

# Genistein in the Diet Reduces the Incidence of Poorly Differentiated Prostatic Adenocarcinoma in Transgenic Mice (TRAMP)<sup>1</sup>

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## ABSTRACT

Latent prostate tumors are commonly found with similar frequency in many countries and ethnic groups. In contrast, aggressive prostate cancer (PC) is significantly less prevalent among Asian men, where the intake of soy products is very high. High consumption of foods containing soy results in high plasma, urine, and prostatic fluid concentrations of phytoestrogens, including genistein. The objective of the present study was to test the hypothesis that dietary genistein might prevent PC progression in a transgenic mouse model of PC (TRAMP). By 28–30 weeks of age, all TRAMP mice in the study had developed prostate tumors, with about half of them displaying well-differentiated prostatic adenocarcinoma (WD, score 4), and the other half divided between moderately differentiated (MD, score 5) and poorly differentiated adenocarcinoma (PD, score 6). Two lines of evidence supported the possibility that prostates with PD may represent a more advanced stage of PC in TRAMP mice: (a) the weight of prostates with PD was two orders of magnitude higher than that of prostates with WD or MD; and (b) expression of androgen receptor transcripts was altered in PD as compared with WD and MD. To test the potential of genistein to prevent the incidence of mice with PD, starting at 5–6 weeks of age, transgenic males were fed a phytoestrogen-free diet (AIN-76A) containing 0, 100, 250, or 500 mg of genistein per kg AIN-76A (25, 10, 17, and 7 mice/group, respectively). Mice were on the diet until they were 28–30 weeks of age. Each mouse was weighed once a week throughout the study. At necropsy, selected organs were weighed and prepared for histopathological evaluation. Serum levels of genistein in mice on diet containing 0, 250, or 500 mg of genistein per kg AIN-76A were  $52.4 \pm 32.7$ ,  $138.9 \pm 69.6$ , and  $397.3 \pm 104.9$  nM, respectively, comparable with those found in Asian men on regular soy diet (276 nM). Using body and organ weights as indicators, dietary genistein had no toxic effect on TRAMP mice. The percentage of transgenic males that developed PD was reduced in a dose-dependent manner by dietary genistein.

## INTRODUCTION

PC<sup>3</sup> is the most commonly diagnosed cancer in North American men (1, 2). Latent or clinically insignificant PC occurs in a large proportion, and at equal rates, in autopsy studies among men from Asian countries and the United States. In contrast, the incidence of clinically significant PC is 15-fold higher in the United States (2–7). Epidemiological studies suggest that this might be attributable to differences in environmental factors and life style, including nutrition (3). Aggressive PC is less prevalent among Asian men where the

intake of soy products is very high (7–9). However, with Westernization and loss of traditional eating habits, the pattern of disease incidence is also changing among Asian men (5, 10–12).

High consumption of food containing soy results in high plasma, urine, and prostatic fluid concentrations of phytoestrogens, including genistein (13–15). On the basis of these findings, it has been postulated that phytoestrogens, especially genistein (16), may have a preventive role in PC. Genistein, an isoflavone (5,7,4'-trihydroxyisoflavone), is found in soybeans as a glycoside conjugate or a methylated derivative, biochanin A (17). These derivatives are metabolized to genistein by enzymes produced by bacteria in the gut (15). Genistein has been shown to inhibit the growth of the rat MAT-LyLu and human PC-3 cell lines *in vitro* (18), consistent with the possibility that it might have chemopreventive potential. More recently, soybean phytochemicals, including genistein, were shown to inhibit *in vivo* growth of PC tumors that resulted from the s.c. injection of PC cells (19–21).

Testing the potential of genistein to prevent advanced PC *in vivo* has been delayed by a lack of appropriate animal models of PC. PC is a disease quite unique to man (22). Naturally occurring prostatic disease has been reported in some canine (23) and rodent (24–26) species. However, PC is less prevalent in dogs than in humans (27), and the use of dogs for large scale preclinical studies poses practical limitations (28). Moreover, these animals have not provided progressive forms of prostatic disease that histologically resemble human PC (22). Through a concerted effort in several laboratories, Greenberg *et al.* (22, 29, 30) established a transgenic mouse model of PC (TRAMP). This mouse model was generated using a construct consisting of the minimal rat probasin promoter (–426 to +28 probasin 5'-flanking DNA) driving expression of the SV40 early genes (T and t; *Tag*). Transgenic mice expressed the transgene in the prostate, in a developmentally and hormonally regulated manner (29, 30). Because SV40 *Tag* is believed to interact with the *p53* and retinoblastoma genes abrogating their function (31–34), expression of the transgene was expected to induce tumorigenesis in the prostate. As expected, TRAMP mice developed progressive forms of PC with lesions ranging from mild PIN to large multinodular malignant neoplasia (22, 29, 30), as well as metastatic spread to lymph nodes and bone (22, 29, 30). TRAMP mice have been recently used to study chemoprevention of prostate carcinogenesis by  $\alpha$ -difluoromethyl ornithine (35). The main objective of the present studies was to test the hypothesis that genistein reduces the incidence of PC in this unique mouse model.

## MATERIALS AND METHODS

**Transgenic Mice.** TRAMP mice in a pure C57BL/6 background were bred in our colony at the UAB School of Medicine. Transgenic females were bred with nontransgenic males because transgenic males tend to develop prostate tumors. All mice were maintained in a climate-controlled environment with a 12-h light/12-h dark cycle, and diet and water were supplied *ad libitum*. Breeders were fed standard pellet mouse feed (Harlan Teklad 7012, Madison, WI). After weaning at 3–4 weeks of age, the gender of offspring was determined, males were separated from females, and a tail biopsy was collected from each mouse. Tail DNA, isolated by standard procedures, was used for determination of transgene incorporation by PCR as described previously (22).

Transgenic males, included in experiments at 5–6 weeks of age, were fed

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<sup>3</sup> The abbreviations used are: PC, prostate cancer; TRAMP, Transgenic Adenocarcinoma Mouse Prostate; *Tag*, T antigen; DLP, dorsolateral prostate; PIN, prostatic intraepithelial neoplasia; AR, androgen receptor; UAB, University of Alabama at Birmingham; HPLC, high-performance liquid chromatography; RT-PCR, reverse transcriptase-PCR; MD, moderately differentiated prostatic adenocarcinoma; PD, poorly differentiated prostatic adenocarcinoma; WD, well-differentiated prostatic adenocarcinoma.

powdered AIN-76A (Harlan Teklad), a semipurified diet containing no detectable phytoestrogens (limit of detection, 5 pmol/ml). Mice were fed AIN-76A diet containing varying levels of genistein throughout the study, until they were 28–30 weeks of age. Genistein used in these studies was chemically synthesized (Roche, Basel, Switzerland) and analyzed by HPLC (98.5% pure, 1.5% methanol) as described previously (36). Throughout the study, each mouse was weighed once a week. Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the University of Alabama Animal Care Committee.

**Preparation and Analysis of Tissues.** At necropsy, conducted at 28–30 weeks of age, all transgenic males were examined for gross organ abnormalities. Kidneys, epididymes, and testes were weighed too. In 3–4 of the mice, the urogenital tract, including the bladder, seminal vesicles, prostate, testes, and epididymes, were removed at necropsy *en bloc* and were prepared for pathological evaluation as described below. In most mice, DLPs were rapidly dissected and weighed, before additional processing. A sample of the DLP was rapidly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ , until additionally processed for mRNA analysis by RT-PCR. The rest of the prostate was fixed in an acid alcohol solution containing 96% ethanol, 1% glacial acetic acid, and 3% distilled water, as described by Folkvord *et al.* (37). The fixed tissue was then embedded in paraffin, and 4–5- $\mu\text{m}$  sections were mounted on Colorfrost/Plus microscope slides (Fisher Scientific). Sections were stained with hematoxylin and eosin or with Gomori trichrome staining (38). Prostate lesions were scored using a 1–6 scale that has been established for TRAMP mice (39). Noncancerous lesions were graded as 1, 2, or 3, indicating normal tissue, low PIN, and high PIN, respectively. Grades 4, 5, and 6 indicated MD, WD, and PD cancerous lesions, respectively.

**Analysis of Relative Steady-state RNA Levels of AR by RT-PCR.** Total RNA was isolated using the Rneasy Mini kit from Qiagen (Valencia, CA).

With total RNA as the starting material, avian myeloblastosis virus reverse transcriptase and the cDNA Cycle kit from Invitrogen (Carlsbad, CA) were used to generate cDNA. AR cDNA was subsequently amplified by PCR using the following primers designed based on the sequence of the mouse AR (40): AR-forward: TGA CAA CAA CCA ACC AGA TTC C; and AR-reverse: CAC TGG AAT AAT GCT GAA GAG C. cDNA for the housekeeping gene  $\beta$ -actin was amplified in the same PCR reaction, using primers designed based on the sequence of mouse  $\beta$ -actin (41):  $\beta$ -actin-forward: CTT TGC AGC TCC TTC GTT G; and  $\beta$ -actin-reverse: TGC CAA TAG TGA TGA CCT G. PCR was performed using an initial denaturing step at  $94^{\circ}\text{C}$  (3 min), followed by 34 cycles consisting of a denaturing step at  $94^{\circ}\text{C}$  (1 min), an annealing step at  $60^{\circ}\text{C}$  (30 s), an extension step at  $72^{\circ}\text{C}$  (30 s), and, finally, one last step at  $72^{\circ}\text{C}$  (5 min). PCR products, AR (390 bp) and  $\beta$ -actin (800 bp), were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide. The intensity of the bands was measured by densitometry. Results are presented as relative steady-state RNA levels of AR, *i.e.*, a ratio of the band intensities of the AR and  $\beta$ -actin PCR products.

**Genistein Analysis.** At necropsy, blood was collected by intracardial puncture. Serum concentrations of total genistein were analyzed by HPLC-multiple reaction ion-monitoring mass spectrometry (36).

**Data Analysis.** Data were analyzed using SigmaStat, version 2.03 (SPSS, Inc). One-way ANOVA was performed if data in multiple groups were normally distributed with equal variance. The Kruskal-Wallis one-way ANOVA on ranks was used if three or more groups were compared, and the data were not normally distributed with equal variance. If these tests indicated that the groups were significantly different, a Tukey or Dunn test were carried out, respectively, to determine which of the groups were significantly different from the others.  $\chi^2$  analysis was used to compare frequencies in multiple groups.

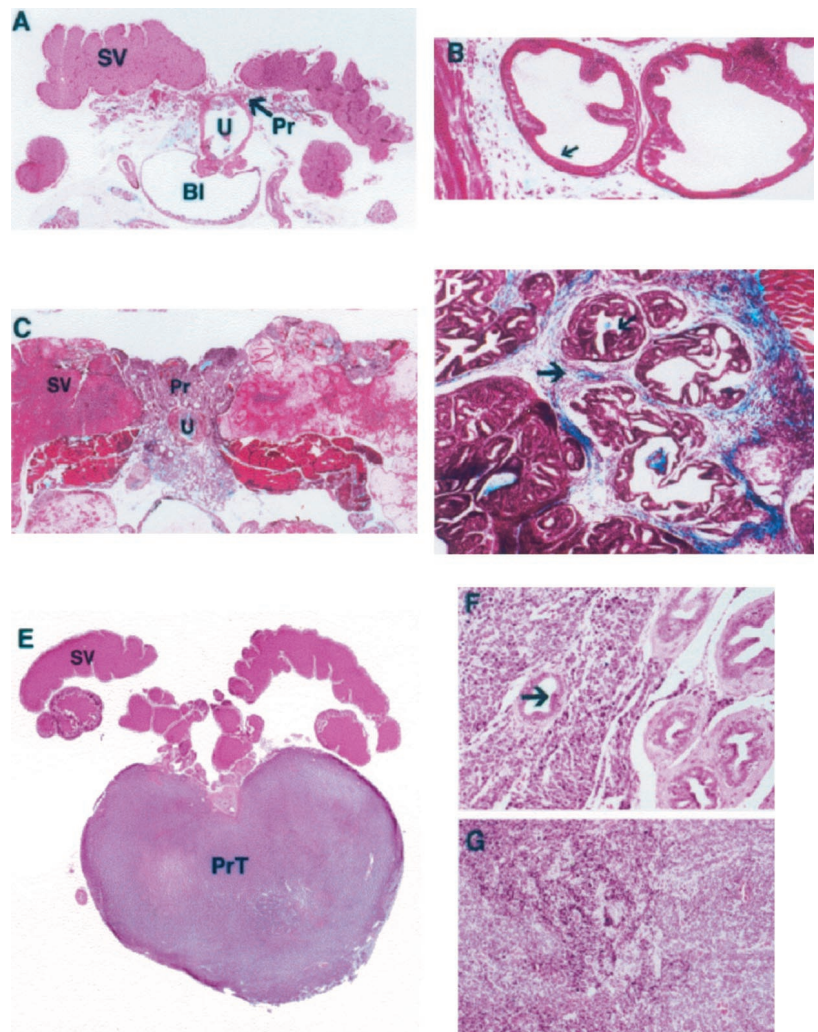


Fig. 1. Histological analysis of the normal prostate and prostate tumors in TRAMP mice. Paraffin sections (4–5  $\mu\text{m}$ ) of the urogenital tract were stained using Gomori's trichrome (A–D) or H&E (E–G) staining. A, section through the urogenital tract of a nontransgenic mouse; B, higher magnification of the section shown in A. Arrow, points to normal epithelium lining a prostate tubule; C, section through the urogenital tract of a TRAMP mouse with WD to MD; D, higher magnification of an area of the prostate in C. Arrow, points to cribriform structures and fibrosis; E, section through the urogenital tract of a TRAMP mouse displaying PD; F, high magnification of an area of the prostate tumor shown in E. Arrow, points to a normal prostate tubule embedded in the tumor mass; G, high magnification of an area of the prostate tumor shown in E. Pr, prostate; PrT, prostate tumor; U, urethra; Bl, bladder; SV, seminal vesicle.



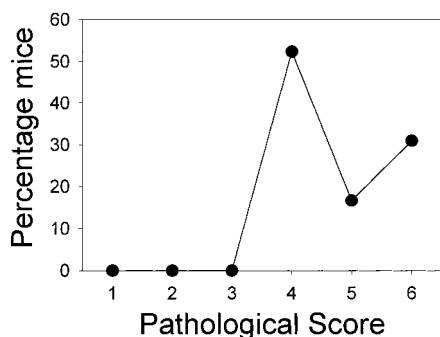


Fig. 2. Frequency distribution of mice as a function of pathological score. The DLPs from 42 TRAMP mice, fed phytoestrogen-free diet from 5 weeks of age until necropsied at 28 weeks of age, were prepared for histopathological examination. Paraffin sections (5  $\mu$ m) stained using Gomori's trichrome were evaluated blindly and scored on a scale of 1–6 by established criteria (30). The DLPs from 22, 7, and 13 TRAMP mice were scored as 4, 5, and 6, respectively. Results given are the percentage of mice as a function of the pathological score.

## RESULTS

**Transgenic Males Produced in Our Colony Display Progressive Forms of PC.** It has been reported that, over their lifetime, TRAMP mice develop progressive forms of PC with lesions ranging from mild PIN to large multinodular malignant neoplasia (22, 29, 30). A section through the normal mouse urogenital tract is given in Fig. 1A. A higher magnification of an area in A is in B, showing the normal epithelium lining prostate tubules. For comparison, an example of a WD to MD prostate tumor of a TRAMP mouse in the UAB colony is shown in Fig. 1C. D is a higher magnification of C displaying fibrosis, hyperplasia, and cribriform structures. An example of PD is in Fig. 1E. A higher magnification of areas in E are in F and G. In F, arrow points to a normal prostate tubule embedded in the mass of PD.

The DLPs from 42 TRAMP mice fed phytoestrogen-free diet from 5 to 28 weeks of age were weighed and prepared for histopathological examination. Sections of the DLP were evaluated blindly and scored using established criteria (30). The frequency distribution of mice with prostate scores of 1–6 is given in Fig. 2. By 28 weeks of age, none of these mice had a normal prostate (score 1), low PIN (score 2), or high PIN (score 3). About half of the mice displayed WD (score 4), with the other half divided between mice with MD (score 5) and PD (score 6; Fig. 2). The weight of prostates with PD (score 6) was two orders of magnitude higher than that of prostates with WD (score 4) or MD (score 5; Fig. 3A;  $P < 0.001$ ). Expression of AR transcripts determined by RT-PCR was lower in prostates with PD (score 6) as compared with prostates with WD (score 4) or MD (score 5; Fig. 3B;  $P = 0.058$ ).

**Evidence Consistent with the Possibility that Genistein Administered in the Diet Is Not Toxic.** Starting at 5–6 weeks of age until they were 28–30 weeks of age, transgenic males (25, 10, 17, and 7 mice/group, respectively) were fed a phytoestrogen-free diet (AIN-76A) containing 0, 100, 250, or 500 mg of genistein per kg AIN-76A. At necropsy, genistein concentrations in the serum were determined in mice from three of the experimental groups and found to be directly proportional to the level of genistein in the diet (Fig. 4). One-way ANOVA, followed by an all pairwise multiple comparison procedure (Tukey test), revealed a statistically significant difference between the treatment groups ( $P < 0.001$ ).

Two criteria were used for gross evaluation of the possibility that genistein might be toxic: (a) changes in body weight; and (b) changes in organ weight.

Results in Fig. 5 are the means of body weights in each experimental group  $\pm$  SD, as a function of age (weeks). Because data were

not normally distributed with equal variance, Kruskal-Wallis ANOVA, followed by an all pairwise multiple comparison procedure (Dunn's method), was used to compare the experimental groups. The median values obtained at each time point, respectively, were not significantly different in the groups fed diets containing various levels of genistein. In conclusion, results indicated that genistein in the diet did not significantly affect body weight at any age.

At necropsy, testes, epididymes, and kidneys of all mice were weighed. The mean  $\pm$  SD of these weights, as a function of the level of genistein in the diet, is given in Fig. 6. Means were compared using one-way ANOVA. No significant difference was found between the weights of kidneys, testes, or epididymes in mice fed AIN-76A diet containing varying concentrations of genistein.

**Genistein in the Diet Reduces the Incidence of 28–30-week-old Mice with PD (Score 6).** The percentage of mice with prostates with pathological scores 4, 5, or 6 was determined in each experimental group. The percentage of mice with prostates with pathological scores 4 or 5 was not affected significantly by feeding genistein in the diet (results not shown). In contrast,  $\chi^2$  analysis revealed that genistein in the diet reduced significantly ( $P = 0.041$ ) the proportion of mice with PD (score 6; Fig. 7).

## DISCUSSION

Several studies show that genistein prevents the growth of PC cells *in vitro* (42, 43). Studies *in vivo* using rat models with carcinogen-induced PC are also consistent with the possibility that genistein

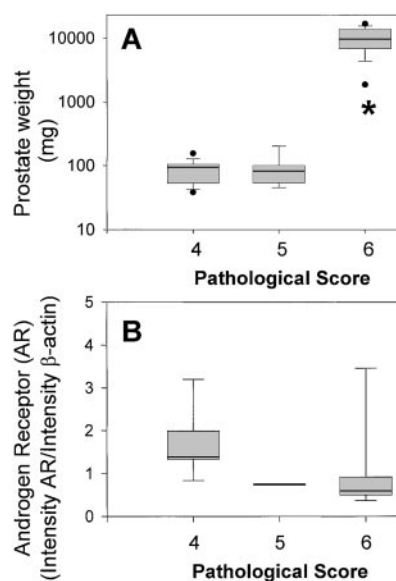


Fig. 3. Evidence suggesting that prostates scored as 6 may represent an advanced stage of PC. TRAMP mice were fed phytoestrogen-free diet from 5 weeks of age until necropsied at 28–30 weeks of age. The DLPs from 22, 7, and 13 TRAMP mice were weighed. Most of the tissue from each mouse was prepared for histopathological evaluation. Samples of prostate tissue, collected at necropsy and rapidly frozen ( $-80^{\circ}\text{C}$ ), were used for evaluation of AR expression. Results are given in box plots, where the box encompasses the 25<sup>th</sup> through 75<sup>th</sup> percentiles of results obtained, with the 50<sup>th</sup> percentile (median) — shown in the box. The 5<sup>th</sup> and 95<sup>th</sup> percentiles are shown as ● below and above the 10<sup>th</sup> and 90<sup>th</sup> percentile whisker caps, respectively. Statistical analysis of differences between the experimental groups was carried out using Kruskal-Wallis one-way ANOVA, followed by an all pairwise multiple comparison procedure (Dunn's method). A, weight of the DLP (mg) as a function of the pathological score. The median of prostate weights measured in the group with pathological score 6 was significantly different from that of the other groups ( $P < 0.001$ ). B, AR mRNA expression as a function of the pathological score. Relative steady-state RNA levels of AR were determined by RT-PCR. The median of relative steady-state RNA levels of AR measured in the group with pathological score 6 was lower than that of the other groups ( $P = 0.058$ ).

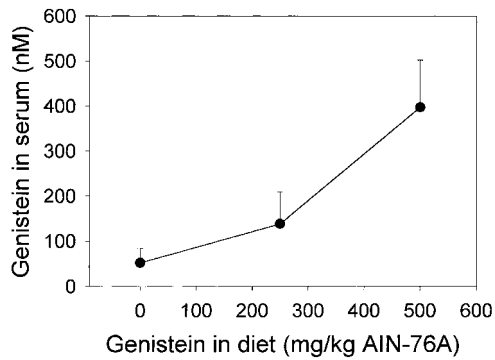


Fig. 4. Serum genistein concentrations in TRAMP mice on diets containing increasing concentrations of genistein. Starting at 5–6 weeks of age until they were 28–30 weeks of age, transgenic males were fed AIN-76A diet containing increasing levels of genistein. Genistein concentrations in the serum were determined in mice fed 250 mg of genistein per kg AIN-76A ( $n = 5$ ), 500 mg of genistein per kg AIN-76A ( $n = 6$ ), and controls on AIN-76A diet without genistein ( $n = 14$ ). A significant difference between mean genistein concentrations in the three experimental groups was found using one-way ANOVA, followed by an all pairwise multiple comparison procedure (Tukey test;  $P < 0.001$ ).

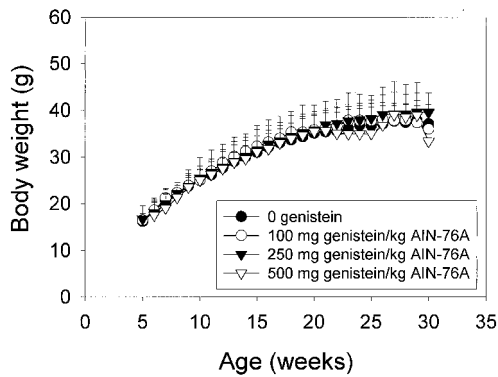


Fig. 5. Genistein administered in the diet does not affect body weight. Starting at 5–6 weeks until they were 28–30 weeks of age, transgenic males were fed AIN-76A diets containing 0, 100, 250, or 500 mg of genistein per kg AIN-76A (25, 10, 17, and 7 mice/group, respectively). Results shown are the means of body weights in each experimental group  $\pm$  SD, as a function of age (weeks). Comparison of mean body weights using Kruskal-Wallis one-way ANOVA, followed by an all pairwise multiple comparison procedure (Dunn's method), did not detect any significant effect of genistein on body weight, at any age.

prevents the growth of prostate tumors (44, 45).<sup>4</sup> Moreover, soybean phytochemicals, including genistein, have been shown to inhibit the growth of transplantable human prostate cells in mice (19–21).

The advantage of the TRAMP mice is that, because of their genetic makeup, initiation of PC occurs spontaneously at puberty. Longitudinal studies in TRAMP mice have shown that over the period of 12–28 weeks of age, this mouse displays progressive stages of PC found in humans (22, 29, 30, 46, 47). As expected, TRAMP mice produced in the UAB colony also developed progressive forms of prostatic disease that resemble human PC (Fig. 1). Careful pathological evaluation revealed that by 28–30 weeks of age (mouse “middle age”), none of the TRAMP mice in our study had normal prostates, low PIN, or high PIN (Fig. 2). Instead, all mice displayed prostatic adenocarcinoma. About half displayed small prostates with WD, with the other half divided between mice with MD and PD (Fig. 2). This was consistent with a recent longitudinal study, in which 60–80% of the population of TRAMP mice was shown to develop palpable prostate tumors by 28–30 weeks of age (48).

PD (score 6) might represent a distinct, more advanced stage of PC

in TRAMP mice. Thus, the median prostate weight was two orders of magnitude larger in prostates scored 6, as compared with prostates displaying WD (score 4) or MD (score 5; Fig. 3A). AR expression was altered in prostates displaying PD as compared with prostates displaying WD (score 4) or MD (score 5; Fig. 3B). We used the incidence of PD in TRAMP mice as an end point to examine the chemopreventive potential of dietary genistein.

Transgenic males were fed from weaning until middle age (28–30 weeks of age) a phytoestrogen-free diet (AIN-76A) supplemented with increasing concentrations of genistein. In the present study, we report only studies with those genistein concentrations in the diet that produced mean serum levels of genistein (Fig. 4) within the range that has been reported in Asian men on a standard soy diet (13). As in humans, these concentrations of genistein did not appear to be toxic in TRAMP mice, as indicated by the fact that weekly body weights (Fig. 5), as well as organ weights at necropsy (Fig. 6), were not affected at all by the presence of genistein in the diet. In contrast, we found a dose-dependent reduction in the incidence of TRAMP mice with PD PC (score 6) as a function of the concentration of genistein in the diet (Fig. 7).

Initiation of PC in TRAMP mice occurs at about 12 weeks of age. Therefore, the reduction observed in the incidence of PD PC at 28–30 weeks of age (Fig. 7) could be attributable to an inhibitory effect of genistein on tumor initiation, despite the fact that TRAMP mice express SV40 *Tag*. In addition, genistein in the diet might prevent development of PD PC by inhibiting mechanisms of promotion. On

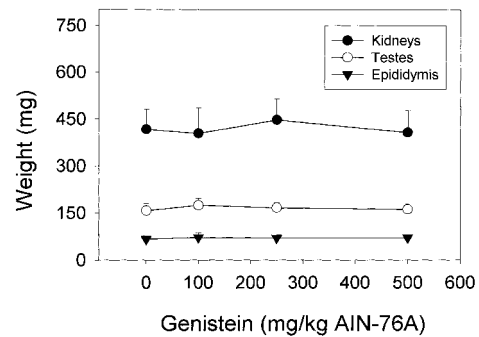


Fig. 6. Genistein administered in the diet does not affect the weight of the testes, epididymides, and kidneys. At necropsy, the weight of testes, epididymides, and kidneys was determined in the TRAMP mice described in Fig. 5. Results are mean  $\pm$  SD of the respective organ weights in mice from each of the four experimental groups. Comparison of mean organ weights by one-way ANOVA revealed no significant difference between mice in groups fed diets that contained increasing concentrations of genistein.

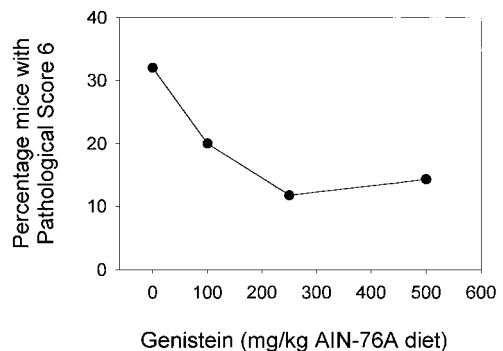


Fig. 7. Genistein reduces the incidence of mice with advanced prostate tumors. The urogenital tract collected at necropsy was prepared for pathological evaluation of the prostate by established criteria (29, 30). Results shown are the number of mice with prostates displaying PD, as a percentage of all mice evaluated in each of the treatment groups described in Fig. 5.  $\chi^2$  test revealed that the frequency of TRAMP mice with PD decreased significantly as a function of genistein in the diet ( $P = 0.041$ ).

<sup>4</sup> J. Wang, I-E. Eltoum, and C. A. Lamartiniere. Dietary genistein suppresses chemically induced prostate cancer in Lobund-Wistar Rats, submitted for publication.

the basis of earlier studies, several molecular mechanisms might be responsible for the preventive action of genistein. Weak plant estrogens are polyphenolic compounds and might therefore act as antioxidants by inhibiting superoxide anion formation (49). Genistein also inhibits topoisomerase II (50–52) and induces cell differentiation (53) and apoptosis (51). Antiproliferative effects of genistein may be related to its ability to inhibit tyrosine kinases *in vitro* (18, 42, 54). At high concentration, genistein is a potent inhibitor of epidermal growth factor receptor tyrosine phosphorylation (54, 55). Genistein has also been shown to inhibit *in vitro* the activity of the phosphotyrosine kinase focal adhesion kinase (56), consistent with the possibility that it might affect cell adhesion-mediated mechanisms. On the basis of these findings *in vitro*, it has been proposed that genistein may also act *in vivo* as a tyrosine kinase inhibitor, reducing the activity of receptors critical for the transduction of mitogenic signals (57). Additional studies are under way to sort out the mechanisms by which genistein might exert its preventive action *in vivo*.

In conclusion, genistein concentrations in the serum of mice fed genistein-containing diets were comparable with those found in Asian men on regular soy diet, a population in which the incidence of advanced PC is lower as compared with that in Western men. On the basis of body and organ weight evaluations, genistein in the diet did not appear to be toxic. Genistein in the diet reduced significantly the incidence of advanced prostate lesions in TRAMP mice, despite their altered genetic makeup. Studies are under way to sort out molecular mechanisms that might underlie the preventive effect of genistein in the diet.

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## Genistein in the Diet Reduces the Incidence of Poorly Differentiated Prostatic Adenocarcinoma in Transgenic Mice (TRAMP)

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