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Original Research

Chlorogenic-induced inhibition of non-small cancer cells occurs through regulation of histone deacetylase 6

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Abstract: Chlorogenic acid (CGA), an ester with various pharmacological effects, is important in cancer therapy. However, the specific antitumor mechanism of CGA is not entirely clear, especially with respect to its suppression of non-small cell lung cancer (NSCLC). The present study was carried out to assess the effect of CGA on NSCLC, and the mechanism involved. Cell viability assay and colony formation assay revealed that CGA blocked the proliferative capacity of NSCLC cells *in vitro*. Results from the migration assay suggested that CGA also inhibited the migration of A549 cells. Other assays further revealed that CGA strongly and selectively inhibited histone deacetylase 6 (HDAC6) activity and suppressed the activity of matrix metalloproteinase-2 (MMP-2) through decreased expression of Ac-NF-κB. Tumorigenicity assay showed that CGA also inhibited the proliferation and metabolism of NSCLC *in vivo*. These results indicate that CGA significantly suppresses the proliferation of NSCLC by regulating the activity of histone deacetylase 6.

Key words: Chlorogenic acid; Antitumor; Non-small cell lung cancer; Histone deacetylase 6.

Introduction

The incidence of lung cancer among many populations is high, and the disease contributes significantly to cancer-based mortalities world-wide (1). Data from diagnosis of lung cancer have revealed that about 80 % of lung cancer pathologies are due to terminal NSCLC (2, 3). Due to the high degree of malignancy and postoperative relapse which are characteristics of lung cancer, more than half of patients are usually diagnosed at advanced stages of the disease, resulting in very short survival time (4, 5). Although chemotherapy is an important treatment strategy for cancer, many cases of the disease are not sensitive to chemotherapy. Fortunately, in the past few years, on account of better knowledge of the molecular basis of cancer, the therapeutic effects have been somewhat improved (6, 7). However, NSCLC-related 5-year survival has not improved appreciably (8, 9). Thus, there is need to reduce the incidence of NSCLC and improve the 5-year survival of patients afflicted with the disease.

The use of natural products for cancer therapy has continued to attract much attention. Chlorogenic acid (CGA) (Figure 1A) is among plant-derived natural products with potent anticancer activities (10, 11). It is an ester derivative of quinic acid and trans-cinnamic acid. Studies have indicated that CGA has various pharmacological effects such as antioxidant, anticancer, antibiosis, antiviral, immune-modulating and hypoglycemic properties (12-14). The anticancer potential of CGA is its most prominent pharmacological property. However, the specific mechanism involved in the antitumor activity of CGA has not been clearly elucidated, especially in so far as it relates to its antitumor effect against NSCLC.

Epigenetics refers to the regulation of gene expression via posttranslational modification of protein complexes associated with DNA, without alterations in the DNA sequence (15). Histone deacetylases (HDACs) deacetylate lysine residues allowing interactions between negatively charged DNA and histone proteins, resulting in a closed chromatin conformation and repressed transcription (16). Once inhibiting HDACs activity, it will induce a broad range of effects on cancer cells, including cell cycle arrest, apoptosis, cell differentiation, autophagy and anti-angiogenic effects, finally terminating tumor development (17).

In the current study, the antitumor activity of CGA against NSCLC was assessed using tumorigenicity and colony formation tests. In addition, we evaluated the antitumor mechanism by cell migration assay and Western blot. The results demonstrated that CGA suppressed NSCLC proliferation and metastasis by regulating histone deacetylase 6.

Materials and Methods

Cell Culture

The NSCLC cells used in this study were human NSCLC cell lines H322, A549, SPC-A1, together with normal HBE cell line, all supplied by KeyGEN BioTECH (Nanjing, China). The cell lines were maintained in DMEM or RPMI culture medium containing 10 % FBS (Hyclone Lab) and 1 % penicillin–streptomycin antibiotic mixture (Gibco BRL) in an incubator with 5 % CO_2 at 37°C. The cells were used in exponential growth phase for the experiments.

Cell Viability Assay

The cells were harvested in logarithmic phase following 0.25 % tryptic digestion. After re-suspending in 100 µL of complete medium, they were plated in 96-well plates at a density of 5×10^4 cells/well, and exposed to different concentrations of CGA (0.25, 0.5, 1 µM; Selleck, USA), and 10% CCK-8. The cells were then cultured at 37 °C for 2 h and read at 450 nm in a plate reader (Bio Rad) (18). All assays were performed in triplicate.

Colony Formation Assay

A549 cells in the different treatment groups (control, 5 µM CGA, and 10 µM CGA) were seeded at a density of 500 cells/well in 35-mm culture dishes and diluted with RPMI medium. Before removing the medium, the cells were cultured for 2 weeks at 37 °C in a 5 % CO_2 incubator. Thereafter, they were subjected to crystal violet (0.1%) staining for 10 min, followed by enumeration of colonies in each well (19).

Cell Migration Assay

Migration assays were performed with A549 cells exposed to varied levels CGA for 24 h in Corning transwell invasion chambers. Cells in serum-free medium (5×10^4 /well; 200 µL per chamber) were seeded onto the atrium, and complete medium (600 µL) containing 10 % FBS was added to the lower chamber. The cells were incubated for 24 h. Thereafter, invading cells were fixed with methanol and subjected to crystal violet staining. Cell migration was randomly taken from 3 microscopic fields of each filter and counted (20, 21).

Apoptosis Assay by Annexin V-FITC/propidium iodide (PI)

In a microtiter plate (12 wells), A549 cells were cultured at a density of 1×10^5 cells/well for 24 h, and then subjected to incubation for 12 h with CGA at doses of 5 µM and 10 µM. The cells were washed twice with PBS and stained with Annexin V-FITC/PI using the apoptosis detection kit (Jiancheng Bioengineering Institute, Nanjing, China). Thereafter, the cells were analyzed using a flow cytometer (BD Bioscience, CA, USA) (22).

Assay of inhibitory effect of CGA against recombinant HDAC activities

The effect of CGA on the activities of HDAC 1, 6 and 8 was assayed with Z-Lys(ε-Ac)-AMC as substrate, while its inhibitory effect on HDAC 2 and 3 activities was determined using Boc-Lys(ε-Ac)-AMC as substrate. Histone deacetylase was assayed using Epigentek assay kits ((Epigentek Incorporated, NY) in line with manufacturer's protocol. The HDAC enzyme was diluted with incubation buffer, pH 8, and then added to the reaction plate (the control contained buffer in place of enzyme). Next, different concentrations of CGA in 10 % DMSO/incubation buffer were added, followed by addition of substrate for HDAC 1, 6, and 8, or HDAC 2 and 3 as appropriate, to start the enzyme reaction. The reaction was allowed to proceed for 2 h at 37°C and stopped by addition of the stop solution as per the kit manufacturer's instruction. The mixture was allowed to stand for 30 min, and the amount of de-acetylated products, which is proportional to the enzyme activity, was determined colorimetrically in a microplate reader at excitation and emission wavelengths of 390 and 460 nm, respectively. The readings were then subjected to analysis using a GraphPad Prism 5.0 software (23-25).

Quantitative RT-PCR analysis

The total RNA was extracted from A549 cells with different treatments, the following qRT-PCR was conducted on basis of previous procedures (26). The level of HDAC-6 mRNA and MMP2 mRNA in cells were determined by comparative Ct method. In this experiment, β-actin was the internal control. All results were conducted in triplicate. The primer sequences used in the present study was as follows, HDAC6 forward 5'–AAGAAGACCTAATCGTGGGACT-3', reverse 5'-GCTGTGAACCAACATCAGCTC-3'; MMP2 forward 5'-TGACTTTCTTGGATCGGGTCG-3', reverse 5'-AAGCACCACATCAGATGACTG-3' β-actin forward 5'-GATGAGATTGGCATGGCTTT-3', reverse 5'-GTCACCTTCACCGTTCCAGT-3'.

Western blotting

The cells were incubated with CGA and SAHA for 72 h to determine their effects on HDAC6 expression. Proteins were extracted from A549 cells using PMSF-containing RIPA buffer and quantified with the bicinchoninic assay method. Following cellular lysis, 100 µg of the lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto PVDF membrane, and blocked with non-fat milk for 1 h. Primary antibodies against MMP2, HDAC6, Ac-NF-κB p65, Ac-NF-κB p65, and β-actin were incubated with the membrane at 4°C overnight. Sequel to washing with TBST buffer (thrice), the membrane was incubated for 2 h with secondary antibody, and then rinsed thrice again with TBST buffer prior to assessment of the protein bands using ECL kit enzyme-linked chemiluminescence. In these assays, β-actin served as internal standards. The intensities of the resultant bands were estimated using Quantity One software. The expression of protein was depicted in terms of ratio of the band intensities of the target protein and reference genes.

The expression levels of Ac-NF-κB p65 and NF-κB p65 were also determined by Western blotting assay in order to study the effect of CGA on downstream pathway of HDAC6 (27).

Assay of tumorigenicity

Non-retrospective ethical approval for the animal experimentations in the present study was obtained from the Animal Research Ethics Committee of No.215 Hospital of Shaanxi Nuclear Industry (20184053). In addition, we confirm that the tumor burden on the experimental animals did not exceed the recommended levels. The animals were anesthetized and sacrificed using acceptable methods/techniques.

Male BALB/c nu/nu mice (immune-deficient) were employed as nude mice xenograft model. The mice had a mean weight of about 19 g and were aged 5 weeks – old. They were maintained on normal mice feed under standard, disinfected laboratory conditions in an atmosphere with equal light-dark periods. The right anterior axillary of each mouse was subjected to subcutaneous

injection of 5×10^6 A549 cells/0.2 mL. After 7 days, the mice were split into 2 groups (10 mice each) and given intraperitoneal injection of 25 mg CGA/kg/day in saline. At 5-day intervals, assessment of tumor size was carried out, and tumor volume (TV) was computed as indicated hereunder:

$TV\ (mm^3) = (width^2 \times length)/2$

Tumor weights were measured in experimental mice 25 days after euthanasia (28).

Statistical analysis

Data are presented as mean ± standard deviation (SD) and subjected to two-tailed *t*-test. Statistical analyses were carried out with GraphPad Prism 5.0. Data on categorical variables were analyzed using chi square test. Values of $p < 0.05$ were taken as indicative of statistical significance.

Results

CGA inhibited proliferation and colony formation in NSCLC cells

The results of CCK-8 assay demonstrated that CGA significantly suppressed lung adenocarcinoma cell growth including A549, H322 and SPC-A1 cell lines, relative to the control group, but had no anti-proliferation effect on normal HBE cells ($p < 0.01$; Figure 1B).

Moreover, CGA at two different concentrations (5 μM, 10 μM) reduced colony formation efficiency in A549 cells, relative to control cells ($p < 0.05$; Figure 2). The results shown in Figure 2 reveal obvious inhibitory effect of CGA on NSCLC cells.

CGA inhibited the migration of NSCLC cells

The influence of CGA on other mitogen-dependent processes was investigated using migration assay so as to compare motilities between CGA group and the control group. The result indicated that A549 cells had significantly decreased migration ability and significantly reduced number of migration cells when exposed to different concentrations of CGA (Figure 3). In addition, the migration of A549 cells cultured with CGA was considerably lower than that of the controls 24 h after plating, and less number of migration cells was observed at higher concentration of CGA.

CGA induced apoptosis in NSCLC cells

As depicted in Figure 4, CGA at different concentrations produced significant increases in the level of early and late apoptotic cells, relative to control after 12h-incubation, and the degree of apoptosis in the 10 μM group was significantly higher than that in the 5 μM group. These results indicate that CGA produced concentration-dependent apoptosis-promoting effects on human NSCLC cell lines A549.

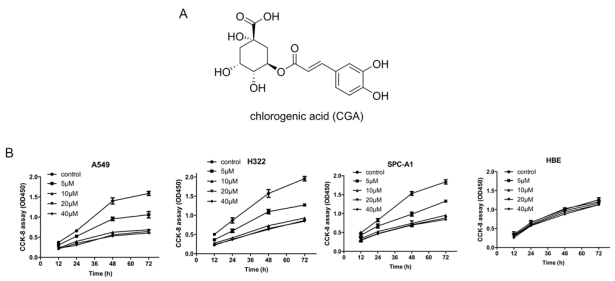

Figure 1. (A) Structure of CGA; (B) Cellular proliferation in treated NSCLC cells measured by CCK-8 assay at different time points. Values are mean ± SD of 3 replicates.

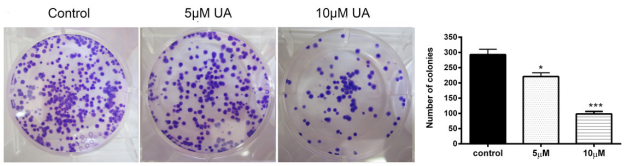

Figure 2. Proliferating colonies in A549 cells with different treatments determined by colony formation assay**.** Values are mean ±SD (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

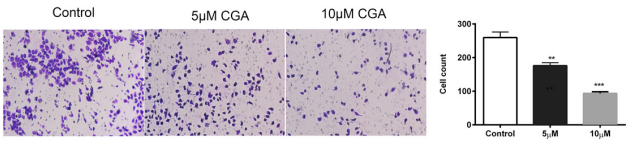

Figure 3. Metastatic condition in A549 cells subjected to the various treatments, as measured by cell migration assay. All data shown are mean ±SD (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

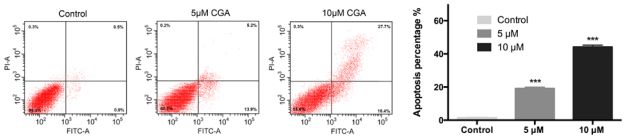

Figure 4. Cell apoptosis in CGA treated A549 cells (determined by Annexin V-FITC and PI staining). Values are presented as mean ±SD (n = 3). *** $p < 0.001$ vs. Control group.

Activities of HDAC 1, 2, 3, 6, and 8 isoforms were inhibited by CGA

Due to the remarkable cytotoxic effects of CGA, its effect on the activities of HDAC-1, 2, 3, 6 and 8 isoforms were determined. The results showed that CGA exerted selective inhibition of HDAC6 with IC_{50} value of 792 nM, which was inferior to the inhibition produced by the positive control vorinostat, with IC_{50} of 131 ± 12.

CGA suppressed the expressions of HDAC6 and HDAC6-associated MMP-2

Based on the report that the NF-κB-MMP-2 pathway is involved in the invasion of lung cell cancer, we decided to investigate if the HDAC6-induced reduction in migration was due to suppressed expression of MMP-2 (29). Using A549 cell lysates treated with 10 μM CGA or 1 μM SAHA, the mRNA and protein expressions of HDAC6 and MMP-2 were determined with qRT-PCR and western blotting. The results obtained indicated that CGA significantly suppressed the expressions of HDAC6 and MMP-2 to extents comparable to the inhibitions produced by the positive control SAHA (Figure 5). This indicated that the tumor inhibitory activity of CGA was caused by decreased expression of MMP-2 protein.

Effect of CGA on Downstream Pathway of HDAC6

In order to further unravel the mechanisms responsible for CGA-induced reduction in the expression of HDAC6, the levels of two key signaling pathway-related factors (Ac-NF-κB p65 and NF-κB p65) were determined in A549 cells after their incubation with

Table 1. Comparison of the effects of CGA and positive control (vorinostat) on the activities of HDAC-1, 2, 3, 6, and 8 isoforms.

Compound	IC_{50} (nM)				
	HDAC1	HDAC2	HDAC3	HDAC6	HDAC8
CGA	3652 ± 210	2130 ±162	4591 ± 387	792 ± 65	3509 ± 325
Vorinostat (SAHA)	50 ± 10	228 ± 17	21 ± 4	131 ± 12	1387 ± 232

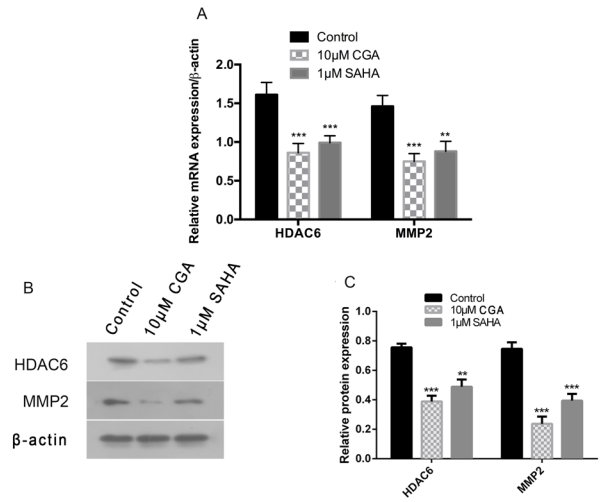

Figure 5. Effect of CGA on the mRNA and protein expressions of HDAC6 and related MMP-2 in A549 cells**.** Values are mean ± SD of 3 replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control.

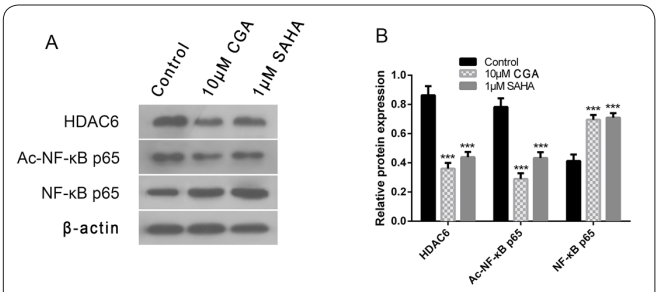

Figure 6. Effect of CGA on downstream pathway of HDAC6. Values are presented as mean ±SD (n = 3)**.** * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$., vs. control.

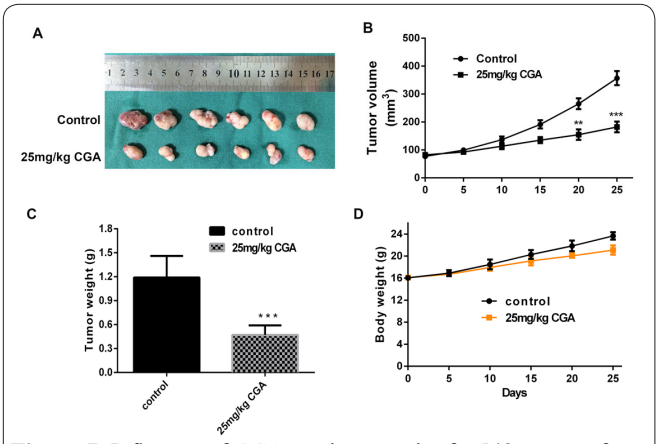

Figure 7. Influence of CGA on the growth of A549 xenograft tumor in mice. (A) Representative images of tumor dimensions at day 25; (B) Volumes of the xenograft tumors at 5-day intervals following exposure to 25 mg CGA/kg/day. Each point indicates a mean value; (C) Terminal tumor weights; (D) Changes in mice body weights throughout the study period. Results are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control.

CGA. The results obtained (Figure 5) revealed significant decreases in MMP-2 levels. Furthermore, due to the HDAC6 down-expression, the expression levels of Ac-NF-κB p65 were decreased, when compared with the control group (Figure 6). These results demonstrated that HDAC6 suppressed the acetylation of NF-κB p65, resulting in the decreased expression of Ac-NF-κB.

CGA inhibited the growth of NSCLC in nude mice model

Results from studies on the tumor-suppressive effect of CGA in nude BALB/c mice subcutaneously injected with A549 cells showed significant CGA-induced suppression of NSCLC growth, relative to saline-treated control. On day 25, A549 cell-injected mice exposed to CGA at the dose of 25 mg/kg/day had tumor size approximately half of that of the control group (Figures 7A and 7B). This demonstrated that CGA suppressed the proliferation and metabolism of NSCLC *in vivo*. After euthanasia, the average tumor weight of experimental mice was measured, the value of the control group was 1.19 ± 0.27 g, while that of the CGA-treated group was much lower (0.47 ± 0.14 g) i.e. CGA caused 60.6 % inhibition in weight (Figure 7C). However, there was no significant difference in body weight between the two groups (Figure 7D).

Discussion

During the last decades, NSCLC has gradually become one of the most lethal malignant tumors. Due to its low 5-year survival rate, it is important and indeed very necessary to discover potential mechanisms for reducing the occurrence and deterioration of NSCLC[20]. Several studies have indicated that a major factor in the etiology of cancers involves multi-function-associated changes in the epigenetic profile (30-32). Investigations have identified HDAC6 as a regulatory protein which may be a promising target more for cancer therapy than for any other disease (33, 34). Indeed, HDAC6 is expressed highly in many cancers, and it may act either as a suppressor or inducer, based on the stage and nature of the cancer (35, 36). Thus, HDAC inhibitors, especially natural products, have gained recognition as potential treatment strategies for malignancies (37). Several studies have suggested that natural HDAC inhibitors act through different mechanisms: some suppress the activity of HDAC6, while some promote the effectiveness of other HDAC6 inhibitors (38-40).

The antitumor activity of CGA has received more attention than that given to any other natural products. Studies carried out in China and elsewhere have shown that CGA exerts a variety of anti-tumor effects on NSCLC, with anti-tumor mechanisms involving inhibition of DNA mutations and cancer initiation; anti-oxidation, cytotoxicity, cell cycle repression, induction of tumor differentiation and apoptosis; anti-invasion, and inhibition of tumor angiogenesis (41-43). However, the

underlying molecular mechanisms responsible for the beneficial effects of CGA in NSCLC remain unclear. It has been discovered that the expressions of MMPs are closely related to lung carcinoma cell invasion, metastasis and recurrence (44-45). The MMPs belong to a group of zinc ion-dependent endopeptidases which are of great importance in many physiological and pathological processes such as tumor etiology wherein their activities are significantly increased. Among these functions, MMP-2 can selectively degrade collagen IV of the basement membrane which is crucial for lung cell cancer migration and metastasis through the basal membrane barrier (46).

We investigated the antitumor effect of CGA using cell viability assay, colony formation assay and tumorigenicity assay, with the aim of unravelling the mechanism involved in the CGA-induced inhibition of NSCLC progression and growth. The mechanism was also investigated via cell migration, HDAC and Western blotting assays. The results revealed that the regulatory mechanism is closely connected with NF-κB. The NF-κB family of transcriptional regulators plays a central role in tumorigenesis and apoptosis, among which P65 with a special transactivation domain (TAD) has been identified as the major active component which regulates the transcriptional activity of a variety of downstream target genes (47, 48). Specifically, NF-κB upregulates the secretion and expression of MMP-2 by binding to the κB sequence of corresponding promoter, which enhances tumor invasiveness and metastasizing potential. Furthermore, HDAC6 promotes Ac-NF-κB p65 deacetylation, and suppresses MMP-2 expression. In the present study, CGA strongly and selectively inhibited HDAC6 activity, and suppressed tumorigenicity.

In summary, the data obtained in this investigation indicate that CGA inhibits NSCLC cell proliferation through down-regulation of the potency of HDAC. This hints at the feasibility of developing CGA as a potential therapy for NSCLC.

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Conflict of interest
The authors have no conflicts of interest to declare.

Authors' contributions
This work was done by the authors named in this article and the authors accept all liabilities resulting from claims relating this article and its contents. The study was conceived and designed by Liang Yingping; Guo Xiaoqi, Liu Junni, Xue Feng, Bai Guodong collected and analysed the data, Liu Hongtao drafted the manuscript. All the authors listed perused the manuscript and gave approval for its publication.

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