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PRINCIPAL INVESTIGATOR: Sanjay Gupta, Ph.D.

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, Ohio 41106-7015

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Sanjay Gupta, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Case Western Reserve University
Cleveland, Ohio 41106-7015

E-Mail: gxs44@po.cwru.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Epidemiological studies, though inconclusive suggest that drinking green tea may lower the risk of prostate cancer (CaP) in humans. Here we report that polyphenols present in green tea especially its major constituent (-) epigallocatechin-3-gallate (EGCG) possesses both cancer preventive and cancer therapeutic properties. Our earlier studies reported in progress report of last year (*Proc. Natl. Acad. Sci. USA 98:10350-5, 2001*) using a transgenic adenocarcinoma of the mouse prostate (TRAMP), a model that mimics progressive forms of human prostatic disease, have shown that oral infusion of a polyphenolic fraction isolated from green tea at a human achievable dose (equivalent to six cups of green tea per day) significantly inhibited CaP development and progression. One significant observation from this study was that oral infusion of green tea polyphenols lead to increased cancer free and overall survival of these mice. Experiments conducted in this reporting period were conducted in athymic nude mice xenograft model implanted with androgen-sensitive 22Rv1 and androgen-insensitive PC-3 CaP cells. We found that intraperitoneal administration of EGCG resulted in significant inhibition in tumor growth and serum PSA levels. Importantly, mice treated with EGCG exhibited a marked decrease in tumor proliferation along with significant increase in apoptosis of both types of cancer cells. These data imply that EGCG can retard the growth of human CaP cells in the widely accepted xenograft model.

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INTRODUCTION

In recent years, green tea and its major polyphenolic constituent (-) epigallocatechin-3-gallate (EGCG) has gained much attention in reducing the incidence of certain human cancers. Extensive laboratory studies in cell culture systems and in animal tumor models have demonstrated that green tea polyphenols and EGCG afford protection against many cancer types. Epidemiological observations, though inconclusive, are suggesting that green tea consumption is associated with reduced risk of some human cancers. Based on the epidemiological evidence where Japanese and Chinese population consuming green tea on a regular basis have the lowest incidence of prostate cancer in the world, we reasoned that green tea polyphenols or EGCG may be an effective agent for chemoprevention and chemotherapy of prostate cancer. The present studies were conducted (as mentioned in specific aims) to investigate the chemopreventive and chemotherapeutic effects of green tea polyphenols and EGCG on prostate tumorigenesis models relevant for human prostate cancer. Employing male TRAMP mice, we have earlier reported (*Proc. Natl. Acad. Sci. USA 98:10350-5, 2001*) that oral infusion of a polyphenolic fraction isolated from green tea at a human achievable dose (equivalent to six cups of green tea per day) significantly inhibited prostate cancer development and progression. One significant observation from this study was that oral infusion of green tea polyphenols lead to increased cancer free and overall survival of these mice. Experiments performed in this reporting period (*July 2001 to August 31, 2002*) were conducted in athymic nude mice xenograft, a widely studied and well accepted model implanted with androgen-sensitive 22Rv1 and androgen-insensitive PC-3 human prostate carcinoma cells. We found that intraperitoneal administration of EGCG (1.0 mg/mouse/daily) resulted in significant inhibition in tumor growth and serum PSA levels. Importantly, mice treated with EGCG exhibited a marked decrease in tumor proliferation along with significant increase in apoptosis of both types of tumors. These data imply that EGCG can retard the growth of human prostate carcinoma cells in the widely accepted xenograft model. Taken together, green tea polyphenols and EGCG are capable of inhibiting prostate cancer development and progression by causing apoptosis to prostate cancer cells in various animal models relevant for human disease. Importantly, green tea polyphenols and EGCG are also capable of modulating serum IGF-1, IGFBP-3 and PSA levels which correlate with inhibition of tumor growth in these mice.

BODY

Green tea, specifically its major constituent EGCG has gained much attention for its cancer chemopreventive properties both in cell culture system and several animal tumor model systems (*Katiyar et al. Int J Oncol. 8:221-38, 1996, Yang et al. Environ Health Perspect. 4:971-6, 1997*). Recent studies are suggesting that EGCG may also possess cancer therapeutic effects (*Mukhtar and Ahmad Toxicol Sci. 1999 52:111-7, Fujiki H. J Cancer Res Clin Oncol. 125:589-97, 1999*). Much interest in green tea polyphenols and EGCG is due to its differential effects on normal *versus* cancer cells (*Ahmad et al. J Natl Cancer Inst. 89:1881-6, 1997*). Importantly, at physiologically attainable concentrations EGCG kills cancer cells through apoptosis but has no effect on normal cells (*Ahmad et al. Arch Biochem Biophys. 376:338-46, 2000, Chen et al. Cancer Lett. 129:173-9, 1998*) Earlier studies from our laboratory and elsewhere have shown that green tea derived polyphenols including EGCG are capable of imparting dose-dependent i) inhibition of cell growth ii) G0/G1 phase arrest of the cell cycle, and iii) induction of apoptosis in several types of human prostate carcinoma cells (*Paschka et al. Cancer Lett.130: 1-7, 1998, Gupta et al. Toxicol. App.Pharmacol.164: 82-90, 2000*). Further *in vivo* studies from our laboratory have shown that oral feeding of 0.2% GTP in drinking water resulted in a significant decrease in testosterone caused induction of ODC activity in sham-operated and castrated rats, respectively. Similar results were obtained with C57BL/6 mice where oral consumption of GTP resulted in significant inhibition in ODC induction in the ventral prostate (*Gupta et al. Cancer Research 59: 2115-20, 1999*). Extending these studies (Specific aim IA) in animal model we have convincingly shown that green tea at human achievable dose significantly inhibits prostate carcinogenesis in **t**ransgenic **a**denocarcinoma of the **m**ouse **p**rostate (TRAMP) model that mimics progressive forms of

human disease. In this study we have shown that oral infusion of a polyphenolic fraction isolated from green tea at a human achievable dose (equivalent to six cups of green tea per day) significantly inhibits prostate cancer development and blocks metastases in these mice. In two separate experiments, the cumulative incidence of palpable tumors at 32 weeks of age in 20 untreated mice was 100% (20 of 20). In these mice, 95% (19 of 20), 65% (13 of 20), 40% (8 of 20) and 25% (5 of 20) of the animals exhibited distant site metastases to lymph nodes, lungs, liver and bone, respectively. However, 0.1% GTP (w/v) provided as the sole source of drinking fluid to TRAMP mice from 8 to 32 weeks of age resulted in (a) significant delay in primary tumor incidence and tumor burden as assessed sequentially by MRI, (b) significant decrease in prostate (64%) and genitourinary (72%) weight, (c) significant inhibition in serum IGF-I and restoration of IGFBP-3 levels, and (d) marked reduction in the protein expression of PCNA in the prostate compared to water-fed TRAMP mice. The striking observation of this study was that GTP infusion resulted in almost complete inhibition of distant site metastases. Furthermore, GTP consumption caused significant apoptosis of prostate cancer cells, which possibly resulted in reduced dissemination of cancer cells thereby causing inhibition of prostate cancer development, progression and metastasis of prostate cancer to distant organ sites.

Development of effective chemopreventive agents against prostate cancer for human requires conclusive evidence of their efficacy in animal models that have relevance to human disease. Since, prostate cancer, at the time of clinical diagnosis represents a mixture of androgen-dependent and androgen-independent cells therefore, agents capable of inhibiting growth of both cell types may be useful in management of prostate cancer. In the present study (Specific Aim IB and II), employing the well accepted and widely used athymic nude mouse xenograft model we have studied the cancer-chemopreventive and -chemotherapeutic potential of EGCG under *in vivo* situation. Androgen-dependent 22Rv1 and androgen-independent PC-3 prostate tumor xenografts were implanted in both the flanks of nude mice. EGCG (1 mg/mouse, *ip*, daily) treatment was given to the animals in three treatment protocols: i) 2 weeks prior to cell inoculation (pre treatment), ii) 6 weeks after cell inoculation (post treatment), and iii) pre+post treatment. Tumor growth was significantly inhibited by pre+post (94%), post (81%) and pre (56%) treatments of EGCG in 22Rv1 tumors. Similar tumor inhibitory trend was obtained with PC-3 implanted tumors. Importantly, EGCG also inhibited serum PSA levels in the mice implanted with 22Rv1 tumors. In addition, EGCG treatment to mice, in all the groups, resulted in a marked decrease in cellular proliferation in tumors as assessed by immunohistochemical analysis of bromodeoxyuridine and proliferation cell nuclear antigen. The anti-proliferative effects of EGCG were found to be mediated by apoptosis as assessed by the cleavage of poly(ADP-ribose) polymerase and ELISA assay in both tumor types. These data suggest that EGCG could be useful against prostate cancer.

Results

a) Oral infusion of GTP inhibits prostate tumorigenesis and genito-urinary (GU) weight in TRAMP mice. To investigate the effect of green tea polyphenol infusion on prostate cancer growth and progression in TRAMP mice, in two separate experiments, 0.1% green tea polyphenols (GTP) was supplied to the animals as the sole source of drinking fluid for 24 weeks starting at the age of 8 weeks. As summarized in table 1 below, in the first experiment, as expected all the ten mice in the water-fed group developed severe prostate cancer with marked local invasiveness in the abdominal region, which was assessed by abdominal pelvic palpation and MRI. In contrast, only three of the ten (30%) GTP-infused TRAMP mice developed palpable tumors. Similarly, in the repeat experiment, all ten mice in the control group developed fully malignant and palpable tumors whereas in GTP-infused group only four of the ten (40%) animals exhibited palpable tumors. Importantly, in these GTP-infused mice the invasiveness of prostate cancer was much less as compared to water-fed mice. Further, we studied the effect of GTP infusion on the metastases to different site organs. The cumulative data at the termination of the experiment (32 weeks of age) from twenty animals in water-fed group showed 100% invasive tumors, which metastasize to lymph (95% animals), lungs (65% animals), liver (40% animals) and bone (25% animals) respectively. In sharp contrast, in the GTP-infused group, none of the twenty

mice exhibited metastases to any of the distant organs studied. Further, to determine the effect of GTP infusion on prostate cancer in TRAMP mice, gross biological indicies (wet weights) were used to assess the tumorigenicity. As observed visibly, GTP infusion resulted in complete absence of hyperplasia in the GU apparatus, especially in the seminal vesicles. An important finding from this experiment was that GTP infusion resulted in a significant decrease in prostate weight (~64%) and GU-weight (~72%) compared to water-fed TRAMP group.

Group[b]	Number of Animals	Palpable Tumor[c]	Animals with Metastasis[d]				Prostate weight (mg)	GU-weight (g)
			Lymph	Lungs	Liver	Bone		
Experiment 1								
Non-TG Control	10	0/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	17.4 ± 1.4	0.45 ± 0.05
TRAMP water-fed	10	10/10	9/10 (90%)	7/10 (70%)	4/10 (40%)	3/10 (30%)	68.2 ± 8.4	3.76 ± 0.48
TRAMP GTP-fed	10	3/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	32.6 ± 2.8*	1.08 ± 0.12*
Experiment 2								
Non-TG Control	10	0/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15.6 ± 1.2	0.52 ± 0.04
TRAMP water-fed	10	10/10	10/10 (100%)	6/10 (60%)	4/10 (40%)	2/10 (20%)	83.7 ± 11.6	4.36 ± 0.58
TRAMP GTP-fed	10	4/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	36.2 ± 3.8*	1.20 ± 0.14*
Cumulative								
Non-TG Control	20	0/20	0/10 (0%)	0/20 (0%)	0/10 (0%)	0/10 (0%)	16.5 ± 1.8	0.49 ± 0.05
TRAMP water-fed	20	20/20	19/20 (95%)	13/20 (65%)	8/20 (40%)	5/20 (25%)	76.0 ± 10.8	4.06 ± 0.64
TRAMP GTP-fed	20	7/20	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	27.5 ± 3.2*	1.14 ± 0.16*

[a] The data represented in each experiment is the mean ± SE of 10 mice.
[b] Mice (8 weeks of age) received plain drinking water (control group) or GTP (0.1% w/v) infusion in drinking water for 24 weeks. At the age of 32 weeks, the animals were sacrificed and studied for prostate tumorigenesis and metastases.
[c] Prostate tumor was assessed by abdominal pelvic palpation.
[d] Metastases in the lymph, liver and bone was examined under the microscope while metastasis in lungs was examined by the India ink method. Details are described in 'Materials and Methods'.
* $p < 0.001$, water-fed, control TRAMP compared with GTP-fed TRAMP, Student's 't' test.

b) Oral infusion of GTP inhibits prostate cancer development in TRAMP mice- An MRI analysis.
To assess the effect of GTP infusion in TRAMP mice on prostate carcinogenesis we measured the prostate growth using magnetic resonance imaging (MRI) (Figure 1).

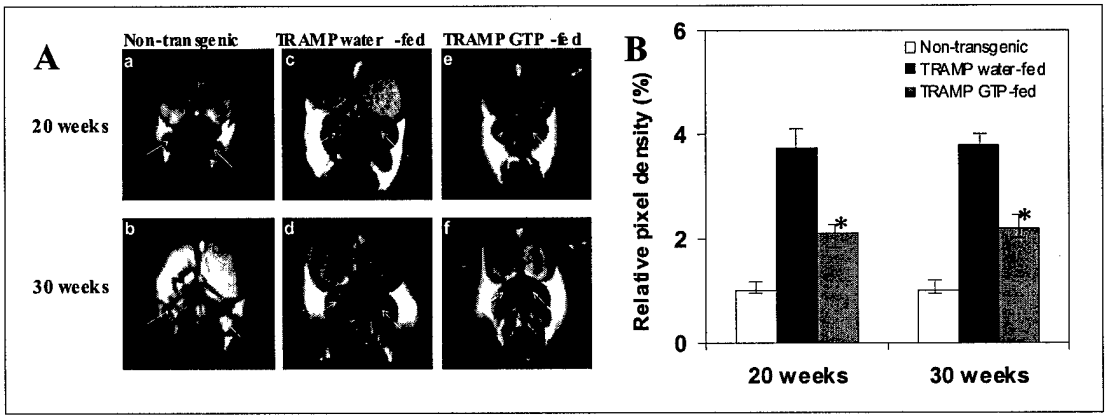

Figure 1: Effect of GTP infusion on prostate cancer development in TRAMP mice evaluated by longitudinal MRI analysis. **(A)** MRI was used to assess the growth of primary tumor in TRAMP mice followed longitudinally in individual animal. A marked reduction in prostate development was observed in these mice after 0.1% (w/v) GTP infusion between 8 to 32 weeks. Representative images of **(a, b)** non-transgenic, **(c, d)** water-fed TRAMP mice and **(e, f)** GTP-fed TRAMP mice are shown here at 20 **(a, c, e)** and 30 **(b, d, f)** weeks of age. Arrows indicate prostate. **(B)** Volumetric analysis of the TRAMP mice prostate after GTP infusion. The data is represented as percent change in relative pixel density observed at 20 and 30 weeks of age where non-transgenic mice prostate is taken as control. Values represent mean ± SE of 5 animals. *p <0.001 compared to TRAMP water-fed.

As shown by MRI scans, water-fed TRAMP mice demonstrated the presence of prostate tumor at 20 weeks of age, when the tumor was also detectable by abdominal pelvic palpation (Figure 1A, panel c). As evident by MRI, at 30 weeks the water-fed TRAMP mice were found to have fully developed tumor (Figure 1A, panel d). In sharp contrast, 0.1% GTP infusion to TRAMP mice was found to result in significant prevention or delay in prostate cancer development (Figure 1A, panel e & f). Compared to water-fed TRAMP mice, GTP-infused animals exhibited a marked reduction in the growth of prostate tumor at 20 weeks of age (44% inhibition) and at 30 weeks of age (42% inhibition) respectively, as observed by the volumetric analysis of the prostate (Figure 1B). This was also evident from abdominal pelvic palpation.

c) Oral infusion of GTP decreases serum IGF-1 and restores IGFBP-3 levels in TRAMP mice. In clinical practice, to monitor prostate cancer progression in humans, levels of prostate-specific antigen (PSA), insulin-like growth factor-I (IGF-I), and insulin-like growth factor binding protein-3 (IGFBP-3) in serum are determined. Since in TRAMP mice, like other mice, the murine equivalent of PSA has not yet been identified and/or isolated, we monitored the effect of GTP infusion on growth and development of prostate cancer by determining the levels of IGF-I and IGFBP-3 in serum. We monitored serum IGF-I and IGFBP-3 after 8, 16 and 24 weeks of GTP infusion. For comparison, these levels were also measured in non-transgenic littermates that did not develop prostate cancer. As shown in figure 2A, compared to non-transgenic animals, increasing levels of IGF-I were observed in water-fed TRAMP mice that were significantly lowered in GTP-infused mice. In contrast, serum IGFBP-3 levels, the major binding protein for IGF-I was lower in water-fed TRAMP mice and was significantly restored in GTP-infused mice (Figure 2B).

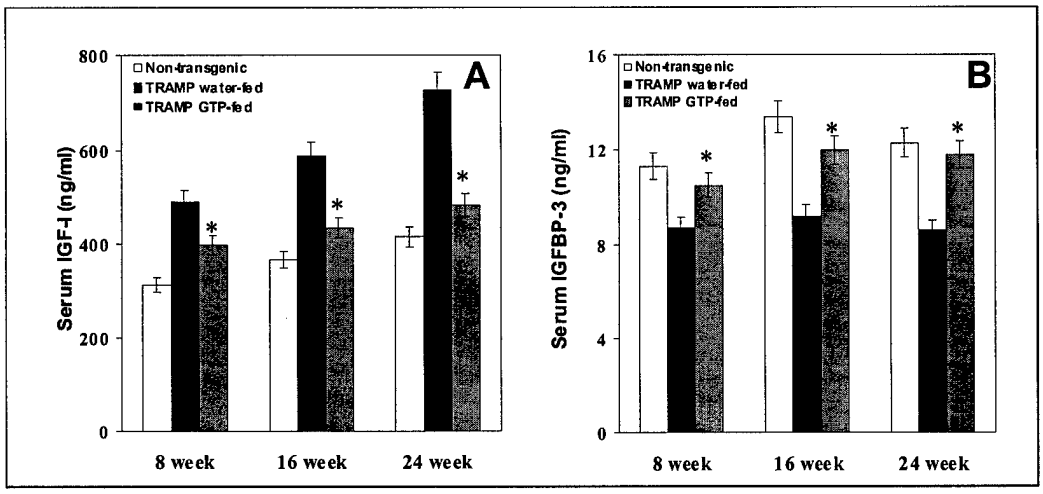

Figure 2: Effect of GTP infusion on serum levels of **(A)** IGF-I and **(B)** IGFBP-3 in TRAMP mice. Eight weeks old TRAMP mice were infused with 0.1% GTP (w/v) as sole source of drinking fluid for 24 weeks. Blood was withdrawn at 8, 16, and 24 weeks after GTP infusion and serum IGF-I and IGFBP-3 levels were analyzed by enzyme linked immunoabsorbant assay. Details are described in '**Appendix-1**'. A marked inhibition in serum IGF-I and restoration in serum IGFBP-3 levels were observed after GTP infusion. Values represent mean $\pm$ SE of 10 animals. *p <0.001 compared to TRAMP water-fed.

d) Oral infusion of GTP decreases proliferation in genito-urinary region and the prostate of TRAMP mice. An important observation of GTP infusion was that it resulted in a significant decrease in GU-weight compared to water-fed TRAMP group (Figure 3, Panel a & b). Further, histological examination of a typical TRAMP mouse prostate tissue at 32 weeks of age revealed prostatic neoplasia characterized by a pronounced proliferation of papillary structures lined by pseudostratified neoplastic cells with marked hyperchromasia and scattered apoptosis. In contrast, the experimental group of GTP-infused mouse exhibited glands lined by uniform columnar cells with dispersed chromatin and minimal luminal infoldings. GTP infusion also resulted in a significant increase in the number of apoptotic cells in the prostate (Figure 3, Panel c & d). GTP infusion for 24 weeks resulted in a marked reduction in PCNA protein expression in the prostate of TRAMP mice compared with the water-fed

group. (Figure 3, Panel B). These results were further confirmed by immunohistochemical analysis of the tissue (Figure 3, Panel B).

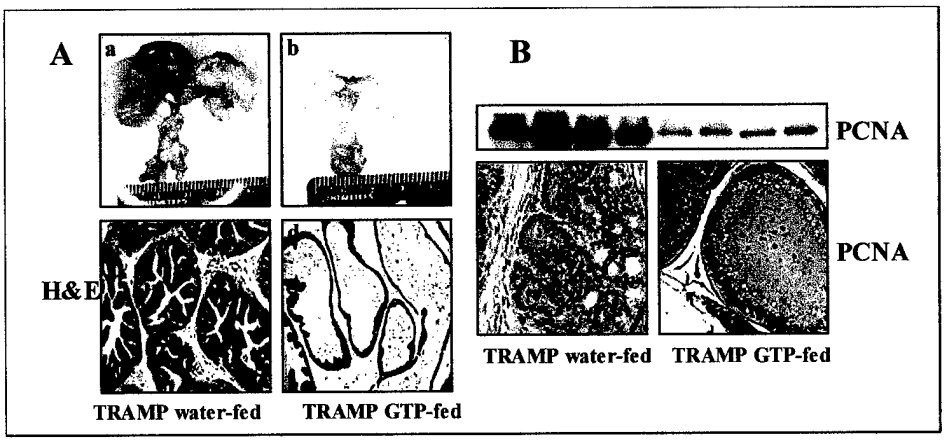

Figure 3: (A) Effect of GTP infusion on GU-apparatus and prostate histology in TRAMP mice. **(a)** Photograph of typical GU-apparatus of TRAMP mice exhibiting hyper-proliferation, **(b)** GU-apparatus of TRAMP mice with 0.1% GTP infusion (w/v) for 24 weeks. A marked decrease in GU-weight and volume was observed in TRAMP mice after GTP consumption. **(c)** Histologic examination of a typical TRAMP mouse prostate at 32 weeks of age revealed moderately-differentiated neoplastic cells with extensive cribriform structures, marked thickening, remodeling and hypercellularity of the fibromuscular stroma. Magnification=40X **(d)** 0.1% GTP infusion (w/v) to TRAMP mice resulted in a marked reduction in epithelial stratification and cribriform structures with little or no glandular formation in the prostate. Magnification=40X. Representative figures are shown here. **(B)** Effect of GTP infusion on the protein expressions of PCNA in the TRAMP mice prostate. Protein expression of PCNA was determined by Western blot, and immunohistochemical analysis. In water-fed TRAMP, an extensive PCNA staining was observed in the nuclei. 0.1% GTP infusion (w/v) resulted in the marked reduction in the protein expression of PCNA in these mice.

e) Oral infusion of GTP causes apoptosis of tumor cells in the prostate of TRAMP mice. Since green tea is known to induce selective apoptosis in cancer cells, we hypothesized that the observed inhibition of prostate tumorigenesis by GTP infusion is mediated by increased apoptosis of cancerous cells. We first performed ELISA for detection of apoptosis. As shown in figure 4A, GTP infusion for 24 weeks resulted in a significant increase in apoptosis in the prostate of TRAMP mice. In the second approach, these results were further confirmed by immunofluorescence detection in the prostate tissue by M30 CytoDEATH antibody that binds to a caspase cleaved formalin-resistant epitope of the cytokeratin 18 cytoskeletal protein, a marker for apoptosis (Figure 4B). A significant increase in apoptotic index (2.12 $\pm$ 0.1 *versus* 27.7 $\pm$ 3.2% control *versus* GTP-infused) was observed in GTP-infused mice prostate compared with water-fed TRAMP mice.

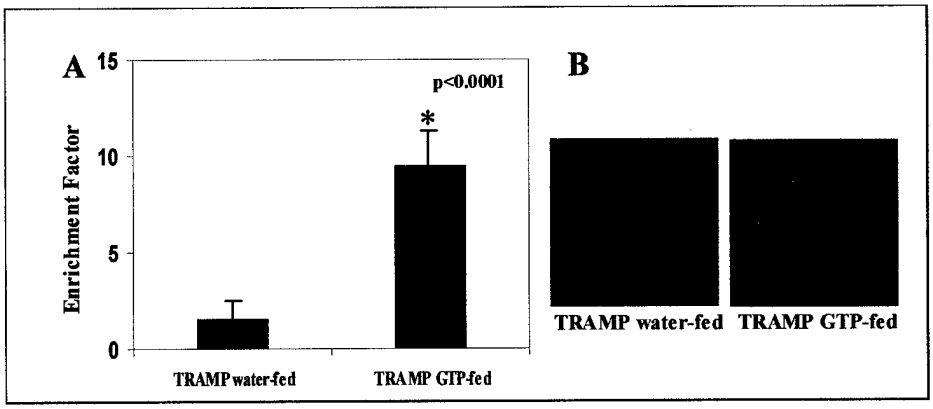

Figure 4: Effect of GTP infusion on the extent of apoptosis in the TRAMP mice prostate. **(A)** Apoptosis was determined by cell-death ELISA^{PLUS} as per vendor's protocol. Data are expressed as Enrichment factor. Values represent mean $\pm$ SE of 10 animals. *p <0.0001 compared to water-fed TRAMP mice. **(B)** Immunofluorescence detection of prostate tissue in water-fed and GTP-fed TRAMP mice by M30 CytoDEATH antibody, a marker of apoptosis. A marked increase in M30 fluorescence was observed after 0.1% GTP infusion (w/v), compared to water-fed TRAMP mice. A representative figure from each group at 80X magnification is shown here.

f) Oral Infusion of GTP increases Tumor Free Survival and Survival Probability in TRAMP Mice. Since extended tumor free survival and survival probability is the most desirable effect of any chemoprevention regimen therefore, we evaluated whether or not GTP infusion leads to tumor free survival and prolong life expectancy of TRAMP mice. Our data indicated (Figure 5) that continuous GTP infusion to TRAMP mice actually resulted in the prolongation of the life span of these mice. The continuous GTP infusion to TRAMP mice significantly increased the tumor free survival ($p<0.001$, log-rank test) inasmuch as 50% of the animals remain tumor-free up to 40 weeks of age (Figure 5A). In addition, GTP-fed TRAMP mice exhibited a significant increase (70% higher) in life expectancy ($p<0.001$, log-rank test) with a median survival of 68 weeks compared to the 42 weeks in water-fed TRAMP mice (Figure 5B).

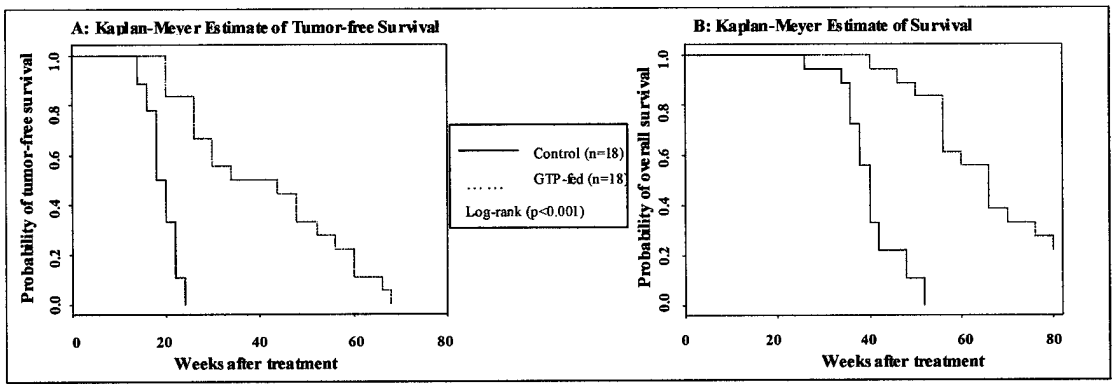

Figure 5: Effect of GTP infusion on **(A)** Tumor free survival and **(B)** Survival probability in TRAMP mice. A significant increase in tumor free survival ($p<0.001$, log-rank test) and survival probability ($p<0.001$, log-rank test) in GTP-fed TRAMP was observed.

(g) EGCG treatment inhibits tumor growth in androgen-sensitive 22Rv1 implanted tumors in athymic nude mouse xenograft model. Consistent with our cell culture studies showing significant cell growth inhibition of both androgen-sensitive LNCaP and androgen-insensitive DU145 human prostate carcinoma cells by EGCG. In the present study, daily administration of EGCG (1 mg/mouse, *ip*) in three different treatment protocols: (i) 2-weeks prior to cell inoculation (pre-treatment), (ii) 6-weeks after cell inoculation (post-treatment), and (iii) pre+post treatment to nude mice implanted with 22Rv1 cells showed a significant inhibition of tumor xenograft growth in terms of tumor volume as well as wet weight of tumors. Tumor growth was significantly inhibited by pre+post (94%), post (81%), and pre (56%) treatment of EGCG in 22Rv1 tumors (Figure 6).

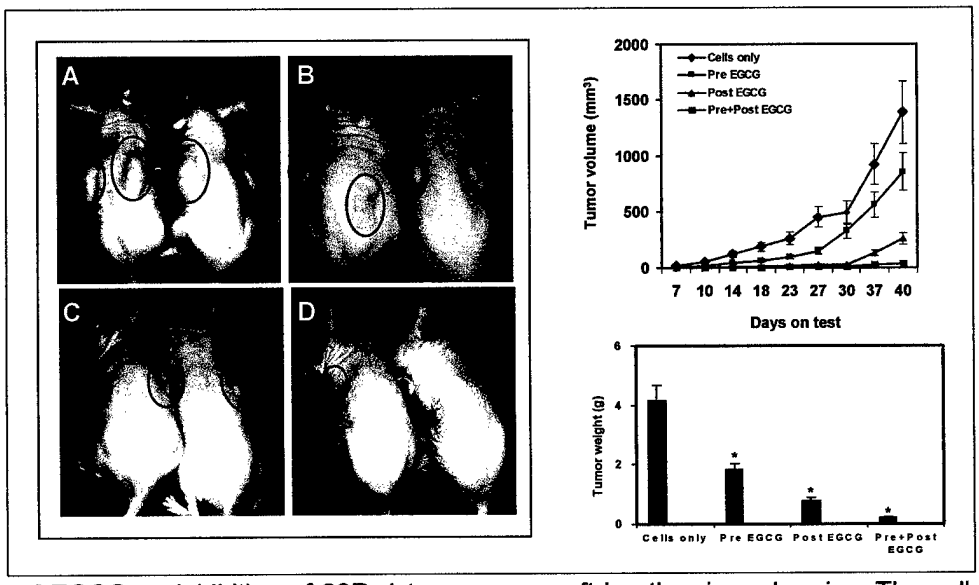

Figure 6: Effect of EGCG on inhibition of 22Rv1 tumor xenograft in athymic nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. **(A)** The control animals received 0.1 ml PBS as vehicle. The animals were

treated with EGCG (1 mg/0.1 ml PBS per mouse, *ip*) in three treatment protocols: **(B)** 2-weeks prior to cell inoculation (pre treatment), **(C)** 6-weeks after cell inoculation (post treatment), and **(D)** pre+post treatment. The photograph was taken on the 30th day after cell implantation. The tumor volume was recorded as indicated in the figure while the tumor weight was recorded at the termination of the experiment. Values represent mean ± SE of 4 to 8 tumors (tumor number differs because at some tumor cell-implanted sites there was complete inhibition) in each group. *p values less than 0.001 were considered as significant, compared to control.

(h) EGCG treatment inhibits tumor growth in androgen-insensitive PC-3 implanted tumors in athymic nude mouse xenograft model. Prostate cancer, like most other solid tumors, represents a very heterogeneous entity. Most prostate cancers, at the time of clinical diagnosis, present themselves as mixtures of androgen-dependent and androgen-independent cells. Also, most prostate cancers respond initially to androgen ablation since the population of androgen-dependent cells undergoes rapid apoptosis upon androgen withdrawal. However, androgen ablation rarely cures patients, most of whom will experience recurrence due to takeover of the tumor mass by androgen-independent tumor cells as well as the emergence of apoptosis-resistant clones as a result of further genetic alterations. On the other hand, although androgen-independent prostate cancer cells do not undergo apoptosis upon androgen blocking, they do maintain the appropriate molecular machinery of apoptosis. Therefore, the key to the control of prostate cancer appears to lie in the selective elimination of both types of cells through mechanism-based preventive/therapeutic approaches. In our earlier studies we have shown that EGCG is capable of causing cell growth inhibition and apoptosis in androgen-insensitive DU145 human prostate carcinoma cells. To further test the relevance of *in vitro* findings under *in vivo* situations we employed athymic nude mice xenograft model implanted with androgen-insensitive PC-3 human prostate carcinoma cells. Daily administration of EGCG (1 mg/mouse, *ip*) in three different treatment protocols: (i) 2-weeks prior to cell inoculation (pre-treatment), (ii) 6-weeks after cell inoculation (post-treatment), and (iii) pre+post treatment to these mice showed a significant inhibition of tumor xenograft growth in terms of tumor volume as well as wet weight of tumors. Tumor growth was significantly inhibited by per+post (79%), post (36%), and pre (25%) treatment of EGCG in PC-3 tumors (Figure 7).

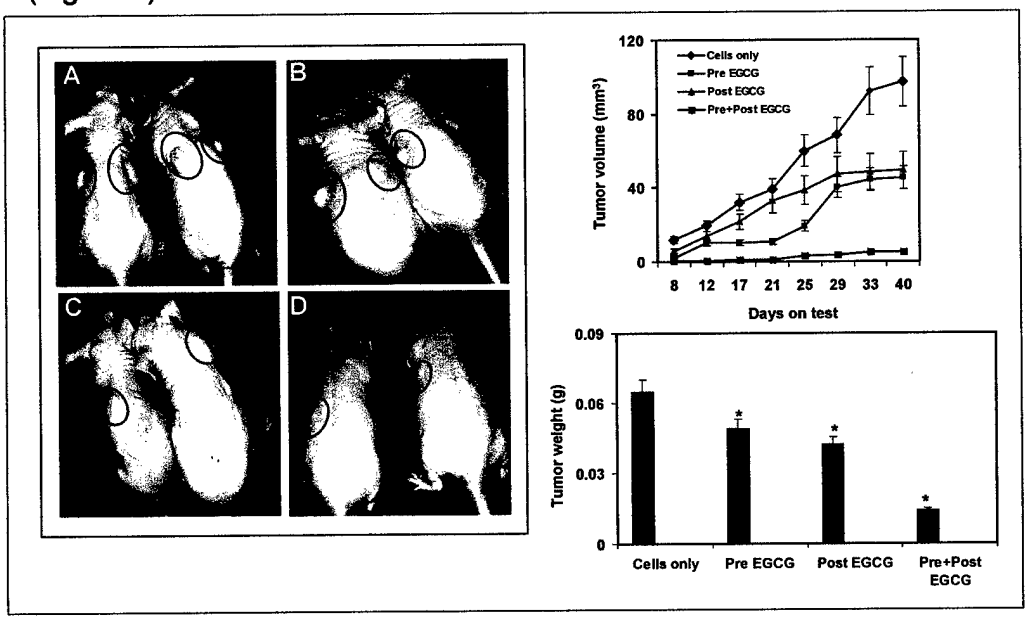

Figure 7: Effect of EGCG on inhibition of PC-3 tumor xenograft in athymic nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. **(A)** The control animals received 0.1 ml PBS as vehicle. The animals were treated with EGCG (1 mg/0.1 ml PBS per mouse, *ip*) in three treatment protocols: **(B)** 2-weeks prior to cell inoculation (pre treatment), **(C)** 6-weeks after cell inoculation (post treatment), and **(D)** pre+post treatment. The photograph was taken on the 30th day after cell implantation. The tumor volume was recorded as indicated in the figure while the tumor weight was recorded at the termination of the experiment. Values represent mean ± SE of 4 to 8 tumors (tumor number differs because at some tumor cell-implanted sites there was complete inhibition) in each group. *p values less than 0.001 were considered as significant, compared to control.

(i) EGCG treatment decreases serum PSA levels in androgen-sensitive 22Rv1 implanted tumors in athymic nude mouse xenograft model. Our cell culture studies has shown the anti-proliferative effect of EGCG against androgen-sensitive human prostate carcinoma LNCaP cells, which is associated with upregulation of PSA (*Gupta et al. Semin Urol Oncol. 17:70-6, 1999*). In the present study, we employed androgen-sensitive human prostate carcinoma cells 22Rv1, which are recently developed at CWRU, and during their growth in nude mice they make PSA that could be measured in serum. EGCG treatment of 22Rv1 tumor bearing mice resulted in a significant decrease in serum PSA levels, which accounted for 5.5%, 30% and 60% change in serum PSA levels in pre+post, -post and –pre treated groups compared to control group of animals (Figure 8).

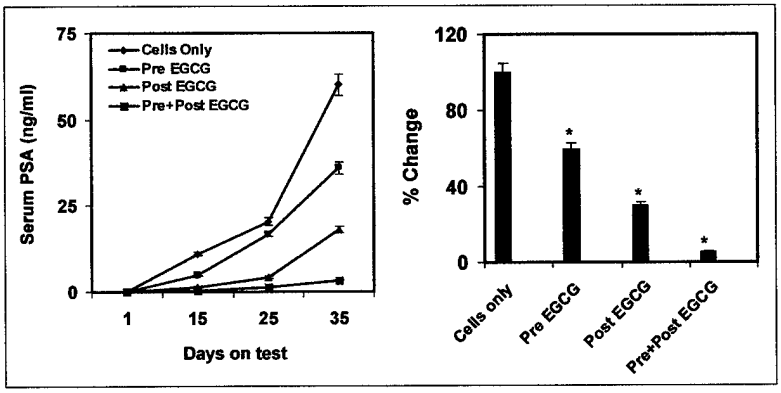

Figure 8: Effect of EGCG on serum PSA levels in 22R*v*1 cell-implanted tumor growth in nude mice. **(A)** Serum PSA levels in 22R*v*1 cell-implantation by treatment with EGCG. The animals on test were anesthetized and blood was drawn from the eye using heparinized tube. Serum was obtained and PSA analysis was conducted. **(B)** Percent change in PSA levels in different EGCG treated groups. Each value represents ± SE from four individual animals. *p values less than 0.001 were considered as significant, compared to control.

(j) EGCG treatment decreases DNA BrdUr incorporation in androgen-sensitive 22Rv1 and androgen-insensitive PC-3 implanted tumors in athymic nude mouse xenograft model. In the next series of experiment we determined the BrdUr incorporation in the DNA of control and various treated groups. In control groups the baseline BrdUrd incorporation was significantly elevated in the tumors compared to pre+post, -post and –pre EGCG treated groups. These results were in agreement with the tumor data (Figure 9).

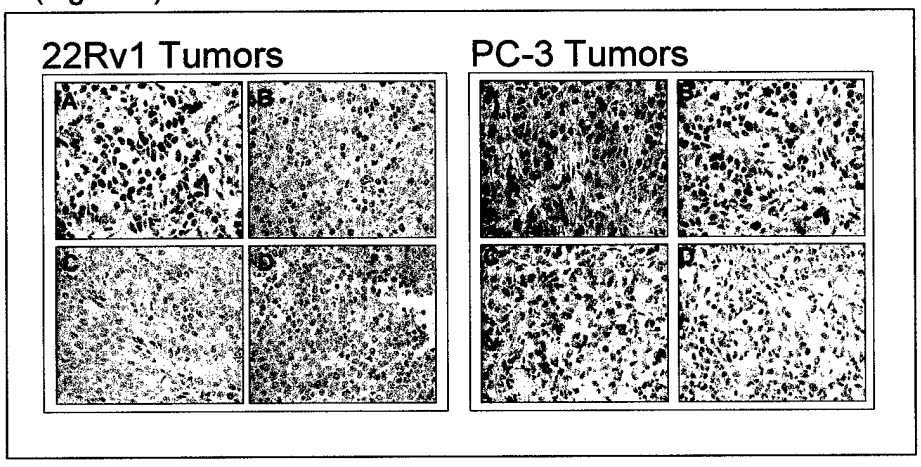

Figure 9: Effect of EGCG on the inhibition of Bromodeoxyuridine (BrdUr) incorporation into DNA of 22R*v*1 and PC-3 cell-implanted tumors in nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. **(A)** The control animals received 0.1 ml PBS as vehicle. The animals were treated with EGCG (1 mg/0.1 ml PBS per mouse, *ip*) in three treatment protocols: **(B)** 2-weeks prior to cell inoculation (pre treatment), **(C)** 6-weeks after cell inoculation (post treatment), and **(D)** pre+post treatment. The animals received BrdUr (50 mg/kg; *ip*) and were sacrificed 1 hr later. The BrdUr incorporation was assessed in sections cut from paraffin-embedded tumor by using a commercial kit (Oncogene Research Products, Cambridge, MA) according to the manufacturer's protocol. Representative figure from each group is shown here.

(k) EGCG treatment decreases proliferating nuclear cell antigen (PCNA) in androgen-sensitive 22Rv1 and androgen-insensitive PC-3 implanted tumors in athymic nude mouse xenograft model. In the next series of experiments, we investigated the effect of EGCG on the ubiquitous and molecular marker for increased proliferation, proliferating cell nuclear antigen (PCNA). PCNA serves as a requisite auxiliary protein for DNA polymerase δ-driven DNA synthesis and is cell cycle regulated. EGCG treatment in various treatment protocols resulted in a marked reduction in PCNA protein expression in EGCG-treated groups compared to the control group. The reduction of PCNA was in the order pre+post, -post and –pre EGCG treated groups compared to the control group (Figure 10).

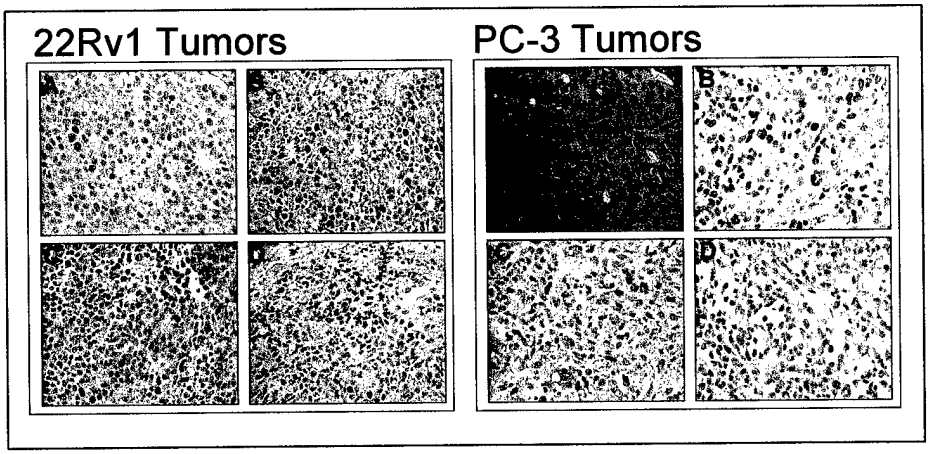

Figure 10: Effect of EGCG on the inhibition of proliferating cell nuclear antigen (PCNA) into DNA of 22R*v*1 and PC-3 cell-implanted tumors in nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. **(A)** The control animals received 0.1 ml PBS as vehicle. The animals were treated with EGCG (1 mg/0.1 ml PBS per mouse, *ip*) in three treatment protocols: **(B)** 2-weeks prior to cell inoculation (pre treatment), **(C)** 6-weeks after cell inoculation (post treatment), and **(D)** pre+post treatment. PCNA was analyzed immunohistochemically in sections cut from paraffin-embedded tumor by standardized protocol. Representative figure from each group is shown here.

(l) EGCG treatment causes apoptosis in androgen-sensitive 22Rv1 and androgen-insensitive PC-3 implanted tumors in athymic nude mouse xenograft model. Since EGCG has shown to induce apoptosis in several human prostate carcinoma cells irrespective of their androgen status, in the next series of experiment we performed ELISA assay to determine the extent of apoptosis caused by EGCG under *in vivo* situations. Daily administration of EGCG (1 mg/mouse, *ip*) in three different treatment protocols: (i) 2-weeks prior to cell inoculation (pre-treatment), (ii) 6-weeks after cell inoculation (post-treatment), and (iii) pre+post treatment to these mice exhibited a significant induction of apoptosis of tumor xenograft growth in both 22Rv1 and PC-3 implanted tumors (Figure 11).

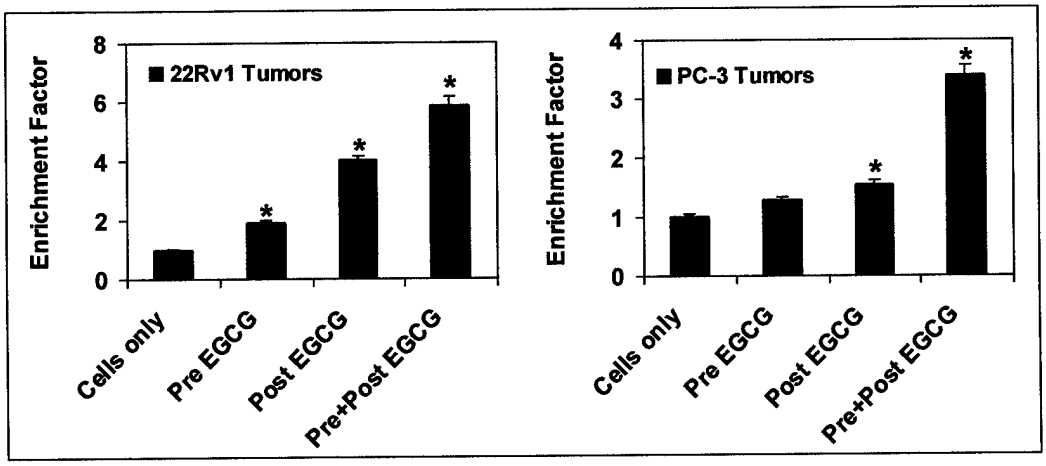

Figure 11: Effect of EGCG on induction of apoptosis in 22R*v*1 and PC-3 cell-implanted tumors in nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with EGCG (1 mg/0.1 ml PBS per mouse, *ip*) under different protocols. The control animals received 0.1 ml PBS as vehicle. After termination of

experiment, tumors were harvested and lysates were prepared. The extent of apoptosis in these samples was determined by Cell Death Detection ELISA PLUS kit according to the manufacturer's protocol. Each value represents ± SE from 4 to 8 tumors. *p values less than 0.001 were considered as significant, compared to control.

(m) EGCG treatment causes (polyADPribose) polymerase (PARP) cleavage in androgen-sensitive 22Rv1 and androgen-insensitive PC-3 implanted tumors in athymic nude mouse xenograft model. In the next series of experiment we determined EGCG induced apoptosis by assessing another marker of apoptosis the cleavage of PARP in various EGCG treated and control group. PARP cleavage analysis showed that the full size PARP (116 KDa) protein was cleaved to yield an 85 KDa fragment after EGCG treatment in both 22Rv1 and PC-3 implanted tumors. The extent of cleavage was in the order pre+post>-post>-pre EGCG treated group, compared to the control group (Figure 12).

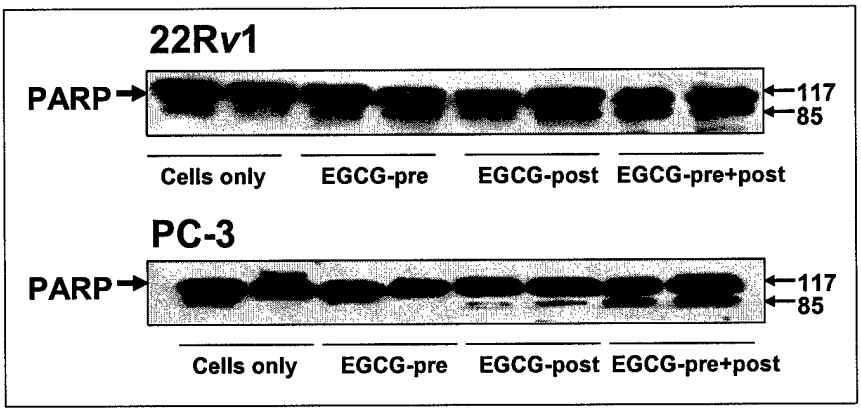

Figure 12: Effect of EGCG on PARP (poly ADP ribose) polymerase cleavage in 22R*v*1 and PC-3 cell-implanted tumors in nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with EGCG (1 mg/0.1 ml PBS per mouse, *ip*) under different protocols. The control animals received 0.1 ml PBS as vehicle. After termination of experiment, tumors were harvested and lysates were prepared. PARP cleavage was assessed in different treated groups by immunoblot analysis.

KEY RESEARCH ACCOMPLISHMENTS:

The work presented in the above report describes that green tea polyphenols and EGCG are capable of

- **inhibiting prostate carcinogenesis in TRAMP mice at physiological achievable dose in humans (equivalent to six cups of green tea per day)**
- **decreasing the growth of primary prostate tumor in TRAMP**
- **reversing the serum levels of IGF-1 and restores IGFBP-3 in TRAMP**
- **reducing proliferation in the TRAMP prostate**
- **complete inhibition of distant site metastases in TRAMP**
- **inducing apoptosis in prostate cancer cells in TRAMP**
- **prolonging life and tumor free-survival of TRAMP**
- **inhibiting tumor growth in both androgen-sensitive 22Rv1 and androgen-insensitive PC-3 human prostate tumor xenograft implanted in athymic nude mice**
- **inhibiting the serum PSA levels in 22Rv1 implanted tumors**
- **decreasing the DNA BrdUr incorporation in both 22Rv1 and PC-3 tumors**

- **decreasing the PCNA protein expression in both 22Rv1 and PC-3 tumors**
- **inducing apoptosis in both 22Rv1 and PC-3 tumors**
- **PARP cleavage in both 22Rv1 and PC-3 tumors**

REPORTABLE OUTCOMES:

Abstract # 3386 presented at 91st Annual Meeting of American Association for Cancer Research on April 1-5, 2000 at San Francisco, CA. *(Appendix-1)*

Chemoprevention of prostate cancer in TRAMP mice by oral infusion of green tea polyphenols

Sanjay Gupta[1], Nihal Ahmad[1], Jonathan S Lewin[2], Norman M Greenberg[3] and Hasan Mukhtar[1]
[1]Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106
[2]Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106
[3]Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Publication:

Inhibition of Prostate Carcinogenesis in TRAMP Mice by Oral Infusion of Green Tea Polyphenols

Sanjay Gupta[1], Kedar Hastak[1], Nihal Ahmad[1], Jonathan S Lewin[2] and Hasan Mukhtar[1]
Proc. Natl. Acad. Sci. USA. 98:10350-5, 2001 ***(Appendix-2)***
[1]Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106
[2]Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106

CONCLUSIONS:

Limited available options for the treatment of prostate cancer and its increasing incidence have spurred the search for novel preventive approaches for the management of this disease. Chemoprevention by the use of dietary agents or synthetic compounds could be one such strategy that may block the neoplastic inception or delay disease progression (1-5). Since prostate cancer is typically diagnosed in men aged 50 years and older, even a slight delay in the onset and subsequent progression of the disease through the use of chemopreventive agent(s) could have important health benefits (2-5). Epidemiological studies, though inconclusive, have suggested the protective role of green tea against prostate cancer development (6,7). Recent laboratory studies have indicated that green tea and its polyphenolic constituents impart inhibitory effects on the activities of many enzymatic, metabolic and signaling pathways that have relevance to cancer development and progression (8-13). A number of studies have shown the growth inhibitory effects of green tea against many animal tumor bioassay systems including lung, skin and forestomach (14-16). Studies from our laboratory have shown that green tea polyphenols show promising testosterone-mediated cell growth inhibitory effects and anchorage-independent growth of human prostate carcinoma cells LNCaP *in vitro* as well as green tea polyphenols-infused Cpb:WU rats and C57BL/6 mice *in vivo* (17). Cell culture studies from this laboratory (18) and elsewhere (19,20) have shown that green tea polyphenols and epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent of green tea, inhibit growth of several types of human prostate carcinoma cells. Our studies in cell culture have shown that EGCG was effective in imparting growth inhibition cell cycle desregulation and apoptosis of both androgen-sensitive as well as androgen-insensitive human prostate carcinoma cells, irrespective of androgen association and p53 status (21). Notably, EGCG has been shown to cause growth inhibition and regression of human prostate tumors in athymic nude mice (22). *The most notable implication of our work was that oral infusion of a human achievable dose of green tea results in significant inhibition in development and progression of prostate cancer along with increased survival in an animal model that emulates human disease. Further, EGCG was also capable of inhibiting the development and growth of prostate cancer*

in athymic nude mouse xenograft model and also repressed the serum PSA levels in these mice. These data, therefore suggest that green tea consumption may have inhibitory effects on prevention and therapy of prostate cancer in humans. This must be investigated in future clinical trials in prostate cancer patients.

In the present study utilizing TRAMP mice we have employed multiple non-invasive techniques to monitor the chemopreventive potential of GTP for the prevention of prostate cancer. Our first effort was to use the non-invasive technique of MRI for the monitoring of prostate cancer development in these mice. Our studies demonstrate for the first time that MRI may be used as an efficient tool for assessing the effectiveness of a chemopreventive agent against prostate cancer in animals. Next, we found that green tea polyphenol infusion to TRAMP mice caused significant inhibition of serum IGF-I and restoration of serum IGFBP-3 levels. This is consistent with recent epidemiological studies implicating deregulation of the IGF axis in prostate cancer progression (23,24) and showing that serum IGF-I could be a better predictor of prostate cancer risk than serum PSA (25,26). This is an important observation because recent studies have shown that high circulating levels of IGF-I are associated with increased risk of several common cancers, including those of the breast, prostate, lung and colorectum (27-29). The level of IGF-binding protein (IGFBP-3), a major IGF-I binding protein in serum that, in most situations, suppresses the mitogenic action of IGF-I has been shown to be inversely associated with the risk of these cancers. Taken together, the IGF axis, particularly IGF-I and IGFBP-3, could be developed as endpoint biomarkers for monitoring prostate cancer chemoprevention.

Increased proliferation of prostate cancer cells ultimately results in tumor invasion and metastasis leading to significant mortality in humans (30). Unfortunately, over 60% of the newly diagnosed cases of prostate cancer develop metastatic forms of the disease (30). In the present study, green tea polyphenols was found to be effective in completely abolishing distant site metastases and cellular proliferation as shown by the proliferation markers *viz.* PCNA. Another important observation of our study was a marked induction of apoptosis in the prostate by green tea polyphenol infusion. In recent years, apoptosis has gained much attention as a preferential way of eliminating the unwanted cancerous cells (31-34). Recent studies from our laboratory have shown that green tea polyphenols selectively induces apoptosis of various human carcinoma cells without affecting the normal cells (18). This observation has been verified from many laboratories worldwide (19,20). Our results show that GTP infusion to TRAMP mice results in massive apoptosis of neoplastic prostatic cells and further suggest that GTP could be an effective agent for a preferential elimination of cancerous and pre-cancerous cells *via* a programmed cell death. Based on our data, we believe that the observed inhibition of prostate tumorigenesis and subsequent metastasis by green tea polyphenol infusion is caused by selective apoptotic death of cancerous cells however further studies are needed to substantiate this suggestion. Further, studies with intervention trials *i.e.* green tea polyphenols and EGCG are capable of restricting tumor growth in established tumor in TRAMP model. Since, consumption of green tea polyphenols resulted in almost complete inhibition of distant site metastases in TRAMP model, further studies on the signaling events that leads to vascularization and angiogenesis are warranted.

Studies indicate that 23% of prostate cancer patients undergoing surgical intervention still show the evidence of disease progression (30). Once the disease becomes hormone refractory, most treatment is palliative and the median life span of these patients is less than 12 months. Therefore, agents that may prolong the survival and quality of life of such patients could have immediate clinical importance. In the present study, GTP infusion to TRAMP mice resulted in a significant increase in tumor free survival and survival probability. Based on these results, we suggest that regular consumption of green tea may prolong life expectancy and quality of life in prostate cancer patients.

In further studies, we have demonstrated the cancer-chemopreventive and -chemotherapeutic potential of EGCG in nude mouse xenograft model against prostate cancer. Growth of human xenografts in immunodeficient mice is well accepted model for studying prostate cancer chemoprevention (35-37). Since it is difficult to obtain long term cultures or cell lines of prostate carcinoma from primary tumors the introduction of nude mouse as a host for hetero-tansplantation provided a new dimension to prostate cancer research. Human xenografts are created by the

introduction of human tissue or cell into immunodeficient rodents. As a consequence of the absence of a functional thymus, the nude mouse had a deficient cell-mediated immune response (38,39). In these mice the humoral antibody formation is only slightly impaired and the activity of natural killer (NK) cells is actually increased in these immunosuppressed animals, subcutaneous grafting of human malignant tissue readily results in tumor development (40). The present study was conducted in athymic nude mice implanted with androgen-sensitive 22Rv1- and androgen-insensitive PC-3 tumors. The uniqueness of the 22Rv1 cells, which are recently developed at CWRU, is that during their growth in nude mice they make PSA that could be measured in serum. Another rationale for the use of two types of carcinoma cells for our studies was that prostate cancer at the time of clinical diagnosis represents a mixture of androgen-dependent and androgen-independent cells. Agents capable of inhibiting growth of both cell types may be useful in management of prostate cancer. Earlier, we have shown the cancer-chemopreventive potential of green tea polyphenols against prostate cancer in a transgenic mouse model that mimics progressive forms of human disease (*Gupta et al. Proc. Natl. Acad. Sci. USA. 98:10350-5, 2001*). In another recent study we showed that EGCG imparts growth inhibition, cell cycle deregulation and apoptosis in androgen-dependent LNCaP and androgen-independent DU145 human prostate carcinoma cells. To test the relevance of *in vitro* findings to *in vivo* situations, in this study we further established the cancer-chemopreventive and -chemotherapeutic potential of EGCG by applying three different treatment protocols in mice that closely mimic prevention as well as intervention in humans. EGCG (1 mg/mouse, *ip*, daily) treatment was given to the animals as follows: i) 2 weeks prior to cell inoculation (pre treatment), ii) 6 weeks after cell inoculation (post treatment), and iii) pre+post treatment. The fourth group received 0.1 ml PBS throughout the experiment and served as control. Tumor growth was significantly inhibited by pre+post (94%), post (81%) and pre (56%) treatments of EGCG in 22Rv1 tumors. Similar tumor inhibitory trend was obtained with PC-3 implanted tumors. Importantly, EGCG also inhibited serum-PSA levels in the mice implanted with 22Rv1 tumors. In addition, EGCG treatment to mice, in all the groups, resulted in a marked decrease in cellular proliferation in tumors as assessed by immunohistochemical analysis of bromodeoxyuridine and proliferation cell nuclear antigen. Further, the anti-proliferative effects of EGCG were found to be mediated by apoptosis as assessed by the cleavage of poly(ADP-ribose) polymerase and ELISA assay in both tumor types. These data suggest that EGCG could be useful against prostate cancer. These studies provide an increase focus on scientific basis of using green tea polyphenols and EGCG in prevention strategy for the people at high risk for prostate cancer. In this regard, our extensive investigations with green tea polyphenols have shown promising results in TRAMP model of prostate carcinogenesis. Furthermore, the present study provides evidence for the efficacy of EGCG against advance prostate carcinoma growth in athymic nude mice. These studies could possibly suggest the therapeutic effects of EGCG against prostate cancer. Importantly, inhibition of serum PSA levels by EGCG could be an important non-invasive marker which could be monitored in further clinical trials in human patients with green tea.

In summary our results suggest that a mixture of various polyphenols derived from green tea and its major constituent EGCG, inhibits the growth and progression of prostate cancer in TRAMP mice and in athymic nude mouse xenograft. Our data support the epidemiologic reports that green tea may reduce prostate cancer risk in humans. It is important to emphasize that during the course of prostate cancer development and progression the effectiveness of green tea is yet not certain in humans. However, based on the present study it is tempting to suggest that green tea in general and polyphenols present therein may prove to be useful supplement in the prevention or slower progress of prostate cancer in humans. We suggest that these experiments should be undertaken as an extension of this paper.

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APPENDICES:

Appendix-1 **Abstract # 3386 presented at 91st Annual Meeting of American Association for Cancer Research on April 1-5, 2000 at San Francisco, CA.**

Chemoprevention of prostate cancer in TRAMP mice by oral infusion of green tea polyphenols

Sanjay Gupta[1], Nihal Ahmad[1], Jonathan S Lewin[2], Norman M Greenberg[3] and Hasan Mukhtar[1]
[1]Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106
[2]Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106
[3]Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Appendix-2 **Publication:**

Inhibition of Prostate Carcinogenesis in TRAMP Mice by Oral Infusion of Green Tea Polyphenols

Sanjay Gupta[1], Kedar Hastak[1], Nihal Ahmad[1], Jonathan S Lewin[2] and Hasan Mukhtar[1]
Proc. Natl. Acad. Sci. USA 98:10350-5, 2001
[1]Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106
[2]Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106

RESEARCH EFFORTS (Received Pay):

Hasan Mukhtar, Ph.D.	Principal Investigator (July 2000, March 2002)
Sanjay Gupta, Ph.D.	Co- & Principal Investigator (April 2002 to September 2002)
Kedar Hastak, M.S.	Ph.D. Student
James D Steele	Research Technician

Appendix-1

Abstract # 3386 presented at 91st Annual Meeting of American Association for Cancer Research on April 1-5, 2000 at San Francisco, CA.

CHEMOPREVENTION OF PROSTATE CANCER IN TRAMP MICE BY ORAL INFUSION OF GREEN TEA POLYPHENOLS. Sanjay Gupta, Nihal Ahmad, Jonathan S Lewin, Norman M Greenberg, and Hasan Mukhtar, Baylor College of Medicine, Dept of Cell Biology, Houston TX, Case Western Reserve Univ, Dept of Dermatology, Cleveland, OH, and Univ Hospitals of Cleveland, Dept of Radiology, Cleveland, OH

Novel chemopreventive approaches are urgently needed for ~ 40,000 human fatalities because of prostate cancer (CaP). To have relevance to human, CaP-chemoprevention studies must be conducted in animal models that emulate human disease, especially where disease progression occurs spontaneously without the administration of unrealistic amounts of chemical carcinogens. Transgenic adenocarcinoma mouse prostate (TRAMP) model spontaneously develops metastatic CaP that mimics human disease. Many studies have demonstrated the cancer chemopreventive effects of a polyphenolic mixture obtained from green tea (GTP). Recently, we showed the antiproliferative effect of GTP in human CaP cells. Employing TRAMP mice, we determined the effect of GTP against CaP development and its subsequent metastasis. In two independent experiments, oral feeding of 0.1% GTP in drinking water for 20 weeks beginning at 8 weeks of age resulted in substantial reduction in tumor burden as assessed sequentially by magnetic resonance imaging during the course of study and at termination of experimennt by measuring size and weight of dorso-lateral prostate and genito-urinary apparatus. Importantly, in GTP-fed mice significant inhibition in serum IGF-1 and restoration of IGFBP-3 was observed at 7, 14 and 20 weeks on test. None out of 20 GTP-fed TRAMP mice exhibited distant site metastases. In sharp contrast, 18 of 20 non-GTP-fed mice exhibited metastases to lymph nodes and lungs. These chemopreventive effects of GTP against CaP development were further confirmed by histopathological examination and proliferation cell nuclear antigen staining in the dorso-lateral prostate. These data demonstrate that green tea could be an effective chemopreventive agent against CaP in humans.

Appendix-2

Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols

Sanjay Gupta*, Kedar Hastak*, Nihal Ahmad*, Jonathan S. Lewin†, and Hasan Mukhtar*‡

Departments of *Dermatology and †Radiology, Case Western Reserve University & The Research Institute of University Hospitals of Cleveland, Cleveland, OH 44106

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Development of effective chemopreventive agents against prostate cancer (CaP) for humans requires conclusive evidence of their efficacy in animal models that closely emulates human disease. The autochthonous *t*ransgenic *a*denocarcinoma of the *m*ouse *p*rostate (TRAMP) model, which spontaneously develops metastatic CaP, is one such model that mimics progressive forms of human disease. Employing male TRAMP mice, we show that oral infusion of a polyphenolic fraction isolated from green tea (GTP) at a human achievable dose (equivalent to six cups of green tea per day) significantly inhibits CaP development and increases survival in these mice. In two separate experiments, the cumulative incidence of palpable tumors at 32 weeks of age in 20 untreated mice was 100% (20 of 20). In these mice, 95% (19 of 20), 65% (13 of 20), 40% (8 of 20), and 25% (5 of 20) of the animals exhibited distant site metastases to lymph nodes, lungs, liver, and bone, respectively. However, 0.1% GTP (wt/vol) provided as the sole source of drinking fluid to TRAMP mice from 8 to 32 weeks of age resulted in (*i*) significant delay in primary tumor incidence and tumor burden as assessed sequentially by MRI, (*ii*) significant decrease in prostate (64%) and genitourinary (GU) (72%) weight, (*iii*) significant inhibition in serum insulin-like growth factor-I and restoration of insulin-like growth factor binding protein-3 levels, and (*iv*) marked reduction in the protein expression of proliferating cell nuclear antigen (PCNA) in the prostate compared with water-fed TRAMP mice. The striking observation of this study was that GTP infusion resulted in almost complete inhibition of distant site metastases. Furthermore, GTP consumption caused significant apoptosis of CaP cells, which possibly resulted in reduced dissemination of cancer cells, thereby causing inhibition of prostate cancer development, progression, and metastasis of CaP to distant organ sites.

prostate cancer | chemoprevention | apoptosis

Prostate cancer (CaP) is an important public health problem, accounting for more than 184,000 estimated new cases and ≈40,000 deaths in the year 2000 alone in the United States (1). In the absence of satisfactory treatment options for CaP, chemoprevention could be an effective approach to reduce the incidence of the disease (2, 3). For a variety of reasons, there is greater emphasis on identifying naturally occurring dietary substances as cancer chemopreventive agents (3–6). Indeed, CaP is an excellent candidate disease for chemoprevention because it is typically diagnosed in elderly men; therefore, even a modest delay in the neoplastic development achieved through pharmacological or nutritional intervention could result in a substantial reduction in the incidence of the clinically detectable disease.

Green tea, a popular beverage consumed worldwide, has been shown to possess cancer chemopreventive effects in a wide range of target organs in rodent carcinogenesis models (4–8). The chemopreventive effects of green tea against tumorigenesis and tumor growth have been attributed to the biochemical and pharmacological activities of its polyphenolic constituents, most notably (−)-epigallocatechin-3-gallate, present therein (7–10). Epidemiological studies, although inconclusive, suggest a protective effect of tea consumption on some cancer types in humans (11, 12). Limited epidemiological studies indicate that people who consume tea regularly may have a lower risk of CaP (13, 14). Further, the Japanese and Chinese populations who regularly consume tea, especially green tea, have one of the lowest incidences of CaP in the world (15, 16). In addition, the incidence of CaP is also low in other Asian men, who consume a traditional low-fat diet and tea (15).

For relevance to humans, CaP chemoprevention studies should be conducted in animal models that closely emulate human disease and possess surrogate endpoint biomarkers for rapid evaluation of chemopreventive and/or therapeutic agents. Recent developments of genetically manipulated animals provide new scope for chemoprevention studies and for developing strategies to offset specific genetic susceptibilities to cancer (17, 18). The major advantage of these models is that in these animals, cancer arises in their natural tissue microenvironment and progresses through multiple stages, as does human cancer.

The *t*ransgenic *a*denocarcinoma of the *m*ouse *p*rostate (TRAMP) is one such model for CaP that closely mimics progressive forms of human disease. In this model, expression of the SV40 early genes (T and t antigen, Tag) are driven by the prostate-specific promoter probasin that leads to cell transformation within the prostate (19). One-hundred percent of male TRAMP mice develop CaP without any chemical or hormonal treatment (19, 20). Further, CaP in this model progresses from prostatic intraepithelial neoplasia to histologic cancer to carcinoma metastatic to lymph nodes, lungs, liver, and bone sequentially over 12–28 weeks with median survival of 42 weeks (20). Recent studies from our laboratory (21) and elsewhere (22, 23) have established the utility of these mice for CaP chemoprevention studies. In the present study, we determined the consequence of oral infusion of a polyphenolic fraction isolated from green tea (hereafter referred to as GTP) on CaP development and progression in this model at a human-achievable dose. Our results demonstrate that oral infusion of GTP causes a significant inhibition in the development, progression, and metastasis of CaP to distant organ sites.

Materials and Methods

TRAMP Mice. The male and female TRAMP mice developed on a pure C57BL/6 background, heterozygous for the probasin-Tag transgene, were bred and maintained in the Animal Care Facility (School of Medicine, Case Western Reserve University). Transgenic males and the nontransgenic littermates were routinely obtained as [TRAMP C57BL/6 × FVB Breeder] F_1. The isolation of mouse-tail DNA and PCR-based screening assay were performed as described (19). All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Abbreviations: CaP, prostate cancer; GTP, green tea polyphenols; TRAMP, transgenic adenocarcinoma mouse prostate; GU, genitourinary; PCNA, proliferating cell nuclear antigen; IFG-I, insulin-like growth factor-I; IGFBP-3, insulin-like growth factor binding protein-3.

‡To whom reprint requests should be addressed. E-mail: hxm4@po.cwru.edu.

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Study Design for GTP Chemoprevention. GTP (>95% enriched preparation) was obtained from Natural Resources & Products (Charlottesville, VA). Chromatographic analysis of this mixture showed that it contains four major polyphenolic constituents: epigallocatechin-3-gallate (62%), epicatechin-3-gallate (24%), epigallocatechin (5%), epicatechin (6%), and caffeine (≈1%). The effect of GTP consumption on prostate carcinogenesis in TRAMP mice was studied in two separate experiments. Throughout each experiment, the animals had access to laboratory chow ad libitum. For each experiment, 20 male TRAMP mice of 8 weeks of age were equally divided into two groups. A freshly prepared solution of 0.1% GTP in tap water was supplied every Monday, Wednesday, and Friday to experimental animals as the sole source of drinking fluid for 24 weeks (GTP-infused group), whereas the control group of animals was supplied with the same tap water throughout the experiment (water-fed group). This feeding regimen has been used in mice in many prior chemoprevention studies from this and other laboratories (24, 25). The feeding protocol mimics an approximate consumption of six cups of green tea per day by an average adult human (25). Additional untreated and treated nontransgenic controls were also included in the study. After completion of the experiment, the animals from both experimental and control groups were killed by cervical dislocation, and the prostate gland was carefully removed under the microscope for further studies.

To investigate the effect of GTP consumption on tumor-free survival, in a third experiment, 36 male mice of 8 weeks of age were equally divided into two groups. The control group of animals was supplied with tap water, whereas animals in the experimental group were infused with 0.1% GTP (wt/vol) in drinking water exactly as in the first two experiments. Animals of both groups were monitored biweekly for tumor development by abdominal pelvic palpation and survival. For these studies, the animals were killed by CO_2 asphyxiation when obviously moribund.

Magnetic Resonance Imaging. Five animals each from both experimental and control groups were randomly selected and monitored for tumor growth and volume by MRI at 20 and 30 weeks of age. Imaging in these animals was performed by using a whole body 1.5 Telsa imager with 25 mT/m gradient strength, 150-ms rise time, and a custom-built 1-cm small animal receiver coil. T1-weighted (TR/TE = 400 ms/14 ms), double-echo T2-weighted (TR/TE = 1900 ms/20, 84 ms), and CISS T2-weighted (TR/TE/Flip angle = 12.3 ms/5.9 ms per 70°) gradient echo volumetric scans with a field of view between 2 and 5 cm and in plane resolution of 78–200 μm were obtained with a slice thickness of 500-2000 μm. Images were filmed for subjective analysis and/or transferred to a free-standing imaging workstation for volumetric analysis of prostate tumor.

Insulin-Like Growth Factor-I (IGF-I) and Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) Assay. Serum was separated from the whole blood obtained from the retro-orbital venous plexus with heparinized capillary tubes, and IGF-1 and IGFBP-3 levels were determined by commercially available ELISA kits (Diagnostic Systems Laboratories, Webster, TX) according to the manufacturer's protocol. The sensitivity of the assay was 0.04 ng/ml, and virtually no crossreactivity was visible with other members of the group.

Preparation and Analysis of Tissue. At the time of sacrifice, the lower GU tract, including the bladder, testes, seminal vesicles, and prostate, was removed *en bloc*. The GU wet weight was recorded to the nearest 0.01 g. Tissues collected at necropsy were fixed in 10% (vol/vol) phosphate-buffered formalin for 12 h and then transferred to 70% ethanol before standard tissue processing. Sections of the prostate (4 μm) were cut from paraffin-embedded tissues and mounted on ProbeOn-Plus slides (Fisher Scientific). Sections were stained with hematoxylin and eosin and were reviewed by light microscopy for the presence of CaP. Distant site metastases were examined as described (20, 21).

Immunoblotting and Immunohistochemistry. Prostate glands were removed from the animals and processed for immunoblotting and immunohistochemistry as described (21) by using appropriate antibodies for PCNA, SV-40 T-antigen, and β-actin obtained from Santa Cruz Biotechnology.

Image Analysis. Sections were visualized on a Zeiss-Axiophot DM HT microscope. Images were captured with an attached camera linked to a computer. Images and figures were composed by using ADOBE PHOTOSHOP 5.5 (Adobe Systems, Mountain View, CA).

Immunofluorescence Analysis and Apoptosis Detection. Four-micrometer-thick sections were cut from paraffin-embedded tissues. Immunofluorescence was performed by using M30 CytoDEATH antibody (Boehringer Mannheim) with a fluorescence microscope (Axiophot, Zeiss). Scoring of apoptotic cells in these sections was done by using the OPTIMAS 6 software program (Optimas, Bothell, WA). Apoptotic index (%) was calculated by dividing the number of apoptotic cells (fluorescence positive) by the total number of cells counted per cross-section of a sample of the prostate.

Apoptosis by ELISA. Apoptosis was also assessed by Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals) according to the manufacturer's protocol.

Statistical Analysis. All statistical analyses were carried out with STATISTICAL ANALYSIS SYSTEM software (SAS Institute, Cary, NC) and P values less than 0.05 were considered significant. The Kaplan–Meier method was used to estimate survival, and differences were analyzed by the log-rank test.

Results

MRI Analysis of TRAMP Mice Infused with GTP. To assess the effect of GTP infusion in TRAMP mice on prostate carcinogenesis, we first measured the prostate growth by using MRI. MRI is considered a powerful tool for imaging internal organs and for diagnosis of certain cancer types in humans (26). We used this technique for monitoring the effect of GTP infusion on CaP development and progression in TRAMP mice (Fig. 1*A*). As shown by MRI scans, water-fed TRAMP mice demonstrated the presence of prostate tumor at 20 weeks of age, when the tumor was also detectable by abdominal pelvic palpation (Fig. 1*Ac*). As evident by MRI, at 30 weeks the water-fed TRAMP mice were found to have fully developed tumor (Fig. 1*Ad*). In sharp contrast, 0.1% GTP infusion to TRAMP mice was found to result in significant prevention or delay in prostate cancer development (Fig. 1*A e* and *f*). Compared with water-fed TRAMP mice, GTP-infused animals exhibited a marked reduction in the growth of prostate tumor at 20 weeks of age (44% inhibition) and at 30 weeks of age (42% inhibition), respectively, as observed by the volumetric analysis of the prostate (Fig. 1*B*). This was also evident from abdominal pelvic palpation.

Effect of GTP Infusion on Serum IGF-I and IGFBP-3 Levels. In clinical practice, to monitor CaP progression in humans, levels of prostate-specific antigen, IGF-I, and IGFBP-3 in serum are determined. Because in TRAMP mice, like other mice, the murine equivalent of prostate-specific antigen has not yet been identified and/or isolated, we monitored the effect of GTP infusion on growth and development of CaP by determining the levels of IGF-I and IGFBP-3 in serum. Recent studies have demonstrated that elevated levels of IGF-I with concomitant lowering of IGFBP-3 levels in serum is associated with CaP risk and could be excellent predictors of CaP progression in humans (27). We monitored serum IGF-I and IGFBP-3 after 8, 16, and 24 weeks of GTP infusion. For comparison, these levels were also measured in nontransgenic littermates that did not develop CaP. As shown in Fig. 2*A*, compared with nontransgenic animals, increasing levels of IGF-I were

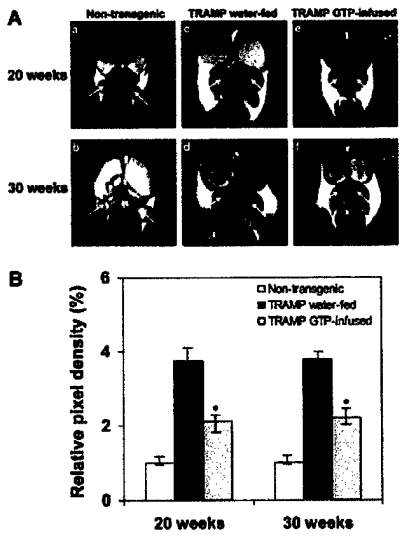

Fig. 1. Effect of GTP infusion on prostate cancer development in TRAMP mice evaluated by longitudinal MRI analysis. (*A*) MRI was used to assess the growth of primary tumor in TRAMP mice followed longitudinally in individual animal. Details are described in *Materials and Methods*. A marked reduction in prostate development was observed in these mice after 0.1% (wt/vol) GTP infusion between 8 to 32 weeks. Representative images of nontransgenic (panels a and b) and water-fed (*c* and *d*) TRAMP mice and GTP-infused TRAMP mice (e and *f*) are shown here at 20 (*a*, *c*, and e) and 30 (*b*, *d*, and *f*) weeks of age. Arrows indicate prostate. (*B*) Volumetric analysis of the TRAMP mice prostate after GTP infusion. The data are represented as percent change in relative pixel density observed at 20 and 30 weeks of age where nontransgenic mice prostate is taken as control. Values represent mean ± SE of five animals. *, $P < 0.001$ compared with TRAMP water-fed mice.

observed in water-fed TRAMP mice that were significantly lowered in GTP-infused mice. In contrast, serum IGFBP-3 levels, the major binding protein for IGF-I, were lower in water-fed TRAMP mice and were significantly restored in GTP-infused mice (Fig. 2*B*).

Effect of GTP Infusion on Prostate Tumorigenesis. GTP infusion for 24 weeks to TRAMP mice did not exhibit any symptoms of toxicity or apparent signs of ill health. No significant affect was observed in the body weight profile in nontransgenic littermates infused with 0.1% GTP when compared with the water-fed nontransgenic controls. However, TRAMP mice receiving GTP infusion regis-

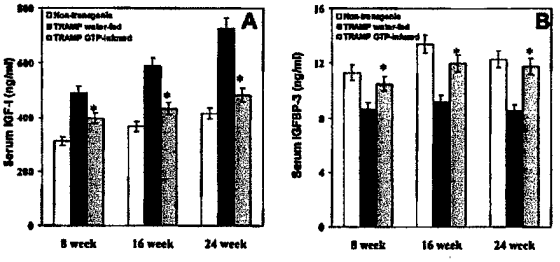

Fig. 2. Effect of GTP infusion on serum levels of IGF-I (*A*) and IGFBP-3 (*B*) in TRAMP mice. Eight-week-old TRAMP mice were infused with 0.1% GTP (wt/vol) as sole source of drinking fluid for 24 weeks. Blood was withdrawn at 8, 16, and 24 weeks after GTP infusion, and serum IGF-I and IGFBP-3 levels were analyzed by enzyme-linked immunoabsorbant assay. Details are described in *Materials and Methods*. A marked inhibition in serum IGF-I and restoration in serum IGFBP-3 levels were observed after GTP infusion. Values represent mean ± SE of 10 animals. *, $P < 0.001$ compared with TRAMP water-fed mice.

tered a slight decrease in body weight (≈5%) compared with their corresponding control group (data not shown). This difference may be the result of more tumor growth and hyperproliferation of the accessory sex organs in the abdominal region that occurs in control TRAMP mice.

To investigate the effect of GTP infusion on CaP growth and progression in TRAMP mice, in two separate experiments, 0.1% GTP was supplied to the animals as the sole source of drinking fluid for 24 weeks starting at the age of 8 weeks. As summarized in Table 1, in the first experiment, as expected all of the 10 mice in the water-fed group developed severe CaP with marked local invasiveness in the abdominal region, which was assessed by abdominal pelvic palpation and MRI. In contrast, only 3 of the 10 (30%) GTP-infused TRAMP mice developed palpable tumors. Similarly, in the repeat experiment, all 10 mice in the control group developed fully malignant and palpable tumors, whereas in the GTP-infused group only 4 of the 10 (40%) animals exhibited palpable tumors. Importantly, in these GTP-infused mice, the invasiveness of CaP was much less as compared with water-fed mice. Further, we studied the effect of GTP infusion on the metastases to different site organs. The cumulative data at the termination of the experiment (32 weeks of age) from 20 animals in the water-fed group showed 100% invasive tumors, which metastasize to lymph (95% animals), lungs (65% animals), liver (40% animals), and bone (25% animals), respectively. In sharp contrast, in the GTP-infused group, none of the 20 mice exhibited metastases to any of the distant organs studied. Further, to determine the effect of GTP infusion on CaP in TRAMP mice, gross biological indicies (wet weights) were used to assess the tumorigenicity (Table 1). As observed visibly, GTP infusion resulted in complete absence of hyperplasia in the GU apparatus, especially in the seminal vesicles. An important observation in this experiment was that GTP infusion resulted in a significant decrease in prostate weight (≈64%) and GU weight (≈72%) compared with the water-fed TRAMP group (Table 1; Fig. 3 *A* and *B*).

Effect of GTP Infusion on Prostate Histology. Histological examination of a typical TRAMP mouse prostate tissue at 32 weeks of age revealed prostatic neoplasia characterized by a pronounced proliferation of papillary structures lined by pseudostratified neoplastic cells with marked hyperchromasia and scattered apoptosis. In contrast, the experimental group of GTP-infused mouse exhibited glands lined by uniform columnar cells with dispersed chromatin and minimal luminal infoldings. GTP infusion also resulted in a significant increase in the number of apoptotic cells in the prostate (Fig. 3 *C* and *D*).

Effect of GTP Infusion on Proliferation Marker. We next determined the effect of GTP infusion on cellular proliferation in prostate as assessed by following the ubiquitous and molecular proliferation marker PCNA. PCNA serves as a requisite auxiliary protein for DNA polymerase δ-driven DNA synthesis and is cell cycle regulated (28, 29). GTP infusion for 24 weeks resulted in a marked reduction in PCNA protein expression in the prostate of TRAMP mice compared with the water-fed group (Fig. 4*A*). These results were further confirmed by immunohistochemical analysis of the tissue (Fig. 4*B*). Further, the effect of GTP infusion on Tag expression (T-antigen) was determined in prostates of TRAMP mice. GTP infusion did not result in any significant alteration in the levels of Tag protein expression, and they are detectable in both GTP-infused and water-fed groups (data not shown).

Effect of GTP Infusion on the Extent of Apoptosis. Because green tea is known to induce selective apoptosis in cancer cells (30), we hypothesized that the observed inhibition of prostate tumorigenesis by GTP infusion is mediated by increased apoptosis of cancerous cells. To test our hypothesis, we used multiple approaches of apoptosis determination. In our first approach, ELISA was per-

Table 1. Effect of oral infusion of GTP on the morphology of prostate and genito-urinary (GU) weight in TRAMP mice and their nontransgenic littermates

Group*	Number of animals	Palpable tumor†	Animals with metastasis‡				Prostate weight mg	GU weight g
			Lymph	Lungs	Liver	Bone		
Experiment 1								
Non-TG control	10	0/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	17.4 ± 1.4	0.45 ± 0.05
TRAMP water-fed	10	10/10	9/10 (90%)	7/10 (70%)	4/10 (40%)	3/10 (30%)	68.2 ± 8.4	3.76 ± 0.48
TRAMP GTP-infused	10	3/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	32.6 ± 2.8§	1.08 ± 0.12§
Experiment 2								
Non-TG control	10	0/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15.6 ± 1.2	0.52 ± 0.04
TRAMP water-fed	10	10/10	10/10 (100%)	6/10 (60%)	4/10 (40%)	2/10 (20%)	83.7 ± 11.6	4.36 ± 0.58
TRAMP GTP-infused	10	4/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	36.2 ± 3.8§	1.20 ± 0.14§
Cumulative								
Non-TG control	20	0/20	0/10 (0%)	0/20 (0%)	0/10 (0%)	0/10 (0%)	16.5 ± 1.8	0.49 ± 0.05
TRAMP water-fed	20	20/20	19/20 (95%)	13/20 (65%)	8/20 (40%)	5/20 (25%)	76.0 ± 10.8	4.06 ± 0.64
TRAMP GTP-infused	20	7/20	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	27.5 ± 3.2§	1.14 ± 0.16§

The data represented in each experiment are the mean ± SE of 10 mice.

*Mice (8 weeks of age) received plain drinking water (control group) or GTP (0.1% wt/vol) infusion in drinking water for 24 weeks. At the age of 32 weeks, the animals were killed and studied for prostate tumorigenesis and metastases.

†Prostate tumor was assessed by abdominal pelvic palpation.

‡Metastases in the lymph, liver, and bone were examined under the microscope, whereas metastasis in lungs was examined by the India ink method. Details are described in *Materials and Methods.*

§, $P < 0.001$, water-fed, control TRAMP compared with GTP-infused TRAMP, Student's t test.

formed for detection of apoptosis. As shown in Fig. 5*A*, GTP infusion for 24 weeks resulted in a significant increase in apoptosis in the prostate of TRAMP mice. In the second approach, these results were further confirmed by immunofluorescence detection in the prostate tissue by M30 CytoDEATH antibody that binds to a caspase-cleaved formalin-resistant epitope of the cytokeratin 18 cytoskeletal protein, a marker for apoptosis (Fig. 5*B*). A significant increase in apoptotic index (2.12 ± 0.1 vs. 27.7 ± 3.2% control vs. GTP-infused) was observed in GTP-infused mice prostate compared with water-fed TRAMP mice.

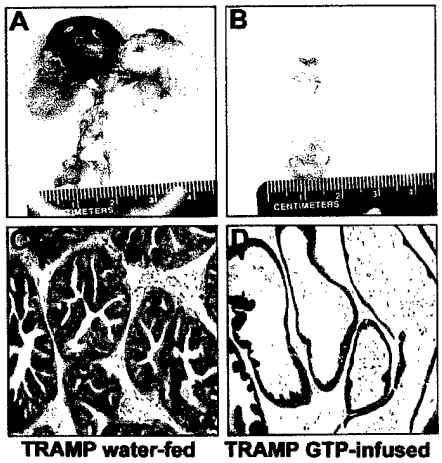

Fig. 3. Effect of GTP infusion on GU apparatus and prostate histology in TRAMP mice. (*A*) Photograph of typical GU apparatus of TRAMP mice exhibiting hyperproliferation. (*B*) GU apparatus of TRAMP mice with 0.1% GTP infusion (wt/vol) for 24 weeks. A marked decrease in GU weight and volume was observed in TRAMP mice after GTP infusion. (*C*) Histologic examination of a typical TRAMP mouse prostate at 32 weeks of age revealed moderately differentiated neoplastic cells with extensive cribriform structures, marked thickening, remodeling, and hypercellularity of the fibromuscular stroma. (Magnification, ×40.) (*D*) GTP infusion (0.1%, wt/vol) to TRAMP mice resulted in a marked reduction in epithelial stratification and cribriform structures, and the glands remained simple without epithelial thickening or surface complexity. (Magnification, ×40.) Representative figures are shown.

Effect of GTP Infusion on Tumor-Free Survival and Survival Probability in TRAMP Mice. Extended tumor-free survival and survival probability is the most desirable effect of any chemoprevention regimen. Therefore, in the next series of experiments, we evaluated whether or not GTP infusion leads to tumor-free survival and prolongs life expectancy of TRAMP mice. Our data indicated (Fig. 6) that continuous GTP infusion to TRAMP mice actually resulted in the prolongation of the lifespan of these mice. The continuous GTP infusion to TRAMP mice significantly increased the tumor-free survival ($P < 0.001$, log-rank test) inasmuch as 50% of the animals remain tumor-free up to 40 weeks of age (Fig. 6*A*). In addition, GTP-infused TRAMP mice exhibited a significant increase (70% higher) in life expectancy ($P < 0.001$, log-rank test) with a median survival of 68 weeks compared with the 42 weeks in water-fed TRAMP mice (Fig. 6*B*).

Discussion

Limited available options for the treatment of CaP and its increasing incidence have spurred the search for novel preventive ap-

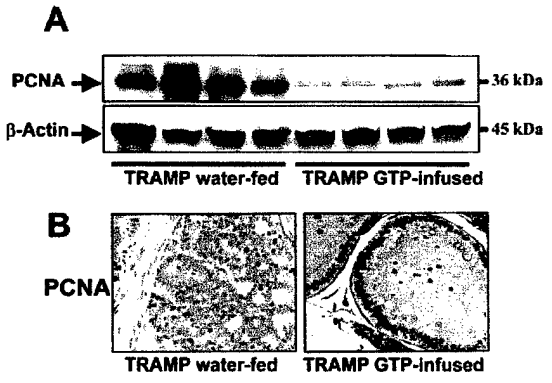

Fig. 4. Effect of GTP infusion on the protein expressions of PCNA in the TRAMP mice prostate. (*A*) Protein expression of PCNA by Western blot. (*B*) Immunohistochemical analysis is shown. In water-fed TRAMP mice, an extensive PCNA staining was observed in the nuclei. GTP infusion (0.1%, wt/vol) resulted in the marked reduction in the protein expression of PCNA in these mice. Equal loading of the protein in the lanes was confirmed by stripping the membrane and reprobing it with β-actin. Details are described in *Materials and Methods.*

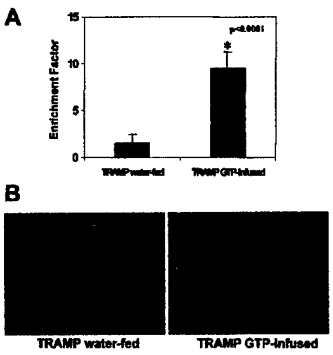

Fig. 5. Effect of GTP infusion on the extent of apoptosis in the TRAMP mice prostate. (*A*) Apoptosis was determined by cell-death ELISA^{PLUS} as per vendor's protocol. Data are expressed as Enrichment factor. Values represent mean ± SE of 10 animals. *, $P < 0.0001$ compared with water-fed TRAMP mice. (*B*) Immunofluorescence detection of prostate tissue in water-fed and GTP-infused TRAMP mice by M30 CytoDEATH antibody that binds to caspase-cleaved epitope of the cytokeratin 18 cytoskeletal protein, a marker of apoptosis. A marked increase in M30 fluorescence was observed after 0.1% GTP infusion (wt/vol), compared with water-fed TRAMP mice. A representative figure from each group at ×80 magnification is shown. Details are described in *Materials and Methods*.

proaches for the management of this disease. Chemoprevention by the use of dietary agents or synthetic compounds could be one such strategy that may block the neoplastic inception or delay disease progression (4–6). Because CaP is typically diagnosed in men aged 50 years and older, even a slight delay in the onset and subsequent progression of the disease through the use of chemopreventive agent(s) could have important health benefits. Ideally, the efficacy of such chemopreventive agents should be verified in animal models that emulate human disease before recommending their use for humans. The most notable implication of our work is that oral infusion of a human-achievable dose of green tea results in significant inhibition in development and progression of CaP along with increased survival in an animal model that emulates human disease. These data, therefore, suggest that green tea consumption may have inhibitory effects on prostate carcinogenesis in humans.

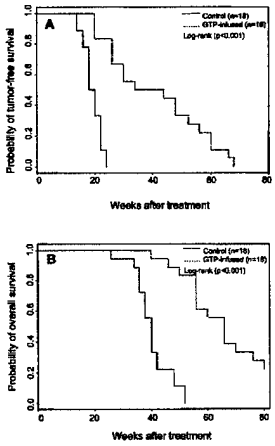

Fig. 6. Effect of GTP infusion on tumor-free survival (*A*) and survival probability (*B*) in TRAMP mice. A significant increase in tumor-free survival ($P < 0.001$, log-rank test) and survival probability ($P < 0.001$, log-rank test) in GTP-infused TRAMP mice was observed.

Epidemiological studies, although not conclusive, have suggested the protective role of green tea against CaP development (10–14). Recent laboratory studies have indicated that green tea and its polyphenolic constituents impart inhibitory effects on the activities of many enzymatic, metabolic, and signaling pathways that have relevance to cancer development and progression (31–36). A number of studies have shown the growth-inhibitory effects of green tea against many animal tumor bioassay systems including lung, skin, and forestomach (37–40). Studies from our laboratory have shown that green tea polyphenols show promising testosterone-mediated cell growth inhibitory effects and anchorage-independent growth of human prostate carcinoma cells LNCaP *in vitro* as well as GTP-infused Cpb:WU rats and C57BL/6 mice *in vivo* (41). Cell culture studies from this laboratory (42) and elsewhere (43–45) have shown that GTP and epigallocatechin-3-gallate, the major polyphenolic constituent of green tea, inhibit growth of several types of human CaP cells. Our studies in cell culture have shown that (−)-epigallocatechin-3-gallate was effective in imparting growth inhibition cell-cycle deregulation and apoptosis of both androgen-sensitive as well as androgen-insensitive human CaP cells (46). Notably, (−)-epigallocatechin-3-gallate has been shown to cause growth inhibition and regression of human prostate tumors in athymic nude mice (47). Earlier *in vivo* studies have not assessed the effect of green tea infusion on CaP chemoprevention in a prostate carcinogenesis model, partly because of the lack of appropriate animal models that could mimic the progressive forms of human prostatic disease. The TRAMP model possesses similarity to human disease in the development and progression to metastatic CaP. Recent studies from our laboratory and elsewhere have established the utility of TRAMP mice for CaP chemoprevention studies (21–23). In the present study, we assessed the chemopreventive potential of GTP against prostate carcinogenesis in the TRAMP model. Our results suggest that GTP infusion was significantly effective in inhibiting CaP development and completely abolished distant site metastases. Prior published studies have shown that polyphenols present in green tea and caffeine possess cancer chemopreventive effects (48). Although the role played by caffeine in observed CaP chemopreventive effects could not be ruled out, we believe that the observed effects in this study may be because of the polyphenolic constituents rather than caffeine because of its presence in low concentration (≈1%) in the GTP mixture.

In the present study, we have used multiple noninvasive techniques to monitor the chemopreventive potential of GTP for the prevention of CaP. Our first effort was to use the noninvasive technique of MRI for the monitoring of CaP development in these mice. Our studies demonstrate for the first time that MRI may be used as an efficient tool for assessing the effectiveness of a chemopreventive agent against CaP in animals. Next, we found that GTP infusion to TRAMP mice caused significant inhibition of serum IGF-I and restoration of serum IGFBP-3 levels. This is consistent with recent epidemiological studies implicating deregulation of the IGF axis in CaP progression (49) and showing that serum IGF-I could be a better predictor of CaP risk than serum prostate-specific antigen (50). Our results are also consistent with the previous observation where prostate-specific IGF-I was found to be increased during prostate cancer progression in TRAMP mice (51). This is an important observation because recent studies have shown that high circulating levels of IGF-I are associated with increased risk of several common cancers, including those of the breast, prostate, lung, and colorectum (49). The level of IGF-binding protein (IGFBP-3), a major IGF-I binding protein in serum that, in most situations, suppresses the mitogenic action of IGF-I, has been shown to be inversely associated with the risk of these cancers. Taken together, the IGF axis, particularly IGF-I and IGFBP-3, could be developed as endpoint biomarkers for monitoring CaP chemoprevention.

Increased proliferation of CaP cells ultimately results in tumor invasion and metastasis leading to significant mortality in humans (52). Unfortunately, over 60% of the newly diagnosed cases of CaP develop metastatic forms of the disease (53). In the present study, GTP was found to be effective in completely abolishing distant site metastases and cellular proliferation as shown by the proliferation markers *viz.* PCNA. An important observation of the study is that GTP infusion to TRAMP mice did not alter the expression of t/T-antigen, as they are readily detectable in both GTP-infused and water-fed groups. These results confirm that mechanism of CaP inhibition by GTP infusion was not through down-regulation of the transgene.

Another important observation of our study was a marked induction of apoptosis in the prostate by GTP infusion. In recent years, apoptosis has gained much attention as a preferential way of eliminating the unwanted cancerous cells (42–46, 53). At present, only a few agents are known to possess the potential for selective elimination of cancer cells (ref. 54 and references therein). Recent studies from our laboratory have shown that GTP selectively induces apoptosis of various human carcinoma cells without affecting the normal cells (42). This observation has been verified from many laboratories worldwide (43–46). Our results show that GTP infusion to TRAMP mice results in massive apoptosis of neoplastic prostatic cells and further suggest that GTP could be an effective agent for a preferential elimination of cancerous and precancerous cells via a programmed cell death. Based on our data, we believe that the observed inhibition of CaP tumorigenesis and subsequent metastasis by GTP infusion is caused by selective apoptotic death of cancerous cells; however, further studies are needed to substantiate this suggestion.

Studies indicate that 23% of CaP patients undergoing surgical intervention still show the evidence of disease progression (52). Once the disease becomes hormone refractory, most treatment is palliative and the median lifespan of these patients is less than 12 months. Therefore, agents that may prolong the survival and quality of life of such patients could have immediate clinical importance. In the present study, GTP infusion to TRAMP mice resulted in a significant increase in tumor-free survival and survival probability. Based on these results, we suggest that regular consumption of green tea may prolong life expectancy and quality of life in CaP patients.

In summary, our results suggest that GTP, a mixture of various polyphenols inhibit the growth and progression of CaP in TRAMP mice. Our data support the epidemiologic reports that green tea may reduce CaP risk in humans. It is important to emphasize that during the course of CaP development and progression the effectiveness of green tea is not yet certain in humans. However, based on the present study, it is tempting to suggest that green tea in general and polyphenols present therein may prove to be a useful supplement in the prevention or slower progress of CaP in humans.

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