

Chemopreventive effects of oral gallic acid feeding on tumor growth and progression in TRAMP mice

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Abstract

Our recent studies have identified gallic acid as one of the major constituents of grape seed extract showing strong *in vitro* anticancer efficacy against human prostate cancer cells. Herein, for the first time, we established the *in vivo* chemopreventive efficacy of gallic acid against prostate cancer by evaluating its activity against prostate tumor growth and progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) model. At 4 weeks of age, male TRAMP mice were fed with drinking water supplemented with 0.3% and 1% (w/v) gallic acid until 24 weeks of age. Positive control group was fed with regular drinking water for the same period. Our results showed that gallic acid-fed groups had a higher incidence of differentiated lower-grade prostatic tumors at the expense of strong decrease (~60%; $P < 0.01$) in poorly differentiated tumors. Immunohistochemical analysis of prostate tissue showed a decrease in proliferative index by 36% to 41% ($P < 0.05$) in 0.3% to 1% gallic acid-fed groups, with an increase in the apoptotic cells by 3-fold ($P < 0.05$). Further, both doses of gallic acid completely diminished the expression of Cdc2 in the prostatic tissue together with strong decrease in the expression of Cdk2, Cdk4, and Cdk6. The protein levels of cyclin B1 and E were also decreased by gallic acid feeding. Together, for the first time, we identified that oral gallic acid feeding inhibits prostate cancer growth and progression to advanced-stage adenocarcinoma in TRAMP mice via a strong suppression of cell cycle progression and cell proliferation and an increase in apoptosis. [Mol Cancer Ther 2008;7(5):1258–67]

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Introduction

Statistical estimates released by the American Cancer Society for the year 2007 indicate that there would be an estimated 218,890 new cases of prostate cancer and 27,050 associated deaths in the United States alone. Prostate cancer is the most frequently diagnosed malignancy in elderly American men and second only to lung cancer in being the leading cause of cancer-related deaths (1). The onset of preclinical prostate cancer may occur even in young men (~30 years old) and could take considerable time for progression to detectable malignancy and then to a hormone-refractory stage, rendering antiandrogen therapy ineffective (2, 3). Accordingly, a considerable window of time may allow for various prevention strategies to be employed (2–4). Chemoprevention for the management of cancer may not necessarily eliminate the lesions; however, it is expected to delay the neoplastic progression to a more advanced state of the disease, which would certainly improve the survival time in prostate cancer patients (2–5). Various research groups have directed considerable efforts toward the identification of dietary or nondietary naturally occurring chemical agents for both prevention and intervention of prostate cancer (3, 4, 6–8). One such dietary agent is grape seed extract (GSE), which is marketed as a dietary supplement (with 95% standardized procyanidins) in the United States, owing to its several health benefits (9–11).

GSE is a complex mixture of polyphenols, also known as procyanidins, mostly containing dimers, trimers, and other oligomers of catechin and epicatechin and their gallate derivatives (12–15). Studies conducted by us and others have shown that GSE inhibits growth of many cancers of epithelial origin, including prostate cancer (16–18). For example, our studies have shown that GSE possesses strong anticancer efficacy against both androgen-dependent and androgen-independent prostate cancer, wherein it inhibits cell growth and causes cell cycle arrest in human prostate cancer LNCaP and DU145 cells (19–21) and induces their apoptotic death as well (20, 22). Regarding the *in vivo* efficacy of GSE, we have also shown that GSE inhibits the growth of DU145 xenograft in nude mice via an inhibition of cell proliferation and an induction of apoptosis (17). One of our recent studies also showed that oral GSE blocks prostate cancer growth and progression at neoplastic stage in transgenic adenocarcinoma of the mouse prostate (TRAMP) model via induction of apoptosis and inhibition of cell proliferation, mediated in part by modulation of cell cycle regulatory molecules (23).

All these studies together indicate the presence of biologically active phytochemicals in the crude mixture of GSE. To identify the biologically active components of GSE, our research group has focused its efforts on the fractionation of GSE, employing a combination of chromatographic

separations and biological efficacy screening of the fractions (15). Completed studies by us have shown that the polyphenolic compound gallic acid accounts for a significant fraction of GSE in causing growth inhibition, death, and apoptosis induction in human prostate cancer DU145, PC3, and 22Rv1 cells (15, 20, 24). This was an important observation as gallic acid, in both free and ester forms, is abundantly found in fruits, vegetables, and green tea and has been reported to inhibit growth and induce apoptosis in various cancer cell lines (25–31). Based on the anticancer efficacy of gallic acid against prostate cancer cells and the fact that the parent agent GSE exhibits anticancer efficacy against prostate cancer in xenograft and TRAMP model, here, for the first time, we evaluated the chemopreventive efficacy of gallic acid feeding against prostate cancer growth and progression and associated molecular alterations in TRAMP model.

TRAMP model was developed in C57BL/6 mice using minimal rat probasin promoter to drive the expression of SV-40 early genes (T/t; Tag) specifically in prostatic epithelium (32, 33). The transgene is hormonally regulated and expressed at sexual maturity, which results in the induction of spontaneous neoplastic epithelial transformation (34). The expressed SV-40 large T antigen then abrogates p53 and Rb function, and as a result, TRAMP male mice develop spontaneous progressive stages of prostatic disease with time from early lesions of prostatic intraepithelial neoplasia (PIN) to late-stage metastatic adenocarcinoma, which closely mimic the progressive forms of human prostatic carcinoma (32, 35–37). Because of the close relevance of the TRAMP model to human prostate cancer, our present findings showing chemopreventive efficacy of gallic acid and associated mechanisms in TRAMP model might have potential clinical significance.

Materials and Methods

Animals and Treatment Protocol, Necropsy, and Histopathology

Heterozygous TRAMP females, developed on a pure C57BL/6 background, were cross-bred with nontransgenic C57BL/6 breeder males. Tail DNA was subjected to PCR-based screening assay for PB-Tag as described previously (34). The routinely obtained 4-week-old TRAMP male mice were randomly distributed into positive control and treatment groups. Positive control mice were supplied with regular drinking water and the treatment groups were fed with gallic acid solutions [0.3% and 1% gallic acid (w/v) in regular drinking water] for 20 weeks. Gallic acid was purchased from Sigma, and the freshly prepared solutions (as the only source of drinking water) were supplied every Monday, Wednesday, and Friday; the fluid consumption by different groups was also recorded. Our earlier laboratory studies have established by high-performance liquid chromatography analysis that the aqueous solutions (0.03–1%, w/v) of gallic acid stored at room temperature are totally stable over 96 h⁴; however, as precautionary measure, fresh solutions were made at 48-h intervals. There

were 11 mice in positive control and 10 mice in both 0.3% and 1% gallic acid-fed groups. As overall controls, non-transgenic mice ($n = 5$ mice per group) were fed with regular drinking water or highest gallic acid dose for same duration. During the study, animals were permitted free access to AIN-76A rodent diet. Food consumption and animal body weight were recorded weekly, and the animals were monitored daily for their general health. Animal care and treatments were in accordance with Institutional guidelines and approved protocol.

At the time of sacrifice, the animals were anesthetized by ketamine injection and then euthanized by exsanguinations. Each mouse was weighed and lower urogenital tract (LUT), including bladder, seminal vesicles, and prostate, was removed *en bloc*. Animals were also examined for gross pathology, and any evidence of edema, abnormal organ size, or appearance in nontarget organs was also noted. LUT wet weight was recorded, and prostate gland was harvested and microdissected whenever possible (when a tumor obscured the boundaries of the lobes, it was taken as such). One portion of dorsolateral prostate was snap-frozen and stored at -80°C . Tissues were fixed overnight in 10% (v/v) phosphate-buffered formalin and processed conventionally. Briefly, the fixed tissues were dehydrated in ascending grades of ethanol, cleared in toluene, and embedded in paraffin wax. Sections (5 μm) were cut with microtome and mounted on superfrost slides (Fisher Scientific) coated with 0.01% poly-L-lysine (Sigma-Aldrich). Sections (5 μm) of paraffin-embedded tissues were stained with H&E for routine histopathologic evaluation.

Immunohistochemical Analysis

Paraffin-embedded sections were deparaffinized and stained using specific primary antibody followed by 3,3'-diaminobenzidine (DAB) staining as described previously (38). Primary antibodies used were anti-SV-40 large T antigen (1:400; BD PharMingen) and anti-proliferating cell nuclear antigen (PCNA) (1:250; DakoCytomation). Biotinylated secondary antibody used was rabbit anti-mouse IgG (1:200; DakoCytomation). Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using Dead End Colorimetric TUNEL System (Promega) as per vendor's protocol. PCNA- and TUNEL-positive cells were quantified by counting brown-stained cells within total number of cells at 10 randomly selected fields at $\times 400$ magnification.

Immunoblot Analysis

The dorsolateral prostate samples dissected out from control and gallic acid-fed groups of mice were homogenized in nondenaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP-40, 5 units/mL aprotinin]. Protein concentration in

⁴ Rajesh Agarwal, unpublished data.

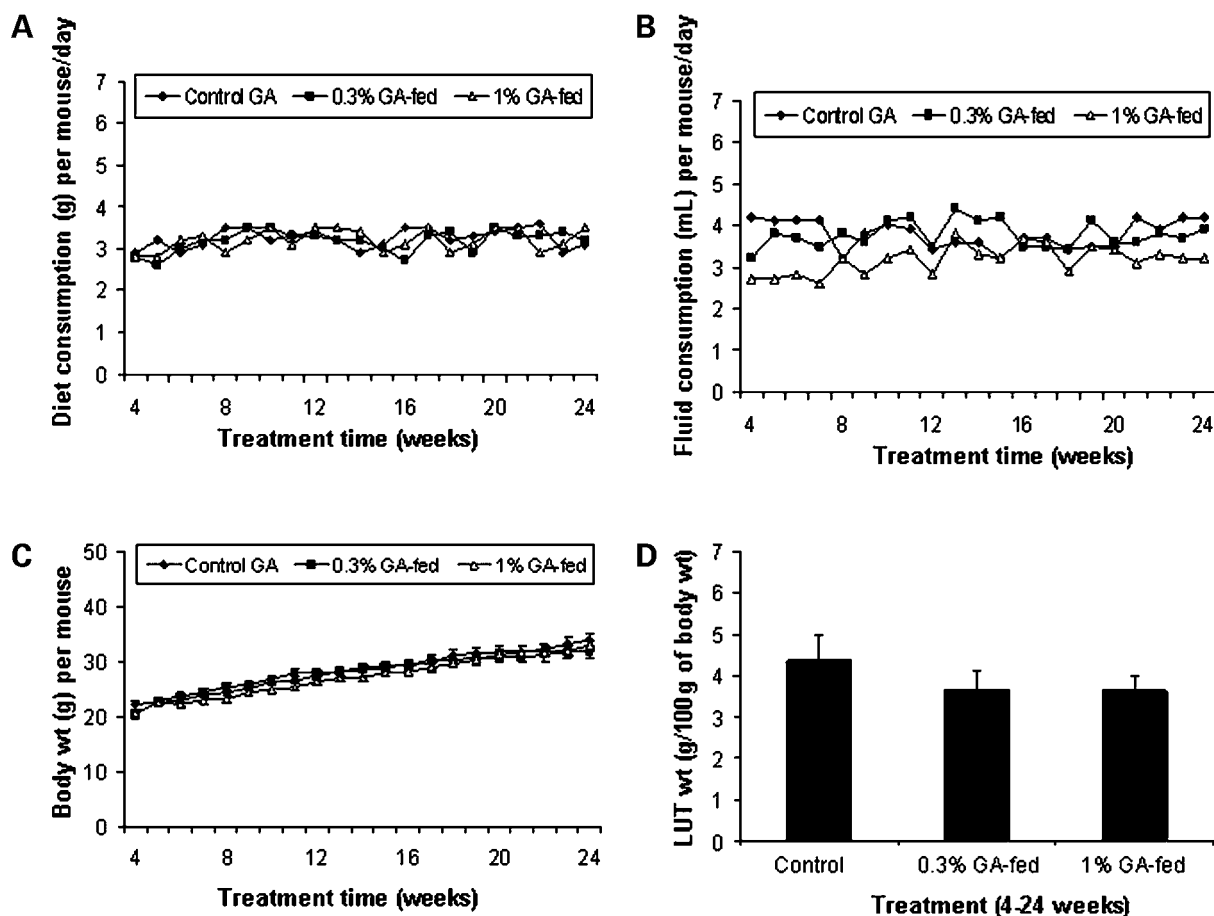


Figure 1. **A**, effect of oral feeding of gallic acid on the daily diet consumption of TRAMP mice. Food consumption by each mouse was recorded weekly throughout the feeding regimen in each group. Diet consumption (g/d) per mouse is plotted as a function of time (weeks) for each group. **B**, effect of oral feeding of gallic acid on the daily fluid consumption of TRAMP mice. Control mice were supplied with regular drinking water and the treatment groups were fed with gallic acid solutions [0.3% and 1% gallic acid (w/v) in regular drinking water] for 20 wk. The freshly prepared solutions (as the only source of drinking water) were supplied every Monday, Wednesday, and Friday; the fluid consumption by different groups was also recorded. Fluid consumption (mL/d) per mouse is plotted as a function of time (weeks) for each group. **C**, effect of oral feeding of gallic acid on the body weight of TRAMP mice. Body weight of each mouse was recorded weekly throughout the experiment. Body weights of mice are represented as average of each group and plotted as a function of time (weeks) for each group. **D**, effect of gallic acid feeding on the weight of the LUT organs. At the time of necropsy, after 20 wk of gallic acid feeding, starting from the fourth week of age, each mouse was weighed and the LUT, including the bladder, seminal vesicles, and prostate, was removed *en bloc* and weighed [$n = 11$ (positive control) and $n = 10$ (0.3% and 1% gallic acid fed)] mice per group. Bars, SE. The statistical significance of difference between positive controls and gallic acid-fed group was analyzed by unpaired two-tailed Student's *t* test. *P* values < 0.05 were considered significant. Control, positive control (TRAMP mice); GA, gallic acid.

lysates was determined using Bio-Rad DC protein assay kit (Bio-Rad Laboratories) by the Lowry method. For immunoblot analyses, protein (50–80 μ g) per lysate sample was denatured in $2\times$ SDS-PAGE sample buffer and subjected to SDS-PAGE on 12% or 16% Tris-glycine gel as needed. The separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% (w/v) nonfat milk powder in TBS (10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20) overnight at 4°C. Membranes were probed with different primary antibodies, including anti-Cdk2 (sc-163), anti-Cdk4 (sc-749), anti-Cdk6 (sc-177), anti-Cdc2 (sc-54), anti-cyclin A (sc-751), anti-cyclin B1 (sc-245; all from Santa Cruz Biotechnology); anti-cyclin E (Ab-1) anti-Kip1/p27 (Neomarkers); and

anti-Cip1/p21 (Upstate). Secondary antibodies were anti-rabbit IgG (Cell Signaling Technology) or anti-mouse IgG (Amersham). Equal protein loading was confirmed by stripping and reprobing membranes with anti- β -actin primary antibody (Sigma-Aldrich).

Statistical and Microscopic Analyses

All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific), and two-sided *P* values < 0.05 were considered significant. Fisher's exact test was used to compare incidence of PIN and adenocarcinoma in positive control versus gallic acid-fed groups. For other data, the difference between positive control versus gallic acid-fed groups was analyzed by unpaired two-tailed Student's *t* test. Histopathologic evaluation

was done by K.R. followed by a blinded examination conducted by M.S. Any disagreement in the diagnosis between the two evaluators was discussed/resolved by mutual consensus and the results are reported as mean of both these observations. Densitometric analysis of immunoblots (adjusted with β -actin as loading control) was done by Scion Image program (NIH). All microscopic histopathologic and immunohistochemical analyses were done by Zeiss Axioscope 2 microscope (Carl Zeiss) and photomicrographs were captured by Carl Zeiss AxioCam MrC5 camera.

Results

Gallic Acid Feeding Reduces LUT Weight without Any Apparent Toxicity

Gallic acid feeding did not show any significant change in diet (Fig. 1A) and fluid consumption (Fig. 1B) between the positive control and gallic acid-fed mice during the entire treatment regimen. On average, the positive control mice consumed 3.7 ± 0.9 mL water/d, whereas the 0.3% and 1% gallic acid-fed mice consumed 3.7 ± 0.8 and 3.2 ± 0.8 mL/d, respectively. In the 1% gallic acid-fed group, however, there was an initial lag phase during which the mice showed low fluid consumption compared with the other groups (we attributed this to the time required for the mice to get adjusted to the new taste). In addition, gallic acid feeding did not show any considerable difference in body weight between the positive control and gallic acid-fed mice during the entire treatment period

(Fig. 1C). At the time of necropsy, all animals were examined for gross pathology, and there was no evidence of edema, abnormal organ size, or appearance in nontarget organs. At necropsy, the LUT weights indicated that there was no significant difference between the LUT weights in gallic acid-fed groups in comparison with the positive control group (Fig. 1D). Gallic acid-treated groups did not show any considerable decrease in the levels of SV-40 T antigen in different stages of prostate tumorigenesis compared with the positive control group as observed by immunohistochemical analysis of the transgene expression (Fig. 2). In nontransgenic mice, gallic acid did not show any change in LUT weight (data not shown).

Gallic Acid Feeding Reduces Poorly Differentiated Adenocarcinoma Incidence

H&E-stained sections were microscopically examined and classified as follows: (a) PIN having foci with two or more layers of atypical cells, with elongated hyperchromatic nuclei and basement membrane-bound gland profiles, increased epithelial stratification may also fill the lumen of the ducts leading to luminal obstruction; (b) well-differentiated adenocarcinoma showing invasion of basement membrane, loss of intraductal spaces, and increased quantity of small glands; (c) moderately differentiated adenocarcinoma showing total loss of intraductal spaces and relatively solid growth; and (d) poorly differentiated adenocarcinoma showing sheets of poorly differentiated cells with remnants of trapped glands. Using these classifications, the histopathologic analysis of the H&E-stained (Fig. 3A) dorsolateral prostate showed that, on average, $78 \pm 12\%$ and $86 \pm 9\%$ area of prostate gland were covered by PIN lesions in 0.3% and 1% gallic acid-fed groups, respectively, compared with $68 \pm 13\%$ in the positive control group (Fig. 3B). Although most of the PIN lesions in all the groups were associated with luminal obstruction, $\sim 10\%$ of mice in both gallic acid-fed groups had PIN lesions displaying only focal stratification with no luminal obstruction. There was no difference in the area covered by well-differentiated lesions between all the groups; on average, 4% to 5% area were covered by these lesions. However, there was a marked difference in the area covered by more aggressive tumors between the gallic acid-fed and positive control groups. The 0.3% and 1% gallic acid-fed groups had 63% and 67% less poorly differentiated area, respectively, compared with the positive control group (Fig. 3B). Whereas 27% of mice in the positive control group had 100% area of their prostate tissue replaced by poorly differentiated adenocarcinoma, none of the animals in the 1% gallic acid-fed group displayed absolute poorly differentiated characteristics. Further, histopathologic analysis revealed that there was a marked difference in tumor incidence between the positive control and gallic acid-fed groups (Fig. 3C). There was an increase in the incidence of more differentiated tumors in the 1% gallic acid-fed group as indicated by 70% incidence of well-differentiated tumors in this group compared with 60% and 55% incidence in 0.3% gallic acid-fed and positive control group of mice, respectively

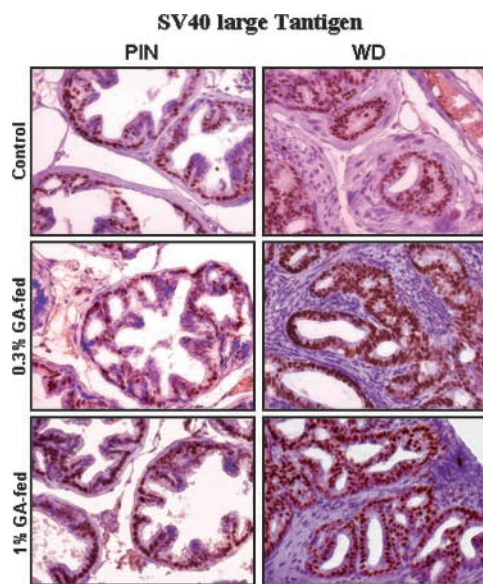


Figure 2. Effect of oral feeding of gallic acid on the expression of SV-40 large T antigen in the dorsolateral prostate of TRAMP mice. Immunohistochemical staining was based on DAB staining as detailed in Materials and Methods. Representative DAB-stained tissue specimens are illustrated here from positive control and 0.3% and 1% gallic acid-fed groups (magnification, $\times 400$) showing brown staining for the expression of SV-40 large T antigen in PIN and well-differentiated (WD) adenocarcinoma.

(Fig. 3C). Also in the 1% gallic acid-fed group, ~10% of the differentiated tumors exhibited features characteristic of microinvasive carcinoma (indicative of lesser extent of the invasive focus) with penetration of PIN involved glands into the surrounding stroma to form small nests of cells. The increase in the incidence of well-differentiated tumors in the 1% gallic acid-fed group was accompanied with a concomitant decrease in the incidence of poorly differentiated tumors. Strikingly, the incidence of poorly differentiated tumors decreased by 63% ($P < 0.01$) in both gallic acid-fed groups compared with the positive control mice (Fig. 3C). In nontransgenic mice, prostate histopathology did not show any difference in control and gallic acid-fed groups (data not shown). These results suggest that gallic acid feeding causes a dose-dependent increase in the incidence of more differentiated tumors in the prostate of TRAMP mice at the expense of strong decrease in the incidence of more aggressive forms of adenocarcinoma.

Gallic Acid Feeding Reduces Proliferation Index and Increases Apoptosis

To assess the *in vivo* effect of gallic acid feeding on proliferation index in the dorsolateral prostate, tissue

samples from both positive control and gallic acid-fed groups were analyzed for PCNA immunostaining. Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in gallic acid-fed groups compared with the positive control (Fig. 4A). Quantification of PCNA staining showed $37 \pm 5\%$ and $34 \pm 3\%$ PCNA-positive cells in 0.3% and 1% gallic acid-fed groups of mice compared with $58 \pm 8\%$ in the positive control (Fig. 4A), accounting for a decrease in proliferation indices by 36% and 41% ($P < 0.05$ for both), respectively. These results suggest the *in vivo* antiproliferative effect of gallic acid feeding during tumor growth and progression in dorsolateral prostate of TRAMP mice.

Further analysis of the tissues was done to assess the *in vivo* apoptotic response of gallic acid feeding on prostate tumorigenesis in TRAMP mice. Microscopic examination of tissue sections showed an increased number of TUNEL-positive cells in gallic acid-fed groups (Fig. 4B). The number of TUNEL-positive apoptotic cells were $12 \pm 3\%$ and $13 \pm 3\%$ in 0.3% and 1% gallic acid-fed groups of mice, respectively, compared with $4 \pm 2\%$ in the positive controls, accounting for a ~3-fold ($P < 0.05$)

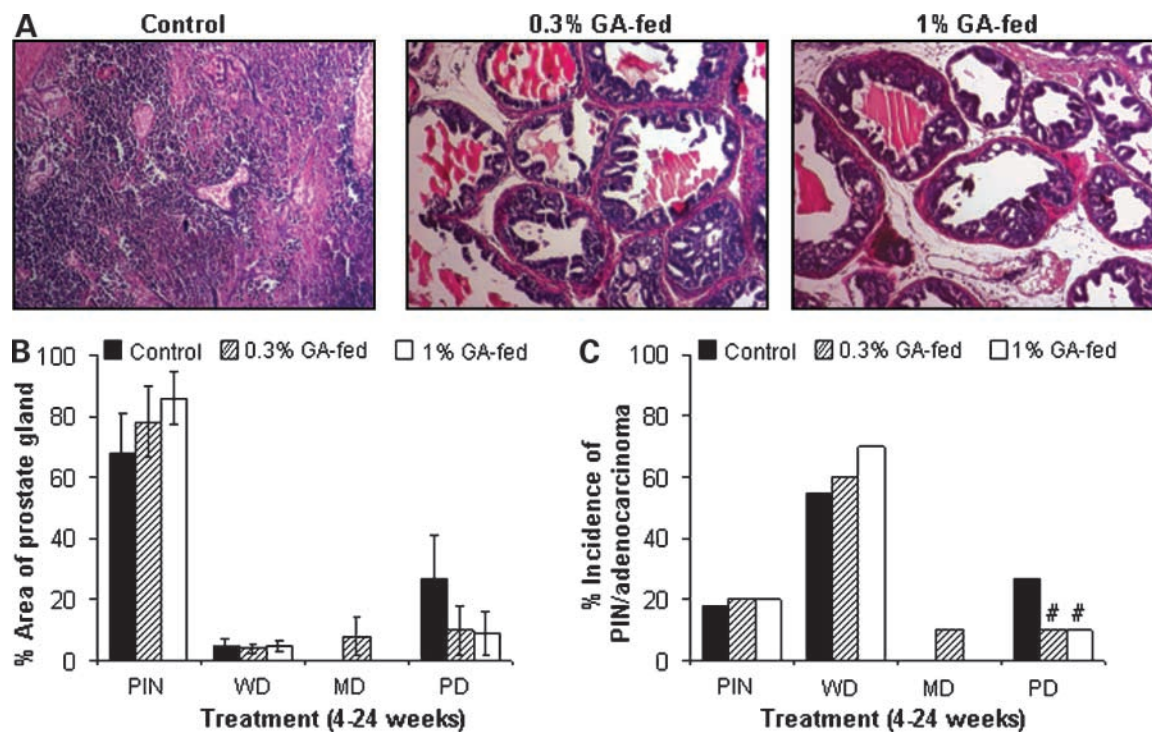
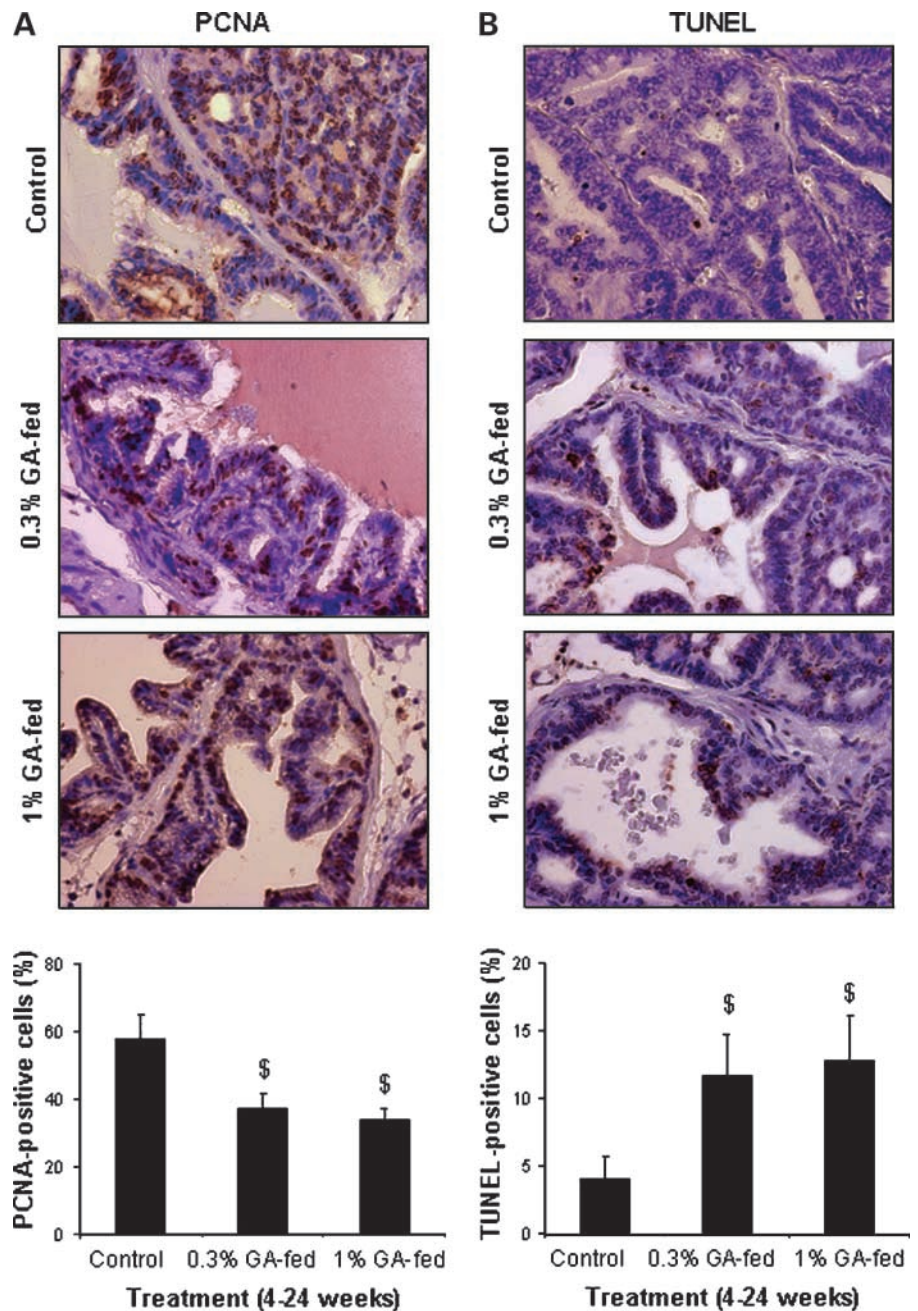


Figure 3. Inhibitory effect of gallic acid feeding on prostate tumorigenesis in TRAMP mice. At the time of necropsy, after 20 wk of gallic acid feeding, starting from the fourth week of age, each mouse was weighed and the LUT, including the bladder, seminal vesicles, and prostate, was removed *en bloc*. The dorsolateral prostate gland was histopathologically analyzed for the different stages of the neoplastic progression. **A**, photomicrographs (magnification, $\times 100$) representative of a treatment group show the H&E staining of the dorsolateral prostate of positive control and gallic acid-fed mice sacrificed after 24 wk of age. **B**, percentage of area of prostate gland of positive control and gallic acid-fed groups having histologically diagnosed PIN, well-differentiated, moderately differentiated (MD), and poorly differentiated (PD) adenocarcinoma characteristics. Bars, SE. The statistical significance of difference between the specific pathologic states of positive control and gallic acid-fed groups was analyzed by unpaired two-tailed Student's *t* test. P values < 0.05 were considered significant. **C**, effect of gallic acid feeding on the incidence of PIN/adenocarcinoma lesions in TRAMP mice. Fisher's exact test was used to compare incidence of PIN and adenocarcinoma in positive control versus gallic acid-fed groups. P values < 0.05 were considered significant. #, $P < 0.01$.

Figure 4. Antiproliferative and proapoptotic effects of gallic acid feeding in TRAMP mice. **A**, *in vivo* antiproliferative effect of gallic acid feeding on dorsolateral prostate of TRAMP. Immunohistochemical staining for PCNA in prostate was based on DAB staining as detailed in Materials and Methods. Representative DAB-stained tissue specimens from positive control and gallic acid-fed groups showing brown-colored PCNA-positive cells are depicted at $\times 400$ magnification. Quantification of PCNA-positive cells for determination of proliferation index is shown as mean \pm SE in each group. $\$, P < 0.05$. **B**, *in vivo* proapoptotic effect of gallic acid feeding on dorsolateral prostate in TRAMP mice. Apoptosis was analyzed by TUNEL staining in prostate tissues as detailed in Materials and Methods. Representative DAB-stained tissue specimens from positive control and gallic acid-fed groups showing brown-colored TUNEL-positive cells are depicted at $\times 400$ magnification. Apoptotic index was calculated as the number of positive cells $\times 100$ / total number of cells counted under $\times 400$ magnification in 10 randomly selected areas in each sample. Bars, SE. $\$, P < 0.05$. **A** and **B**, statistical significance of difference between positive control and gallic acid-fed groups was analyzed by unpaired two-tailed Student's *t* test. *P* values < 0.05 were considered significant.



increase in apoptotic cells by both doses of gallic acid (Fig. 4B). This finding suggests that, in addition to antiproliferative effect, proapoptotic effect could be another potential mechanism underlying the chemopreventive effect of gallic acid on prostate tumorigenesis in TRAMP model.

Gallic Acid Feeding Modulates Cell Cycle Regulators

The decrease in proliferation index in the mouse prostate by gallic acid feeding prompted us to investigate its effect on cell cycle regulatory molecules. Immunoblot analysis of the prostate tissue lysates for the expression of cyclin-

dependent kinases (Cdk), cyclins, and Cdk inhibitors was carried out. Western blots for Cdks, cyclins, and Cdk inhibitors with densitometric data (adjusted with β -actin as loading control) are shown in Fig. 5. Membranes were stripped and reprobbed for β -actin for each blot (data not shown). Results showed that gallic acid strongly decreased protein levels of Cdk2 by 96% and 100% ($P < 0.01$ for both doses), Cdk4 by 72% ($P < 0.05$) and 90% ($P < 0.01$), and Cdk6 by 69% ($P < 0.01$) and 74% ($P < 0.001$), respectively, in 0.3% and 1% gallic acid-fed groups of mice compared with the positive controls (Fig. 5). Strikingly, both doses of gallic

acid strongly decreased the protein levels of Cdk2 by 100% ($P < 0.001$) compared with the positive control (Fig. 5). Because cyclins act as the regulatory subunits that mediate the activation of the Cdks, we also examined the expression levels of different cyclin molecules in the prostate tissue lysates. Immunoblot analysis showed that gallic acid feeding also down-regulates the expression of cyclin A, B1, and E (Fig. 5). The expression of cyclin A was decreased

by ~80% in both gallic acid-fed groups of mice. However, this difference in the expression of cyclin A in control versus gallic acid-fed groups was not significant, which can be attributed to a large degree of variability (>50%) in the expression levels of cyclin A in the individual prostate samples within the positive control group (Fig. 5). The expression of cyclin B1 was decreased by 62% ($P < 0.01$) in 1% gallic acid-fed groups of mice compared with the

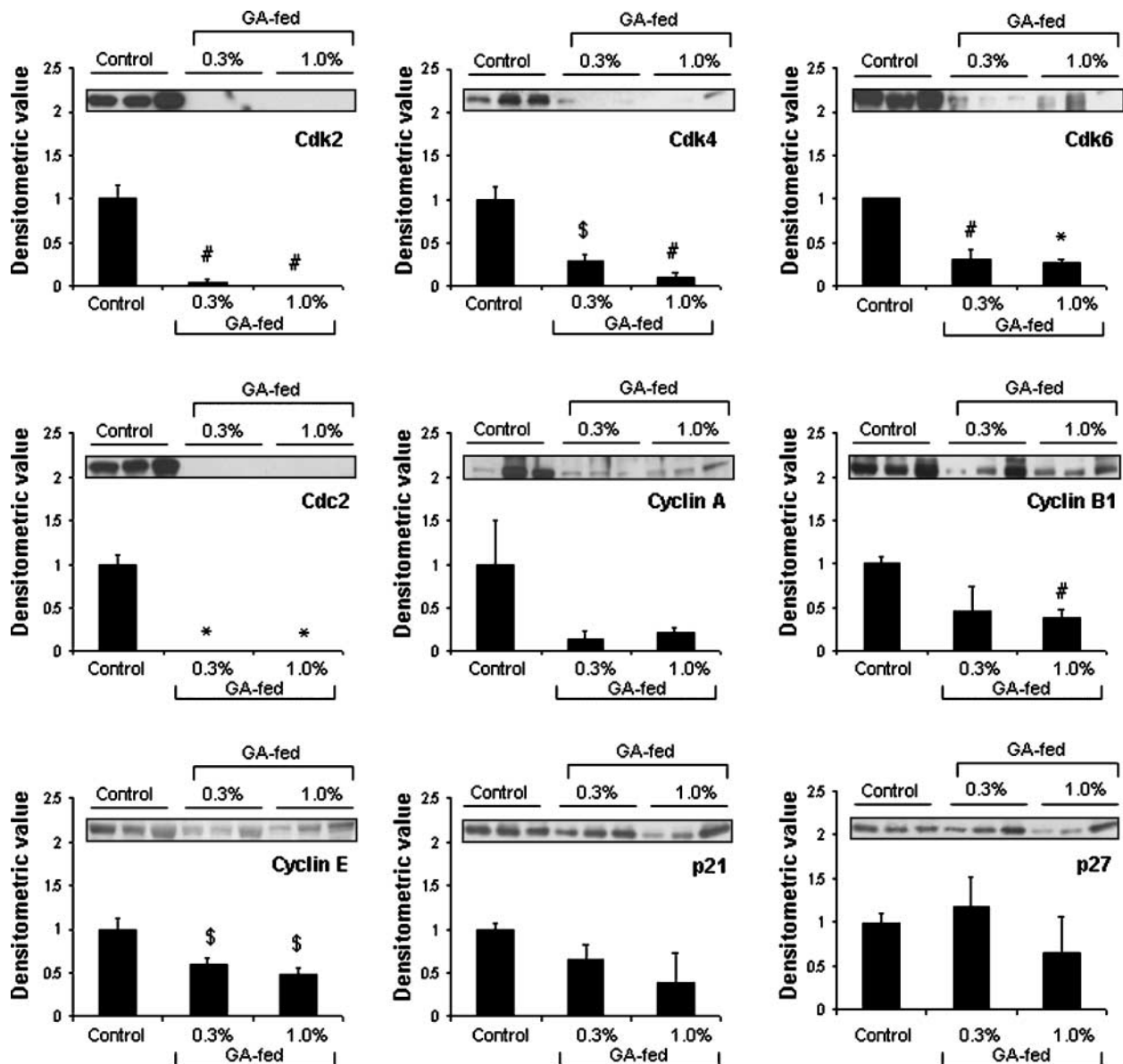


Figure 5. Gallic acid feeding alters the expression levels of cell cycle regulatory molecules in the dorsolateral prostate of TRAMP mice. Randomly, three prostate tissue samples from individual mice were selected from each group for immunoblot analyses detailed in Materials and Methods. Reactive protein bands for the expression of Cdk2, Cdk4, Cdk6, Cdc2, cyclin A, cyclin B1, cyclin E, Cip1/p21, and Kip1/p27 were visualized by enhanced chemiluminescence detection system, and in each case, the membrane were stripped and probed with β -actin as loading control. Densitometric analysis of band intensity for each protein was adjusted with β -actin (blots not shown) and is shown as mean \pm SE of the three bands from individual mouse prostate in each group. The statistical significance of difference between positive control and gallic acid-fed groups was analyzed by unpaired two-tailed Student's *t* test. *P* values < 0.05 were considered significant. *, $P < 0.001$; #, $P < 0.01$; \$, $P < 0.05$.

positive controls (Fig. 5). However, feeding with 0.3% gallic acid did not significantly decrease the expression levels of cyclin B1 compared with the positive control. On the other hand, cyclin E protein levels were decreased by 41% to 52% ($P < 0.05$) in 0.3% to 1% gallic acid-fed groups of mice compared with the positive control (Fig. 5). We did not observe any significant difference in the expression levels of Cdk inhibitors, Cip1/p21, and Kip1/p27 between the gallic acid-fed groups and the positive control (Fig. 5). Together, these results indicate that gallic acid strongly decreases the expression of cyclins and Cdks regulating G₁-S and G₂-M checkpoints in cell cycle progression, which could potentially inhibit prostate tumor progression in TRAMP mice.

Discussion

Progression of prostate cancer in humans is a multistage process involving the initial development of a small carcinoma of low histologic grade, which progresses slowly to the aggressive lesions of higher grade. Prostate cancer development and progression in TRAMP model closely mimics the human type of disease in a stochastic fashion and hence serves as an ideal model to study the chemopreventive efficacy of an agent against prostate cancer (37, 39). Chemopreventive strategies involving naturally occurring dietary/nondietary agents for prostate cancer intervention are being extensively studied because epidemiologic evidences suggest that dietary habits and lifestyle are among the major factors in prostate cancer growth and progression (8, 40, 41). In the last few decades, several research groups have directed their efforts in this direction, leading to identification of many cancer chemopreventive agents, one of them being gallic acid, which has shown anticancer effects in various cancer cell types (42–44).

Gallic acid and its esters are hydroxybenzoic acid derivatives ubiquitously present in fruits, vegetables, green tea, and also red wine (26, 28, 42). In several studies, gallic acid (free/esters) has been isolated and identified as the major active fraction in herbal medicinal plants, with growth-inhibitory effect in various cancer cell lines (25–30, 43–44). Also in our laboratory studies, while identifying the biologically active components of GSE, we recently isolated and identified gallic acid as an active agent in GSE (15, 45). We observed that gallic acid causes cell cycle arrest in prostate cancer cells, and this effect was mediated via a decrease in Cdks and cyclins protein levels and by an inactivating phosphorylation of cdc25A/cdc25C-cdc2 via ATM-Chk2 activation (15, 45). Further, gallic acid treatment was also shown to induce apoptosis by caspase-dependent and caspase-independent pathways via the activation of p38 mitogen-activated protein kinase (24). These *in vitro* findings were the basis for the present *in vivo* efficacy study of gallic acid and associated molecular mechanisms in TRAMP model. The novel findings in the present study are (a) oral gallic acid inhibits prostate tumor growth and progression in TRAMP mice without

any toxicity and (b) chemopreventive efficacy of gallic acid is accompanied by increase in the incidence of more differentiated tumors possibly at the expense of a concomitant decrease in poorly differentiated tumors together with a decrease in cell proliferation and an increase in apoptotic cell death. Potential molecular mechanisms of gallic acid efficacy, as identified here, are decreased expression of Cdks and cyclins.

Our earlier laboratory studies assessed the toxic effect of gallic acid by feeding aqueous solutions of gallic acid [0.03%, 0.1%, 0.3%, and 1% gallic acid (w/v) in regular drinking water; the only source of drinking water] to nontransgenic C57BL/6 mice ($n = 5$ mice per group) for 5 weeks starting from the fifth week of age.³ In this pilot study, gallic acid feeding did not show any considerable change in diet/fluid consumption and there was no considerable difference in body weight between the control (fed with regular drinking water) and gallic acid-fed nontransgenic mice during the entire treatment regimen. At the time of necropsy, gross pathologic examination did not show any evidence of edema, abnormal organ size, or appearance in different organs including prostate. Histologic evaluation also revealed that there was no evidence of any pathologic lesion in the prostate of gallic acid fed nontransgenic mice.³ In our present study also, gallic acid feeding for 20 weeks starting from the fourth week of age neither showed any toxic effects in mice nor influenced the normal histology of the prostate in nontransgenic mice, suggesting that gallic acid could be a nontoxic chemopreventive agent to suppress prostate tumor growth and progression.

The anti-prostate cancer effect of gallic acid was accompanied by strong antiproliferative and proapoptotic effects as observed by immunohistochemical analyses of prostate samples for the expression of PCNA- and TUNEL-positive cells. Further, based on our earlier *in vitro* studies in DU145 cells, it was anticipated that antiproliferative effect of gallic acid against prostate tumor progression might also involve cell cycle regulatory mechanisms. The differential expression of cell cycle regulatory molecules and their changing patterns have been well characterized during the progression of prostate cancer in TRAMP model, wherein an up-regulation of mitotic cyclins, including cyclin A, B, and E, and a decrease in cyclin D1 have been observed (46). The levels of cyclin D3 do not vary much during the disease progression, although there is an increased expression compared with normal prostate (46). In our present study, gallic acid down-regulated the expression of Cdk2, Cdk4, Cdk6, and Cdc2 as well as cyclin E and B1 in the prostate of TRAMP mice, suggesting that the antitumor progression effect of gallic acid in TRAMP mice could likely be mediated at least in part via its effect on Cdk-cyclin axis. We also observed that gallic acid feeding did not negatively regulate the expression of transgene in prostate epithelial cells, which initiates the process of tumorigenesis, suggesting that the observed biological effects of gallic acid are not due to its effect on transgene.

In summary, our findings suggest that gallic acid could be a useful agent for prostate cancer prevention and intervention. Whereas more studies are needed in future to assess whether the anti-prostate cancer efficacy of gallic acid also involves other molecular changes, to our knowledge, this is the first study in the TRAMP model with strong prostate cancer chemopreventive efficacy of gallic acid involving the modulation of cell cycle regulatory molecules together with strong antiproliferative and proapoptotic effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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