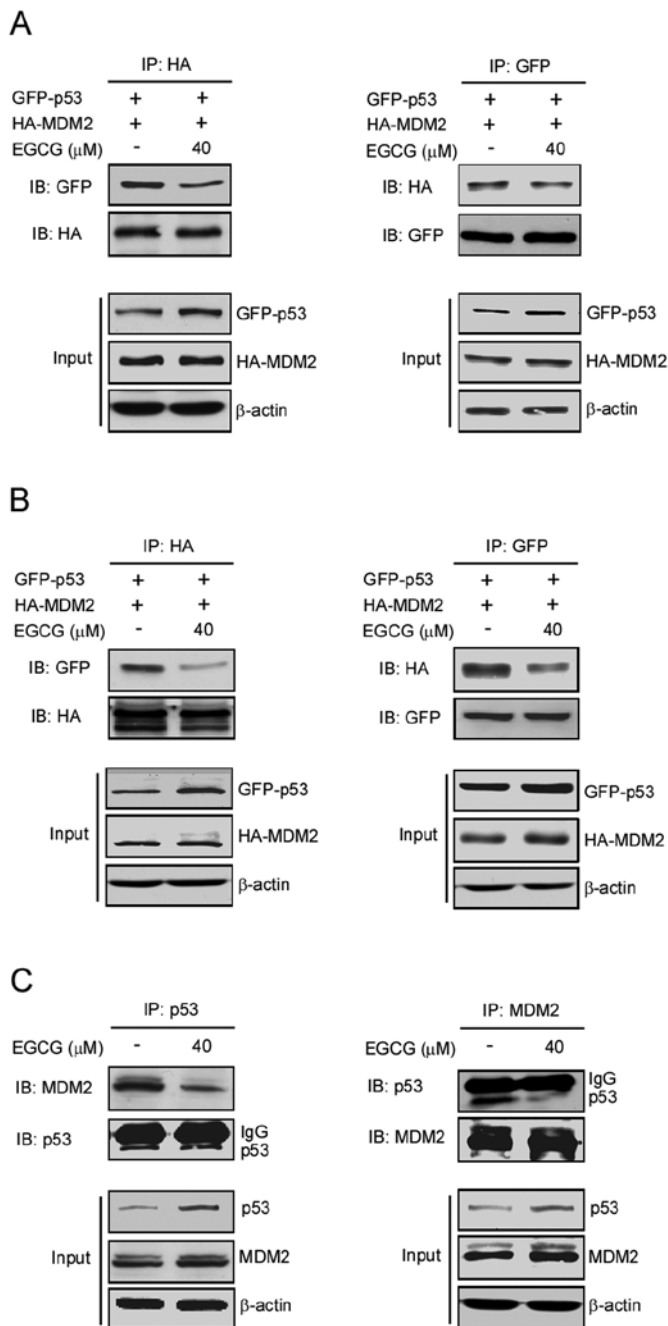


Figure 6. EGCG inhibits the interaction of p53 and MDM2. (A) 293T and (B) H1299 cells were transfected with plasmids and treated with EGCG as indicated. Cell lysates were used for IP of GFP-p53 and/or HA-MDM2. Immunoblotting was performed with the indicated antibodies following SDS-PAGE to detect the binding affinity of p53 and MDM2, and the same cell lysates were subjected to western blotting to determine the expression levels of p53 and MDM2 as indicated. (C) A549 cells were treated with or without 40 μ M of EGCG for 24 h. Cell lysates were immunoprecipitated with 2 μ g of p53 and/or MDM2 antibodies, and the binding activities were analyzed by western blot analysis.

p53. The plasmids GFP-p53 (2 μ g) and HA-MDM2 (2 μ g) were co-transfected into 293T and H1299 cells. Following treatment with 40 μ M EGCG for 24 h, co-immunoprecipitation was performed to detect the interaction between MDM2 and p53. As shown in Fig. 6A and B, in the overexpression systems 293T and H1299, EGCG treatment resulted in substantial inhibition of the interaction between p53 and MDM2. In A549, we

observed similar results in which EGCG treatment inhibited MDM2 binding with p53 (Fig. 6C). These results indicate that EGCG suppresses MDM2-mediated p53 ubiquitination by disrupting their interaction.

Discussion

The natural compound EGCG is the major polyphenol component of green tea. It has been extensively studied due to its relatively high abundance and strong epidemiologic evidence of cancer-preventive activity (2,3). Although several reports have shown that EGCG exerts its anticancer activity by targeting specific cell signaling pathways, the underlying mechanism is not yet fully understood.

Previous reports revealed that EGCG treatment induces lung cancer cell apoptosis or cell cycle arrest (18,35). Other groups also found that the tumor suppressor gene p53 may be involved in the antitumor activity of EGCG since cancer cells expressing wild-type p53 are more sensitive to EGCG treatment compared with p53-null or p53 knock down cancer cells (36,40). In the present study, we first examined the anti-tumor effects of EGCG in several human lung cancer cells that express wild-type p53 by soft agar assay. Our results demonstrated that EGCG inhibits the anchorage-independent growth of human lung cancer cells in a dose-dependent manner. Western blotting data indicated that EGCG promotes the accumulation of wild-type p53 and its target genes, p21 and MDM2. Our data demonstrated and corroborated other studies showing that the effect of EGCG against lung cancer may partly depend on p53 activity.

To further investigate p53 stability mediated by EGCG, the effects of EGCG on the half-life of p53 and MDM2 were examined using CHX treatment. We found that EGCG significantly upregulated the half-life of p53 from approximately 40 min to 90 min, as well as that of its target gene, MDM2, from approximately 40 min to over 90 min. These results indicate that p53 is a potential anticancer target mediated by EGCG.

The activity and localization of p53 are mainly regulated by post-translational modifications, such as phosphorylation, acetylation and ubiquitination (27,28,34). Accumulating evidence demonstrates that p53 phosphorylation at Ser15 and Ser20 attenuates the binding of p53 to MDM2 and disrupts MDM2-mediated p53 ubiquitination both *in vivo* and *in vitro* (42,43). In our study, we demonstrated that the increased p53 expression was accompanied by the phosphorylation of p53 at Ser15 and Ser20. In addition, by extracting the cytosolic and nuclear fractions, we further determined p53 subcellular localization upon EGCG treatment. The results clearly showed that EGCG potently induced nuclear accumulation of p53 in a dose-dependent manner while, at the same time, the expression levels of MDM2 in the nucleus appeared slightly decreased. Therefore, we hypothesized that EGCG-induced stabilization and nuclear accumulation of p53 might induce an increase in its transcriptional activity and result in an upregulation of its target genes, p21 and MDM2. Thus, we tested p53 transcriptional activity by setting up a reporter gene assay. Our data confirmed the hypothesis that EGCG treatment increases p53 transcriptional activity.

MDM2, a downstream target gene of p53, is the major E3 ligase responsible for regulating p53 polyubiquitination

and targeting p53 for proteasomal degradation. Therefore, enhanced p53 activity causes increased expression of its own negative regulator, MDM2, thereby forming an auto-regulatory feedback loop. In the present study, we found that EGCG treatment sustainably increased p53 expression, accompanied by MDM2 upregulation. To further investigate the underlying mechanism, we detected p53 polyubiquitination by immunoprecipitation. Although EGCG treatment increased MDM2 protein levels, MDM2-mediated p53 ubiquitination was markedly decreased. Furthermore, our co-immunoprecipitation data showed that EGCG treatment disrupts the interaction between p53 and MDM2. This observation suggests that upon EGCG treatment, MDM2 acts only as a target gene and is upregulated by p53, and that its activity to induce p53 degradation is suppressed.

Collectively, our study identifies p53 as a potential target of EGCG to execute its cancer-preventive activity, and that the natural compound EGCG, inhibits the anchorage-independent growth of human lung cancer cells by promoting p53 stability/activity and by inhibiting MDM2-mediated p53 ubiquitination and degradation. Our study provides a new mechanism to explain the chemopreventive effect of EGCG on cancer; further research is required to provide evidence for the clinical use of EGCG in cancer prevention and therapy.

Acknowledgements

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