Clinical Cancer Research

Lifetime Genistein Intake Increases the Response of Mammary Tumors to Tamoxifen in Rats

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Abstract

Purpose: Whether it is safe for estrogen receptor–positive (ER+) patients with breast cancer to consume soy isoflavone genistein remains controversial. We compared the effects of genistein intake mimicking either Asian (lifetime) or Caucasian (adulthood) intake patterns to that of starting its intake during tamoxifen therapy using a preclinical model.

Experimental Design: Female Sprague-Dawley rats were fed an AIN93G diet supplemented with 0 (control diet) or 500 ppm genistein from postnatal day 15 onward (lifetime genistein). Mammary tumors were induced with 7,12-dimethylbenz(*a*) anthracene (DMBA), after which a group of control diet–fed rats were switched to genistein diet (adult genistein). When the first tumor in a rat reached 1.4 cm in diameter, tamoxifen was added to the diet and a subset of previously only control diet–fed rats also started genistein intake (post-diagnosis genistein).

Results: Lifetime genistein intake reduced *de novo* resistance to tamoxifen, compared with post-diagnosis genistein groups. Risk of recurrence was lower both in the lifetime and in the adult genistein groups than in the post-diagnosis genistein group. We observed downregulation of unfolded protein response (UPR) and autophagy-related genes (GRP78, IRE1α, ATF4, and Beclin-1) and genes linked to immunosuppression (*TGFβ* and *Foxp3*) and upregulation of cytotoxic T-cell marker *CD8a* in the tumors of the lifetime genistein group, compared with controls, post-diagnosis, and/or adult genistein groups.

Conclusions: Genistein intake mimicking Asian consumption patterns improved response of mammary tumors to tamoxifen therapy, and this effect was linked to reduced activity of UPR and prosurvival autophagy signaling and increased antitumor immunity. *Clin Cancer Res; 23(3); 814–24. ©2017 AACR.*

Introduction

High soy food intake among women living in Asian countries is thought to contribute to their low breast cancer risk (1, 2). Soybeans contain the isoflavone genistein that has physicochemical properties similar to 17β-estradiol (E2). Genistein activates both estrogen receptors ERα and ERβ in a manner comparable with E2 but with a lower affinity (3). Studies done using estrogen receptor–positive (ER+) human MCF-7 breast cancer cells indicate that physiological doses of E2 or genistein stimulate the growth of these cells *in vitro* and *in vivo* in athymic nude mice (4). Because estrogenic compounds, including hormone replacement therapy, are not recommended for patients with breast cancer, oncologists often advise their patients not to take isoflavone supplements or to consume soy foods (5). Also, genistein intake can reduce the efficacy of both tamoxifen and aromatase inhibitors in eliminating breast cancer cells in athymic nude mice (6–8).

Studies in women show no evidence of adverse effects of soy intake on breast cancer outcome (9). Indeed, patients with breast cancer consuming more than 10 mg isoflavones daily, corresponding to one third cup of soymilk, have the lowest risk of recurrence, both among Caucasian and Asian populations (10–12). The protective effect is seen in ER+ and ER− patients with breast cancer (11, 12) and in patients treated with endocrine therapy (12). However, it is unclear whether the protective effect reflects high lifetime soy intake or whether a similar outcome can be reached by starting soy intake for the first time during endocrine therapy.

Resistance to endocrine therapies is a significant problem in treating ER+ breast cancers (13). While about 50% of ER+ patients with breast cancer respond to tamoxifen, tumors can either exhibit *de novo* resistance (never respond to the treatment) or acquire resistance after initially responding and recur (14, 15). Factors involved in determining which patients will respond to endocrine therapy and which will recur remain largely unknown (16, 17). Tamoxifen induces endoplasmic reticulum (EnR) stress and the unfolded protein response (UPR); when prodeath signaling dominates cancer cells are eliminated by apoptosis (16, 18). However, UPR which involves GRP78 as a key activator of the process, and 3 signaling arms consisting of IRE1α, PERK, and ATF6 pathways can also activate autophagy-related genes to induce cancer cell survival. Earlier *in vitro* studies indicate that genistein suppresses UPR signaling (19, 20), but as these findings

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Because of its apparent estrogenicity, the safety of genistein in soy foods for estrogen receptor–positive (ER+) patients with breast cancer who are treated with antiestrogen therapy remains controversial. We show here using a preclinical rat model that lifetime or adult genistein intake improved tamoxifen responsiveness and reduced the risk of recurrence, compared with starting genistein intake during tamoxifen therapy. The positive effects of lifetime genistein intake were linked to reduced unfolded protein response and autophagy and improved antitumor immune responses. If true for women, our results suggest that patients with breast cancer should continue consuming soy foods after diagnosis but not to start if they have not consumed genistein previously.

were obtained using 50 to 200 times higher doses of genistein than can be achieved by consuming soyfoods, their clinical relevance is not known.

The UPR regulates inflammatory responses in antigen-presenting macrophages and dendritic cells (21). Specifically, activation of UPR reduces antigen processing and presentation and consequently suppresses CD8+ cytotoxic T cells (22), leading to evasion of antitumor immunity (23). UPR activation is linked to upregulation of FOXP3, a master transcription factor that participates in the development and function of regulatory T cells (Treg; ref. 23). High FOXP3 expression is associated with promotion of immunosuppression. Autophagy also modulates immune responses by increasing Tregs (24) and downregulating CD8+ cells (25). Genistein has several effects on the immune system *in vitro* and *in vivo* (26), including increasing CD8+ T cells and reducing Tregs (27). Because high FOXP3 levels are predictive of poor survival among ER+ patients with breast cancer (28, 29), genistein intake may promote antitumor immunity and prevent recurrence.

More than 40 years ago, Jordan and colleagues (30) used 7,12-dimethylbenz[*a*]anthracene (DMBA) to induce ER+ mammary tumors in rats and showed that these tumors stopped growing and many disappeared when animals were treated with tamoxifen. Using this same model, with some modifications (31), we compared the effects of lifetime genistein exposure that mimics the soy food intake of Asian women, with genistein intake starting during adult life (mimicking soy food intake among some Western women), or intake that began during tamoxifen treatment. Most breast cancers in the United States likely fall into the latter category, if patients start using soy supplements or soy foods to alleviate menopausal symptoms induced by antiestrogens. Our results show that either lifetime or adult genistein intake inhibits tamoxifen resistance and reduces local mammary cancer recurrence. Starting to consume genistein during tamoxifen treatment has the opposite effect. We also found changes in tumor UPR and immune markers that are indicative of lower levels of EnR stress and improved antitumor immune responses in the lifetime genistein-exposed group.

Materials and Methods

Animals

Sixteen female Sprague-Dawley rats, each nursing ten 10-day-old female pups, were obtained from Charles River Laboratory (Frederick, MD) and housed in the Department of Comparative Medicine at Georgetown University. Rats were kept in a temperature- and humidity-controlled room with free access to water and food under a 12:12-hour light:dark cycle. All experimental procedures were approved by Georgetown University Animal Care and Use Committee.

Dietary exposures and DMBA administration

When pups were 15 days of age, 16 litters were divided into 2 groups: (i) 7 dams with a total of 70 female pups were fed AIN93G laboratory diet supplemented with 500 ppm genistein (genistein diet) and (ii) 9 dams with a total of 90 female pups were fed an AIN93G diet (control diet). Pups were weaned on postnatal day (PND) 21 and kept on the genistein or control diet until PND 30. Between PND 31 and 55, all rats were fed the control diet to avoid the potential effects of genistein on the metabolism and activation of DMBA.

On PND 48, mammary tumors were initiated by administering 1 mL of peanut oil containing 10 mg DMBA by oral gavage. One week later, rats were divided into 5 groups. (i) Those previously fed genistein diet received 500 ppm genistein from PND 55 onward until the end of the study (lifetime genistein group mimicking Asian women; $n = 35$). (ii) Those fed genistein between PNDs 15 and 30 received control diet until they developed mammary tumors and started antiestrogen treatment. Once antiestrogen treatment started, these rats were again fed 500 ppm genistein (prepubertal genistein group; $n = 35$). This group does not represent either Caucasian or Asian soy intake patterns; this group was included as a control group for the lifelong genistein intake group. (iii) Those animals previously fed control diet received 500 ppm genistein from PND 55 onward until the end of the study (adult genistein group mimicking Caucasian women; $n = 35$). (iv) Rats previously fed control diet remained in the control diet until they developed mammary tumors and started antiestrogen treatment at which point they were fed for the first time 500 ppm genistein (antiestrogen treatment only genistein group; $n = 20$). Finally, (v) a control group composed of rats kept on the control diet throughout the study (control; $n = 15$). This control group was not included to assessing tumor responses to tamoxifen treatment. Instead, a historical control group (31) was utilized to compare tumor responses to tamoxifen. All the diets were provided by Harlan Laboratories and the ingredients are listed in Supplementary Table S1. Outline of dietary exposures are provided in Fig. 1.

To determine the effect of genistein intake on UPR, autophagy, and immune parameters in the mammary tumors prior to antiestrogen treatment, we euthanized 15 rats per group when their tumor reach a size of 14 ± 1 mm in diameter.

Mammary tumor latency, incidence, and burden

Tumor latency was the length of the tumor-free period (in weeks) from the date of DMBA administration until a rat developed the first measurable mammary tumor. Incidence was assessed weekly and was defined as the percentage of animals per group that had developed at least one tumor. Tumor burden was the overall tumor area, calculated by measuring the width $\times$ length of each tumor per animal, and was assessed when rats started receiving antiestrogen treatment.

Antiestrogen treatment

Mammary tumor growth was monitored weekly by palpation, and tumor locations and sizes (measured by a caliper) were

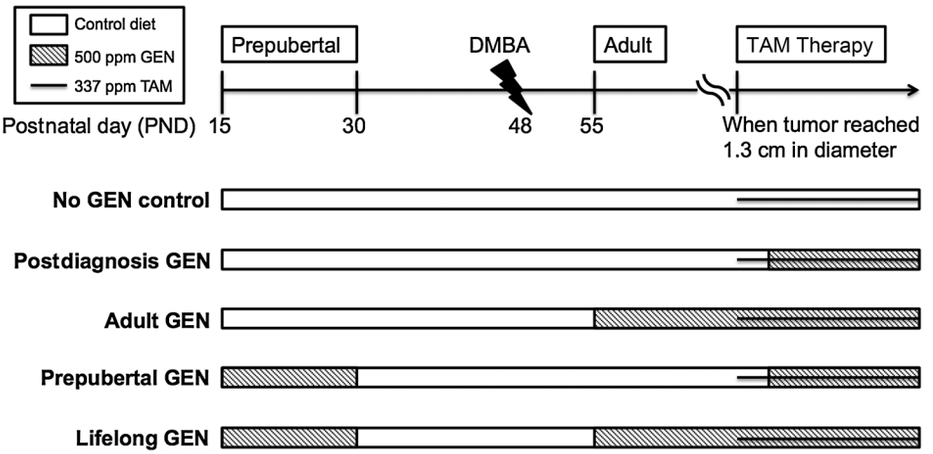

Figure 1.
Study design. Rats were fed 0 or 500 ppm genistein (GEN)-containing diet either before puberty, adult life or both, or rats started consuming it for the first time during tamoxifen (TAM) treatment. Mammary tumors were initiated with DMBA at PND 48, and when a first tumor per rat reached a size of 1.4 cm in diameter, 337 ppm tamoxifen citrate (TAM) was added to the diet. Response of tumors to TAM was monitored for up to 38 weeks.

recorded. When a tumor reached a size of 14 ± 1 mm in diameter, a rat was switched to a diet containing 337 ppm tamoxifen citrate. This dose results in a daily exposure of 15 mg/kg tamoxifen. The response of the tumors to tamoxifen was assessed by the criteria outlined in Supplementary Table S2.

Tissue collection

At the end of the tumor monitoring period, rats were euthanized by CO_2; earlier if burden reached 10% of the body weight, or if animals lost weight or were sick, as required by the Georgetown University Animal Care and Use Committee. At necropsy, blood was collected by cardiac puncture, and mammary glands and tumors were removed and flash-frozen in liquid N_2 for future analysis or were fixed in 10% formalin for histopathology purpose.

Tumor pathologic evaluation

Formalin-fixed mammary tumors were embedded in paraffin and cut into 5-μm sections. Hematoxylin and eosin (H&E)-stained tumor sections were used for the evaluation. Tumors were classified according to their histopathology as evaluated by an experienced pathologist (ARUP Laboratory). In all the molecular biology assays done using mammary tumors, only tumors that were malignant adenocarcinomas were included to the analysis.

RNA extraction and cDNA synthesis

One hundred grams of frozen mammary tissue was ground using a mortar and pestle in liquid nitrogen. Total RNA was isolated from the ground mammary glands by RNeasy Lipid Tissue Mini Kit (Qiagen), following the manufacturer's instructions. Total RNA from malignant mammary tumors was extracted using TRIzol reagent (Life Technologies) followed by one step of DNase I treatment to prevent gDNA contamination (Roche), as the manufacturer instructed. Quantity and quality of RNA were determined according to the optical density ratio (OD_{260}:OD_{280}) using a ND1000 Nanodrop spectrophotometer (Thermo Scientific). A total of 2 μg RNA per sample was used to generate cDNA via reverse transcription in a PTC-100 thermal cycler (Bio-Rad) using the following steps: initiation at 25°C for 10 minutes, reverse transcription at 37°C for 2 hours, and deactivation at 85°C for 5 minutes.

Quantitative real-time PCR

To measure the relative mRNA abundance of UPR and immune-related genes, quantitative real-time PCR (qRT-PCR) was conducted. Briefly, 12.5 μg cDNA was used as template with primers specific for *PgR*, *Ki67*, *Tgfβ1*, *Foxp3*, *Cd8a*, spliced *Xbp1* (*Xbp1s*), unspliced *Xbp1* (*Xbp1us*), *Hspa5* (GRP78), and *Hprt* using 5 μL Absolute QPCR SYBR Green ROX Mix in a 10 μL reaction (Thermo Scientific). Serially diluted cDNA samples (20 to 0.625 ng/μL) were included with each primer. To determine the relative quantity of the gene, the expression was normalized to the level of the housekeeping gene *Hprt*. RT-PCR reactions were carried out in an ABI Prism 7900 Sequence Detection System (Life Technologies) with the following thermocycler setting: activation of the enzyme at 95°C for 15 minutes, 40 cycles of denaturing at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, followed by one step of dissociation to ensure the purity of the product. Primers used in the RT-PCR were designed using Vector NTI software (Life Technologies) and are listed in Supplementary Table S3. The result of the reaction was checked and exported using SDS 2.3 software (Life Technologies). The highest efficiency of the machine was confirmed by ensuring that the R^2 of the standard curve was >0.98 and that the slope was within 3.3 ± 0.3.

Protein isolation and immunoblotting

Protein isolated from mammary glands and malignant tumors was used to determine whether genistein intake and tamoxifen treatment affected the expression of ERα, ERβ, progesterone receptor (PgR), Erb2/HER2, GRP78, IRE1α, XPB-1, sXBP-1, PERK, ATF4, ChOP, ATF6, Beclin-1, ATG7, LC3I, LC3II, and p62. Detailed description of the procedure and antibodies used can be found in Supplementary Text.

Serum isoflavone quantification

Blood was drawn by cardiopuncture at necropsy and serum was separated and kept at −80°C until analysis. Serum levels of total isoflavone (aglycones and conjugated forms) were determined by LC-ES/MS/MS. Briefly, complete enzymatic hydrolysis was performed by incubating the serum samples overnight with *Helix pomatia* preparation containing glucuronidase, sulfatase, and glucosidase, followed by isotope dilution quantification of

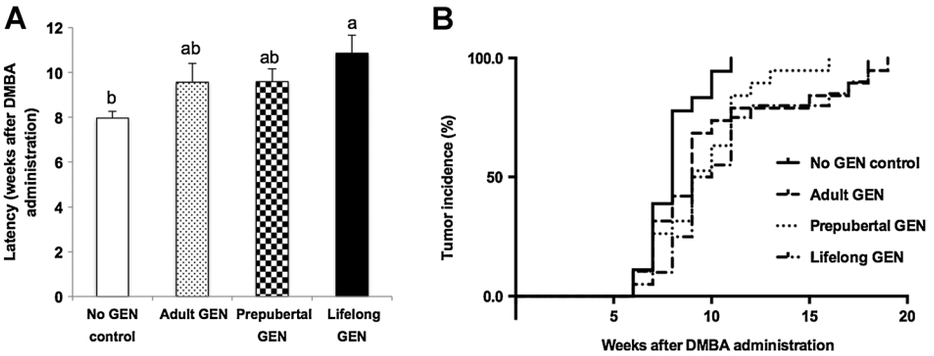

Figure 2.

Mammary tumor latency and incidence. **A,** Time between DMBA administration and appearance of first measurable mammary tumor (tumor latency) per rat consuming control diet, fed genistein (GEN) through adulthood, fed GEN during prepuberty, and fed GEN both before puberty and adulthood (lifelong). Lifelong GEN intake lengthened tumor latency. Means ± SEM are shown. Bars with different letters are significantly different from each other, $P < 0.05$. **B,** Mammary tumor incidence, shown as percentage of rats between weeks 5 and 20 after DMBA administration that developed at least one measurable mammary tumor. It was significantly lower in the lifelong GEN group than in the controls ($P = 0.0071$). $n = 20$–35 rats per group.

genistein, daidzein, and equol. Inter- and intraday analysis was performed, and the precisions of measurements were ensured with 1% to 6% relative SD. For each sample, the method detection limit for genistein was approximately 0.005 μmol/L per aliquot of 10 μL. Quality control samples were included for the analysis of every sample test including the analysis of blank and spiked serum samples (glucuronidase/sulfatase), blank injections, and injections of authentic standards.

Serum cytokine levels

To investigate possible changes in serum cytokine levels in rats fed genistein at different times of their life, a rat cytokine multiplex array (#110449RT) was carried out by Quansys Biosciences. This analysis includes the following 9 cytokines: IL1α, IL1β, IL2, IL4, IL6, IL10, IL12p70, IFNγ, and TNFα. Serum was obtained from rats that were euthanized before tamoxifen treatment. Samples were run in triplicates using ELISA-based chemiluminescent assay, and the mean value was calculated per animal to determine the cytokine levels.

Statistical analysis

Statistical analysis of the tumor latency, serum cytokine levels, mRNA expression, and protein levels were performed using one-way ANOVA followed by *post hoc* analysis using Fisher least significant difference (LSD) test. If not otherwise specified, *P* values given in Results represent those obtained from the LSD test. Kaplan–Meier survival analysis and the log-rank test were used to compare the differences in tumor incidence. χ^2 analysis was applied to determine the statistical significance in tumor response to tamoxifen treatment and in the rate of recurrence among the five groups. Repeated one-way ANOVA over each tumor monitoring week was applied to the tumor burden data. All statistical analyses were carried out using SPSS SigmaStat software, and differences were considered significant if *P* was less than 0.05. Data are expressed as mean ± SEM.

Results

We measured the serum genistein concentration by LC-ES/MS/MS: they were 4.14 ± 0.35 μmol/L in all of the genistein-exposed groups and did not differ by the duration of genistein intake. These levels are comparable with those seen in humans consuming 2 to 4 servings of soyfoods daily (32). We did not measure tamoxifen or its metabolite levels here. However, on the basis of previously published study in Sprague-Dawley rats receiving either 13.3 or 22.5 mg/kg/d of tamoxifen and having blood tamoxifen levels of about 120 or 180 ng/mL, respectively (33), we estimate that the levels in our study in rats consuming 15 m/kg/d tamoxifen were about 120 to 130 ng/mL. These levels are comparable to those reported in breast cancer patients (∼84 ng/mL) who took 20 mg of tamoxifen per day for 28 days (34).

Tumor latency and incidence

Lifelong genistein intake (10.9 ± 0.8 weeks) significantly delayed mammary tumorigenesis ($P < 0.05$), when compared with control rats that never consumed genistein (7.9 ± 0.3 weeks). No significant differences were observed among the other groups (Fig. 2A). Survival analysis revealed that the cumulative tumor incidence in the lifelong genistein consumption group was significantly lower than in the control rats ($P = 0.0071$). Because most animals in each group developed mammary tumors, tumor incidence at the end of the study did not differ among the groups (Fig. 2B).

Tumor responses to tamoxifen treatment and the risk of recurrence

Responses to tamoxifen treatment were monitored for up to 30 weeks from DMBA administration; that is, until rats were 37 weeks of age. No difference in the length of the monitoring period was seen among the groups (data not shown). Responses (R) to tamoxifen in a historical non-genistein reference group were seen among 54% of the tumors treated with tamoxifen (ref. 31; Fig. 3A). Similar response rates were seen in rats that consumed genistein during prepuberty (56%) or lifetime (52%). These 2 groups exhibited a higher percentage of partially responding (PR) tumors; tumors that stopped growing upon tamoxifen exposure, 21% and 24%, respectively, compared with the control group (8%; $P = 0.007$) (Fig. 3A). Consequently, *de novo* resistance to tamoxifen was significantly lower in the prepubertal (23%)

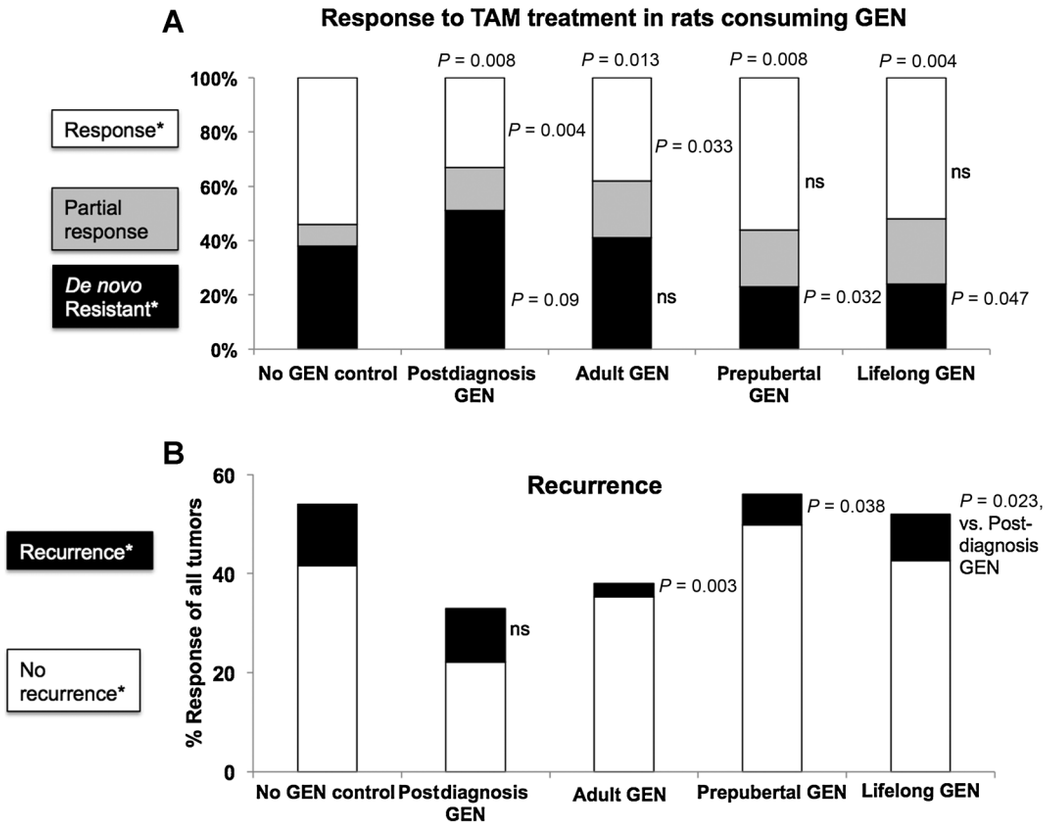

Figure 3.

Responses of mammary tumors to tamoxifen (TAM) therapy. **A,** Percentage of responses, partial responses, and *de novo* resistance in rats that consumed genistein (GEN) during different time periods of their lives. Numbers of tumors per group were 30 in no GEN controls (historical reference group described in ref. 31), 47 in postdiagnosis GEN, 44 in adult GEN, 40 in prepubertal GEN, and 42 in lifelong GEN. Starting GEN intake during TAM treatment ($P = 0.004$) and adult GEN intake ($P = 0.033$) reduced responses, whereas prepubertal ($P = 0.032$) and lifelong GEN intake ($P = 0.047$) reduced *de novo* resistance, compared with no GEN controls. **B,** Percentage of completely responding tumors that recurred locally. Black portion of each bar represents recurrent tumors. Compared with no GEN controls, adult ($P = 0.003$) and prepubertal GEN intakes ($P = 0.038$) reduced recurrence, and recurrence was also significantly lower in the lifelong GEN group than in the postdiagnosis GEN group ($P = 0.023$).

and lifetime genistein (24%) groups than in the control group (38%; $P = 0.03$).

In comparison, tumors were least likely to exhibit a response in rats that started consuming genistein either during tamoxifen treatment ($R = 33\%$; $P = 0.004$) or as adults ($R = 38\%$; $P = 0.03$) when compared with the reference control animals ($P = 0.004$ or $P = 0.03$, respectively), lifetime genistein intake group ($P = 0.01$ or $P = 0.065$), or group that consumed genistein during prepuberty and tamoxifen treatment ($P = 0.002$ and $P = 0.016$). In the adult genistein group, 21% of the tumors exhibited a PR, and thus *de novo* resistance was not increased in this group (Fig. 3A). *De novo* resistance in rats that started consuming genistein during tamoxifen treatment was significantly higher than in the prepubertal ($P = 0.032$) and lifetime ($P = 0.047$) genistein groups.

The rats that started consuming genistein as adults had a significantly higher tumor burden, and the rats that consumed genistein during prepubertal period or lifetime had a significantly lower tumor burden, compared with the reference group (repeated measures ANOVA: $P < 0.001$; Supplementary Fig. S1).

Tumors that exhibited a response and were undetectable for at least 6 weeks, but then regrew at the same location to reach $\geq$1.4 cm in diameter were characterized as recurring tumors with acquired tamoxifen resistance. Prepubertal (11% recurrence rate) and lifetime genistein intake (18% recurrence) decreased the risk of tumor recurrence, when compared with rats that started consuming genistein with tamoxifen treatment (33% recurrence; $P < 0.001$; Fig. 3B). The risk of recurrence was lowest in the rats that started genistein intake as adults (7% recurrence; $P = 0.003$, compared with the reference group).

Hormone receptor expression in the tamoxifen-treated mammary glands and tumors

Western blotting was performed to determine the protein expression of ERα, ERβ, and HER2 in the mammary glands and tumors. qRT-PCR was applied to measure *PgR* mRNA expression. In the mammary gland, protein levels of ERα (Fig. 4A), ERβ (Fig. 4B), and HER2 (Fig. 4D) and mRNA level of *PgR* (Fig. 4C) did not differ among groups. Tumors in tamoxifen-treated rats were all ER-positive, including acquired resistant tumors. ERα

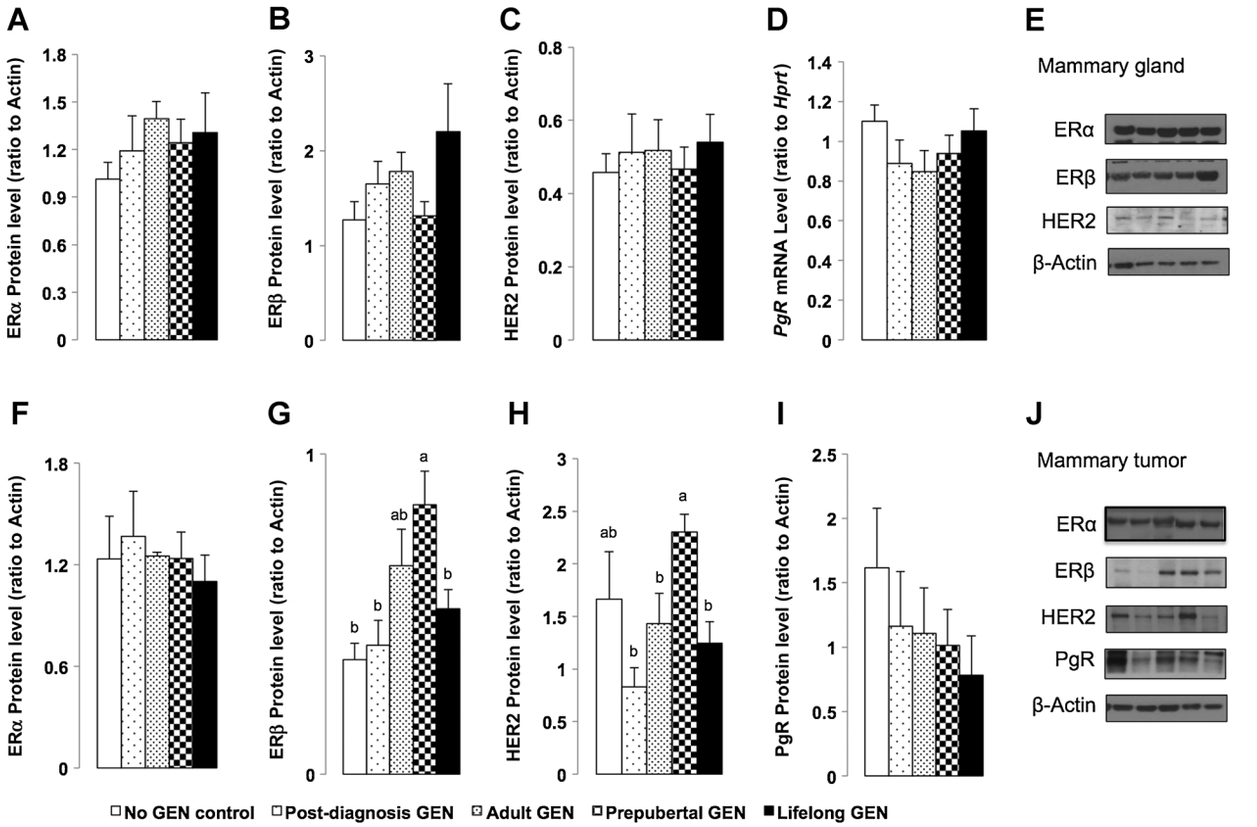

Figure 4.

Expression of ERα, ERβ, PgR, and Erb2/HER2 in the mammary glands and tumors of genistein (GEN)-fed and tamoxifen (TAM)-treated rats. Western blot analysis of (**A**, **E**) ERα, (**B**, **E**) ERβ, and (**C**, **E**) Erb2/HER2 protein levels and (**D**) RT-qPCR analysis of *PgR* mRNA levels in the mammary glands and tumors of rats fed GEN during different periods of their lives. Quantitated data and Western blot analyses are shown. Lifelong GEN intake increased ERβ levels. For the adenocarcinomas (either partially responding or *de novo* resistant), protein levels of (**F**, **J**) ERα, (**G**, **J**) ERβ, and (**H**, **J**) Erb2/HER2 levels and (**I**, **J**) PgR are shown. Data are presented as means ± SEM. Bars with different letters are significantly different from each other, $P < 0.05$. Prepubertal GEN intake increased ERβ levels. $n = 5$–10 mammary glands and 5–10 tumors per group.

(Fig. 4F) or PgR (Fig. 4H) protein levels were not affected by the different timings of genistein exposure. Protein levels of ERβ ($P = 0.02$, Fig. 4G) and HER2 ($P = 0.007$, Fig. 4I) were upregulated in the tumors of prepubertally genistein-exposed rats, when compared with the group that started genistein intake during tamoxifen therapy.

Tumor pathologic type

Histopathology of the mammary tumors was assessed before and after tamoxifen treatment by examining H&E-stained tumor sections. Results are shown in Supplementary Fig. S2. No statistical difference in tumor histopathology was seen in animals that were euthanized prior to tamoxifen treatment. Nearly 0% to 27% of the tumors were benign in the control and all genistein groups and the remaining tumors were either papillary or tubular adenocarcinomas.

Tumor histopathology was assessed after tamoxifen treatment in partially responding, *de novo* resistant, or recurring mammary tumors. Benign tumors constituted 23% of tumors in the controls, 27% in the rats that started to consume genistein during tamoxifen therapy, and 33% in the rats that consumed genistein during adulthood. Prepubertal and lifetime genistein intake significantly increased the rate of benign tumors in tamoxifen-treated rats to 53% ($P < 0.001$ compared with control group). Thus, because of tamoxifen therapy, more than half of the partially responding or growing tumors in these groups were no longer malignant.

UPR and autophagy signaling in the mammary glands and tumors before tamoxifen treatment

Mammary glands. Protein levels, determined using Western blot analyses, of UPR and autophagy markers did not change among genistein groups in rats before tamoxifen treatment (Supplementary Fig. S3).

Mammary tumors. We assessed possible changes in UPR and autophagy using Western blot analyses in the mammary glands and tumors obtained from rats before tamoxifen treatment. All the tumors used here were malignant adenocarcinomas and were approximately 1.4 cm in diameter when harvested. None of the UPR or autophagy genes studied were significantly different among the controls or genistein-fed groups (Supplementary Fig. S3).

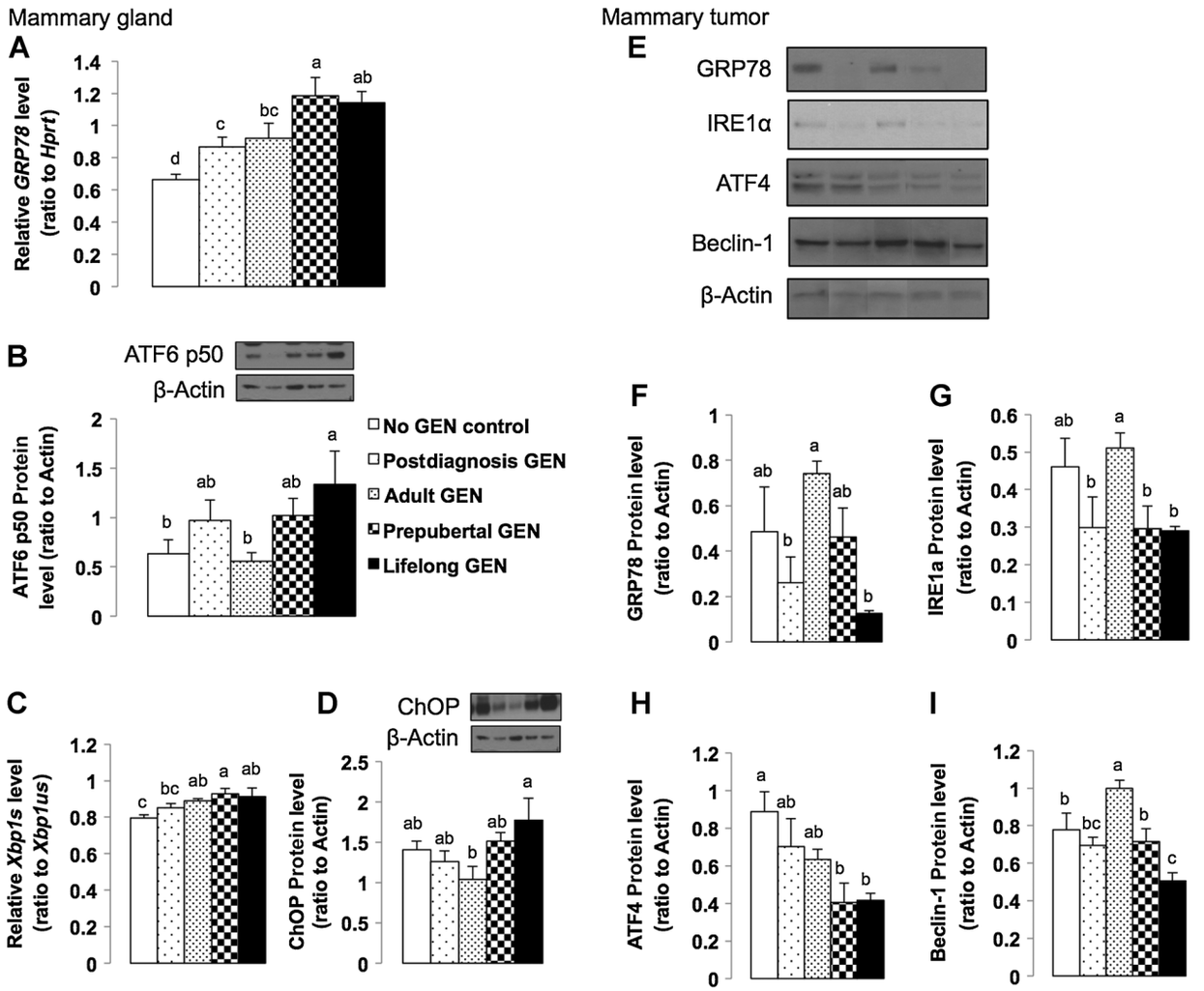

Figure 5.

Protein levels of genes in UPR pathways in the mammary glands and tumors of genistein (GEN)-fed and tamoxifen (TAM)-treated rats. **A,** UPR chaperone *GRP78*, (**B**) ATF6, and its downstream targets (**C**) *Xbp1* (ratio of spliced to unspliced version) and (**D**) ChOP were elevated in the mammary glands of rats fed GEN though the lifetime. In contrast, (**F**) GRP78, (**G**) IRE1α, (**H**) ATF4, and (**I**) Beclin-1 were downregulated in the mammary tumors of rats fed GEN through the lifetime. **E,** Western blot analyses of the genes in the tumors. **B** and **D**, Blots in the mammary tissues. Data are presented as means ± SEM; bars with different letters are significantly different from each other, $P < 0.05$. $n = 6$–10 mammary tissues and $n = 3$–6 tumors per group.

Alterations in UPR and autophagy signaling in the mammary glands and tumors in tamoxifen -treated rats

Mammary glands. Tamoxifen did not affect UPR or autophagy signaling in the DMBA-exposed mammary glands (Supplementary Fig. S4A and S4C–S4H). Consumption of genistein throughout the lifetime increased *GRP78* mRNA expression, compared with the control rats ($P < 0.001$) or rats that started consuming genistein during tamoxifen treatment ($P = 0.007$). *GRP78* mRNA also was elevated in prepubertally genistein-fed rats, compared with the same 2 groups ($P < 0.001$ or $P = 0.02$). No other changes in mRNA levels in UPR or autophagy genes were seen. At the protein level, ATF6 was higher in the lifetime genistein group than in the control ($P = 0.02$) or adult genistein ($P = 0.01$) rats, and CHOP protein was higher in the lifetime group than in the adult genistein rats ($P = 0.05$; Fig. 5A, B, and D).

Because ATF6 acts as a transcription factor, we assessed the mRNA levels of several ATF6 target genes. mRNA levels of total *Xbp1* were significantly higher in the lifetime ($P = 0.01$) and prepubertally genistein-fed rats ($P = 0.02$) than in the control rats. In addition, the ratio of spliced *Xbp1* levels to total *Xbp1* levels was higher in the lifetime ($P = 0.02$) and prepubertally genistein-fed rats ($P = 0.01$) than in the controls (Fig. 5C). These findings show that in the mammary glands with no tumors, UPR was more activated in those groups that received genistein already during pubertal development, suggesting that upregulation of UPR may be involved in protecting against mammary cancer development.

Mammary tumors. Consistent with the earlier studies showing that tamoxifen activates UPR in breast cancer cell lines (18), we found elevated protein levels of GRP78 ($P = 0.01$) and IRE1α ($P = 0.05$),

when compared with tumors obtained from rats not treated with tamoxifen (Supplementary Fig. S4B and S4I–S4N). We also noted that Beclin-1 ($P = 0.05$) protein levels were increased and p62 protein levels were reduced ($P = 0.02$) in the tamoxifen-treated tumors, indicative of higher level of autophagy. These findings are in agreement with the reported effects of tamoxifen on human breast cancer cell lines (18).

In contrast to the mammary glands, several UPR components were significantly downregulated in the mammary tumors in rats that consumed genistein throughout their lifetime. The downregulated genes included GRP78 ($P < 0.001$, compared with adult genistein group), ATF4 ($P = 0.01$, compared with control group), and Beclin-1 ($P = 0.05$ and $P = 0.001$, compared with control and adult genistein group, respectively; Fig. 5E–I). Because ATF4 and Beclin-1 both induce autophagy (35), lifetime genistein intake may promote higher responsiveness to tamoxifen therapy by reducing autophagy.

Alterations in tumor immune system markers before and after tamoxifen treatment

The mRNA level of tumor immune markers *Tgfβ1*, *Foxp3*, and *Cd8a* were determined in the mammary tumors from rats before and after treatment with tamoxifen. Foxp3 is a member of the forkhead-box transcription factor family and induces differentiation of immature $CD4^+$ T cells to $CD4^+CD25^+$ Tregs (36). Before tamoxifen treatment, genistein consumption during childhood ($P = 0.04$), adulthood ($P = 0.005$), or throughout lifetime ($P = 0.02$) reduced *Foxp3* mRNA level, when compared with the control rats (Fig. 6E). Tgfβ1 increases differentiation of immature T cells into Treg lineage, and Foxp3 induces its expression (37). Tgfβ1 mRNA levels in the mammary tumors were significantly lower in all genistein groups than in the control group ($P = 0.05$). Genistein exposure tended to reduce *Tgfβ1* mRNA level in the tumors of rats exposed during childhood and adulthood, but the difference did not reach statistical significance (Fig. 6D). CD8 is

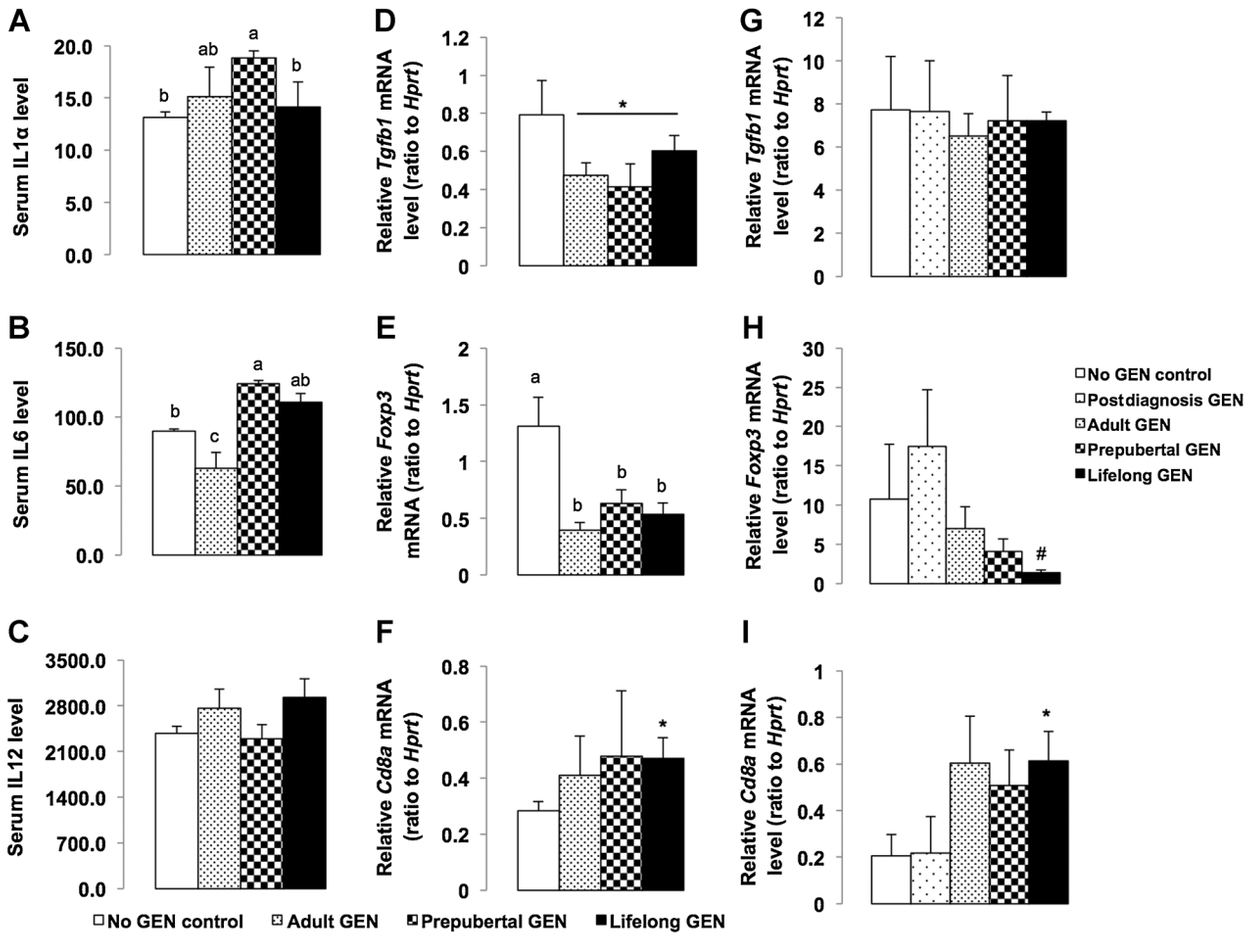

Figure 6.

Cytokine levels and expression of *Foxp3*, *TFGβ1*, and *Cd8a* in the mammary tumors of genistein (GEN)-fed and tamoxifen (TAM)-treated rats. Serum cytokine levels of (**A**) IL1α, (**B**) IL6, and (**C**) IL12 and tumor mRNA levels of (**D**) Treg cell marker *Foxp3* and (**E**) its downstream target *TGFβ1* and (**F**) cytotoxic T-cell marker *Cd8a* in rats fed GEN before puberty, adulthood, or both and not yet treated with TAM ($n = 9$ adenocarcinomas per group). GEN intake, either past or current, reduced *Foxp3* levels and increased *Cd8a* levels. Same endpoints were measured in tumors during TAM treatment (**G–I**) and this assessment included tumors from rats that started GEN intake for the first time during TAM treatment ($n = 4$–6 partially responding or *de novo* resistant adenocarcinomas per group). Lifelong GEN intake reduced *Foxp3* and increased *Cd8a* levels in TAM-treated rats. Data are presented as means ± SEM; bars with different letters are significantly different from each other, $P < 0.05$. *, significantly different from no GEN control; #, statistically different from postdiagnosis GEN-exposed rats, $P < 0.05$.

expressed in cytotoxic T cells. Lifelong genistein exposure increased mRNA level of *Cd8a* in tumors before tamoxifen treatment, compared with control rats ($P = 0.03$; Fig. 6F).

In tamoxifen -treated tumors, mRNA level of *Tgfβ1* did not differ among the 5 groups (Fig. 6G). Lifelong genistein-fed rats exhibited the lowest mRNA level of *Foxp3* ($P = 0.05$ compared with rats starting to consume genistein with tamoxifen therapy) and highest *Cd8a* ($P = 0.02$, compared with controls; Fig. 6H and I). Together, these findings suggest that lifetime genistein intake prevents pathways leading to tumor immunosuppression and promotes antitumor immune responses.

Alterations in circulating cytokine levels

Cytokine levels in serum were assessed by multiplex rat cytokine arrays only before tamoxifen treatment. Thus, rats fed a genistein-containing diet during adulthood and throughout their lifetime were consuming this isoflavone at the time cytokines were measured, unlike the control rats or rats that received genistein before puberty. Of the 9 cytokines tested, only IL1α, IL6, and IL12 had detectable levels in the rat serum. Rats that consumed genistein prepubertally (but not at the time the cytokines were assayed) had higher level of serum IL1α than the control rats ($P < 0.001$; Fig. 6A). Serum IL6 levels also were higher ($P < 0.001$) in this group than in the controls, whereas the levels were significantly reduced in the group consuming genistein during adulthood ($P = 0.04$; Fig. 6B). IL12 levels tended to be elevated in rats consuming genistein at the time of measurements but the difference did not reach statistical significance (Fig. 6C).

Proliferation and apoptosis in the tamoxifen -treated mammary glands and tumors

To determine the level of cell proliferation, qRT-PCR was carried out to test the relative mRNA level of *Ki67* in the mammary glands and tumors. This is a less time-consuming measure of cell proliferation than assessing Ki67 though immunohistochemistry, but equally accurate (38). In the mammary glands, mRNA levels of Ki67 were higher in the rats consuming genistein prepubertally than in the postdiagnosis genistein-fed rats ($P = 0.01$) and control rats ($P = 0.003$; P for ANOVA = 0.02; Supplementary Fig. S5A). However, Ki67 levels in the adenocarcinomas did not differ among the groups (Supplementary Fig. S5C).

Apoptosis was assessed by determining the ratio between the protein levels of proapoptotic marker Bax and antiapoptotic marker Bcl2 in the mammary glands and tamoxifen-treated tumors. Neither the Bax nor Bcl-2 levels were different among the groups (data not shown). The ratio between the 2 also was similar in the glands (Supplementary Fig. S5B) and tumors (Supplementary Fig. S5D) across all groups.

Discussion

In 2012, more than 1.67 million women were diagnosed with breast cancer worldwide (39); about 70% of these patients had an ER^+ tumor. Although endocrine therapies are highly effective in preventing and treating breast cancer (14, 15), approximately half of the treated patients exhibit resistance and recur (13). In the present study, by using a preclinical model of ER^+ breast cancer, we investigated whether genistein intake modifies responsiveness to tamoxifen. Findings obtained *in vitro* and in immunodeficient mice indicate that genistein impairs response to tamoxifen (6, 8), but observational studies in patients with breast cancer show that soy food intake is linked to reduced risk of breast cancer recurrence (10–12). It is unclear how to explain these conflicting findings. Because genistein does not alter the expression of tamoxifen-metabolizing enzymes (40) nor is there any evidence that increased estrogenicity impairs antiestrogen action, as tamoxifen elevates circulating estrogen levels (41), the opposing effects of genistein on human breast cancer cells *in vitro* or *in vivo* and on patients with breast cancer are unlikely to reflect estrogenicity of this isoflavone.

We found that lifelong genistein intake reduced the risk of *de novo* and acquired tamoxifen resistance and local recurrence. Similar results were seen in rats that consumed genistein before puberty and again during tamoxifen therapy, suggesting that genistein intake around puberty is critical in preventing tamoxifen resistance. These finding are consistent with childhood soy intake in humans and prepubertal genistein exposure in animal models reducing later breast cancer risk (42). Starting genistein intake during adulthood did not interfere with the ability of tamoxifen to inhibit the growth of rat mammary tumors and was highly effective in preventing recurrence. In contrast to lifetime genistein intake, rats that started consuming genistein only when they were treated with tamoxifen exhibited an increased risk of recurrence. Thus, genistein has a preventative effect in tamoxifen-treated animals only if it is consumed before tumors start to develop.

To identify pathways that may explain the differing effects of lifetime genistein intake versus starting genistein consumption during tamoxifen treatment, we focused on 3 interconnected biologic systems. First, the effects on UPR and autophagy were investigated, as these are causally linked to the development of antiestrogen resistance (13, 16, 18). Earlier studies have reported downregulation of GRP78 by genistein in prostate and liver cancer cells (19, 20), but using pharmacologic doses that are not achievable by isoflavone or soy intake in humans. In the tamoxifen-treated mammary tumors, GRP78 expression was downregulated in rats consuming genistein through their lifetime or during prepuberty. ATF4 and Beclin-1 protein levels also were significantly reduced in the tumors of lifetime genistein group, compared with controls, indicating that autophagy may be reduced, as these transcription factors both induce autophagy (35). Previous results obtained in cancer cell lines *in vitro* indicated reduced autophagy by physiological doses of genistein (43). The reduced UPR and autophagy in the mammary tumors of rats consuming genistein through their lifetime may be linked to their increased sensitivity to tamoxifen therapy.

In the non–tumor-bearing mammary glands, GRP78 levels were increased in rats consuming genistein through their lifetime, as were the levels of CHOP and ATF6, and ATF6′s downstream target Xbp1. Thus, upregulation of UPR may be a successful defense against malignant transformation. Upregulation of Xbp1 in *Caenorhabditis elegans* was recently found to confer a stress-resistant phenotype and increased longevity (44), raising the possibility that in mammary glands, elevated Xbp1 expression also is linked to increased resistance to EnR stress.

Next, we studied whether genistein affects markers of cytotoxic T cells (CD8a) that drive antitumor immune responses, and Tregs (Foxp3 and TGFβ) that induce immunosuppression and allow cancer cells to escape elimination by the immune system (45, 46). ER^+ mammary tumors in our model are sensitive to immunomodulation.

A previous study found that genistein did not prevent DMBA-induced skin carcinogenesis in immunodeficient mice lacking T lymphocytes, whereas in immunocompetent mice, genistein reduced cancer growth; however, this protective effect was seen only if genistein was given prior to tumor induction (27). These mice, but not those receiving genistein after tumors were detected, exhibited increased activity of cytotoxic T cells and natural killer (NK) cells and decreased presence of Tregs in the spleen (27).

Pro- and anti-inflammatory cytokines are produced by immune cells and they have a profound influence on the development and function of T lymphocytes. Serum IL12 levels were nonsignificantly increased in rats consuming genistein at the time of measurement (lifetime and adult genistein intake groups), compared with control or prepubertal genistein groups. However, tumor-bearing rats fed genistein during prepuberty, but not at the time the cytokine panel was assessed, had significantly higher serum levels of IL1α and IL6 than control rats. Because IL1α or IL6 both can attenuate Treg function (47), these changes may explain the reduced expression of Foxp3 in the mammary tumors of prepubertally genistein-fed rats. Lifetime genistein intake did not modify the levels of either IL1α or IL6, suggesting that the presence of genistein reversed the increase in cytokine levels caused by prepubertal genistein intake. This is supported by the finding that adult genistein intake significantly reduced IL6 levels, in accordance with findings reported by others (48).

In summary, we used a validated preclinical model of ER^+ breast cancer to study the response of tumors to tamoxifen in rats that were fed genistein. Our results show that prepubertal and lifetime genistein consumption improved responsiveness to tamoxifen, indicating that improved response to endocrine therapy was preprogrammed early in life. Furthermore, adult genistein intake almost completely eliminated local recurrences. However, animals that received genistein only during tamoxifen treatment were at an increased risk of recurrence. Because no changes in tumor ERα or PgR levels were seen in any genistein group, the differences cannot be caused by alterations in hormone receptor expression. Rather, we found reductions in tumor UPR and autophagy signaling and markers of tumor immunoevasion in the lifetime genistein intake group. Although translation of findings from animal models to humans should always be done with caution, our results suggest that it is beneficial to continue to consume soy foods after diagnosis to reduce tamoxifen resistance and breast cancer recurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Zhang, I.M. Cruz, M. Rosim, J. Riskin, L. Hilakivi-Clarke
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zhang, R. Clarke, L. Hilakivi-Clarke
Writing, review, and/or revision of the manuscript: X. Zhang, K.L. Cook, A. Warri, J. Riskin, W. Helferich, R. Clarke, L. Hilakivi-Clarke
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