Research Article

Protective Effects of Prepubertal Genistein Exposure on Mammary Tumorigenesis Are Dependent on BRCA1 Expression

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Abstract

This study investigated whether prepubertal dietary exposure to genistein reduces mammary tumorigenesis by upregulating Brca1 expression in mice. Heterozygous Brca1+/− mice and their wild-type (WT) littermates were fed control AIN93G diet or 500 ppm genistein–supplemented AIN93G diet from postnatal day (PND) 15 to PND30 and then switched to AIN93G diet. Prepubertal dietary exposure to genistein reduced 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary incidence (P = 0.029) and aggressiveness of the tumors (P < 0.001) in the WT mice and upregulated the expression of Brca1 in their mammary glands (P = 0.04). In contrast, prepubertal genistein diet neither significantly reduced mammary tumorigenesis nor tumor aggressivity nor increased Brca1 mRNA expression in the Brca1+/− mice. These results may be related to the opposing effects of prepubertal genistein diet on the expression of Rankl and CK5/CK18 ratio (marker of luminal epithelial cell differentiation) in the mammary gland and estrogen receptor (ER-α) and progesterone receptor (PgR) protein levels in the mammary tumor: these all were reduced in the WT mice or increased in Brca1+/− mice. Both the WT and Brca1+/− mice exhibited reduced levels of amphiregulin, CK5, and CK18, delayed ductal elongation and a reduction in terminal end bud number in the normal mammary gland, and reduced HER-2 protein levels in the mammary tumors; however, these effects were not sufficient to significantly reduce mammary tumorigenesis in Brca1+/− mice. Our results show that upregulation of Brca1 may be required for prepubertal dietary genistein exposure to reduce later mammary tumorigenesis, perhaps because in the absence of this upregulation, mice do not exhibit genistein-induced downregulation of ER-α, PgR, and Rankl. Cancer Prev Res; 4(9): 1436–48. ©2011 AACR.

Introduction

Soy consumption during childhood and adolescence has been consistently linked to a marked reduction in breast cancer risk in Asians, American Asians, and Caucasians (1–5). Genistein, a soy-derived isoflavone, is thought to play a key role in the cancer-preventive activity of pubertal soy intake. In agreement with this view, pubertal genistein exposure reduces mammary cancer risk in rodent models (6). The protective effect of soy/genistein may be limited to puberty, as an adult exposure does not alter the risk in mice or rats (6). Consumption of soy limited to adult life may not affect breast cancer risk in humans either (7).

The mechanisms mediating the protective effect of pubertal genistein exposure on mammary tumorigenesis remain to be established. Genistein is a weak estrogen receptor α (ER-α) agonist, but it binds relatively strongly to ER-β (8). However, with the exception of very low doses, genistein activates both estrogen receptors equally, similarly to estradiol (9). Previous studies indicate that genistein modifies the expression of ER-α in vitro and in vivo (10–13) and increases progesterone receptor (PgR) in human breast tumors (14), human breast cancer cells in a mouse model (15), and normal mammary tissue in animal models (16). Genistein also targets many other signaling pathways including tyrosine kinases ErbB2/HER-2 (17). It has been shown that genistein downregulates HER-2 in human breast cancer cells (18), and following pubertal genistein exposure, in rat mammary tumors (19). Pubertal genistein exposure also affects mammary gland morphology. It reduces the number of targets for malignant transformation (terminal end buds, TEB), and this is proposed to explain the cancer-protective effects of genistein (6).

Because genistein targets several biological pathways, it is not clear which one of them may contribute to its breast cancer risk–reducing effects. Our earlier study showed that prepubertal genistein exposure, similarly to prepubertal estradiol exposure, upregulates the tumor suppressor gene...
BRCA1 (breast cancer susceptibility gene 1; ref. 12). Others have reported that genistein upregulates BRCA1 in vitro in human breast and prostate cancer cells (20) and in vivo in the mammary gland of ovariectomized rats (21). BRCA1 expression in the mammary gland is also elevated during pregnancy when estrogen and progesterone levels are high (22) and is reduced by ovariectomy (23). BRCA1 physically interacts with ER-α and PgR to inhibit their transcriptional activity (24). The link between BRCA1 and these steroid receptors as well as the role of BRCA1 in inducing differentiation of stem/progenitor cells to luminal cells (25) may explain why this tumor suppressor reduces breast cancer risk.

To further explore the role of BRCA1 as a mechanism mediating the effects of prepubertal genistein exposure on the mammary gland, we used Brca1<sup>+/−</sup> mice. BRCA1<sup>+/−</sup> mutations are the main known cause for familial breast cancer (26), and women who have inherited a germline BRCA1 mutation have a 66% to 85% risk of developing breast cancer by the age of 70 years (27). The BRCA1-induced changes were also investigated, focusing on ER-α and PgR and their target genes amphiregulin and receptor activator of NF-kB ligand (Rankl), respectively. Amphiregulin is an epithelial growth factor receptor (EGFR) ligand (28, 29). It is transcriptionally regulated by ER-α (30) and BRCA1 (31), and it mediates the proliferative actions of estradiol by activating the EGFR (30). Amphiregulin is required for mammary gland ductal morphogenesis at puberty (28), and its absence prevents duct-limited mammary progenitor cell proliferation but has no effect on lobule-limited mammary progenitor cells (32). It is not known whether genistein alters amphiregulin expression. RANKL is a member of the TNF family and mediates progesterone-induced mammary cell proliferation downstream of PgR (33). Genistein downregulates RANKL in the bone (34) and prostate cancer cells (35), but the effects on the mammary gland have not been explored.

We found that prepubertal dietary genistein exposure reduced mammary tumor incidence in the WT mice but failed to do so in the Brca1<sup>+/−</sup> mice. In contrast to WT mice, prepubertal genistein diet did not increase Brca1 or reduce ER-α, PgR, or Rankl expression in the mammary gland or tumor in these mice. However, prepubertal genistein exposure elevated CK5/CK18 ratio in the mammary gland of the Brca1<sup>+/−</sup> mice, indicative of reduced luminal differentiation (36). Our results show that upregulation of Brca1 may be required for prepubertal dietary genistein exposure to reduce later mammary tumorigenesis.

Materials and Methods

Animals

Brca1<sup>b007</sup>C1d1 heterozygous (+/−) frozen mouse embryos at 129Sv/C57BL/6 background were obtained from the National Cancer Institute mouse repository (Bethesda, MD). Frozen embryos were implanted in ICR female mice and a colony was established at the Georgetown University’s Animal Facility. Mice were fed a semipurified AIN93G (the American Institute of Nutrition) diet upon arrival. Animals were housed in a temperature- and humidity-controlled room under a 12-hour light–dark cycle. All animal procedures were approved by the Georgetown University Animal Care and Use Committee, and the experiments were carried out following the NIH guidelines for the proper and humane use of animals in biomedical research.

Genotyping

Wild-type (WT) and heterozygous Brca1 (+/−) mice were genotyped using DNA extracted from the tail clips using the NaOH method. PCR amplification was carried out using forward and reverse primers to obtain a 450- and 550-bp bands for wild-type and knockout alleles, respectively. Primer sequences were: forward primer (Brca1 004): 5′-CTGGTAGTTTGGTAAAGCATGC-3′ and reverse primer (Brca1 005): 5′-CAATAACTGCTTCTGCGAGG-3′ and (Brca1 007): 5′-ATCGCCCTCCTATCGCGCTGAGTTC-3′. After PCR amplification, products were analyzed by agarose gel electrophoresis. The B004/B005 primer combination generates a 450-bp product that corresponds to the wild-type Brca1, whereas the B004/B007 primer combination generates a 550-bp product that corresponds to the Neo cassette.

Prepubertal dietary exposures

On postnatal day 15 (PND15), WT and Brca1<sup>+/−</sup> female mice were divided into 2 dietary groups (n = 30 mice per genotype per group): AIN93G diet (control) or genistein (500 ppm)-supplemented AIN93G diet obtained from Harlan-Teklad. The mice were kept on these diets until PND30, and all mice were then switched to the non-supplemented AIN93G diet.

Effects on mammary tumorigenesis

Brca1<sup>+/−</sup> mice do not develop spontaneous mammary tumors, and therefore, mammary tumors were induced by administration of a subcutaneous injection of 15 mg in 100 μL of medroxyprogesterone acetate (MPA; DepoProvera, Pfizer) to mice at 6 weeks of age, followed by administration of 1 mg 7,12-dimethylbenz(a)anthracene (DMBA; Sigma) weekly for 4 weeks (weeks 7–10). The carcinogen was dissolved in corn oil and administered by oral gavage in a volume of 0.1 mL. This study included 22 WT mice fed the control diet, 21 WT mice fed prepubertally genistein diet, 25 Brca1<sup>+/−</sup> mice fed the control diet, and 26 Brca1<sup>+/−</sup> mice fed genistein diet. Animals were examined for mammary tumors by palpation once per week. The endpoints for data analysis were (i) latency to tumor appearance, (ii) the number of animals with tumors (tumor incidence), and (iii) the number of tumors per animal (tumor multiplicity). During the follow-up, animals in which tumor burden approximated 10% of total body weight were euthanized, as required by the ethical guidelines of our institution. All surviving animals were sacrificed 20 weeks after the last dose of DMBA administration.
Hypermorphic alveolar nodules

Whole mounts of the mammary glands were obtained from mice 20 weeks after DMBA exposure, processed as described below. Whole mounts were evaluated under the microscope to determine the number of hypermorphic alveolar nodules (HAN) in each gland. Whole mounts of the fourth abdominal mammary glands were obtained from mice 20 weeks after DMBA exposure and processed as previously described (37). Whole mounts were evaluated under the microscope to determine the number of HANs in each gland.

Histopathologic evaluation

Mammary tumors were classified according to its histopathology by a veterinarian pathologist, through ARUP veterinarian services.

Effects on mammary gland morphology and cytokeratin (CK) 5 and CK18 mRNA expression

To assess changes in mammary gland morphology, whole mounts of the fourth abdominal glands obtained on PND50. The total number of TEBs was counted blindly by 2 independent investigators using an Olympus dissecting microscope. Identification of TEBs was based on the guidelines established by Russo and Russo (38). Ductal elongation (epithelial outgrowth) was measured as the growth, in centimeters, from the end of the lymph node to the end of the epithelial tree.

We also determined whether the mRNA expression of CK5 (committed progenitor cells) or CK18 (luminal cells; ref. 36) or CK5/CK18 ratio were different between the WT and Brca1<sup>−/−</sup> mice and whether prepubertal genistein exposure affected the expression of these cytokeratins. The mRNA expression was determined as described below under the section Mammary gland amphiregulin, Rankl, CK5, and CK18 mRNA levels.

Mammary gland and tumor ER-α and HER-2 protein levels

Using immunohistochemistry, PgR protein expression was assessed in mammary glands and tumors collected at the end of the follow-up period. Briefly, tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned (5 μm). Sections were deparaffinized in xylene, hydrated through graded alcohols, and incubated with H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidases. Antigen retrieval was carried out in a Target Retrieval solution (pH = 9; Dako S2368) in a pressure cooker for 20 minutes followed by 2 hours of cool down. Tissue sections were incubated overnight with the primary antibody against PgR at a 1:400 dilution (polyclonal rabbit anti-human PgR; A0908, Dako). After several washes, sections were treated with secondary antibody (anti-rabbit) and developed using the Dako EnVision+ Dual Link System-HRP, DAB<sup>+</sup> (K4065) as instructed by the manufacturer. The PgR status of each sample was evaluated according to a modified version of the scoring system proposed by Allred and colleagues (39). The total score for each sample was calculated as the sum of the estimated proportion of positive-staining cells (0–7) plus the estimated intensity of positive-staining cells (0–4).

Mammary gland amphiregulin, Rankl, CK5, and CK18 mRNA levels

Frozen tissue samples were weighed and homogenized using a PowerGen 35 handheld homogenizer (Fisher Scientific) with RNase-free disposable OMNI-Tips (Fisher Scientific). RNA extraction was then carried out according to the Qiagen's RNeasy Lipid Tissue Kit (Qiagen Inc.) instructions. The quantity and quality of RNA were measured by comparing the optical density ratios (OD<sub>260</sub>/OD<sub>280</sub>) obtained using a Nanodrop (ND-1000) Spectrophotometer (Thermo Scientific). RNA samples were stored at −80°C until use.

cDNA synthesis and quantitative real-time PCR analysis

A total of 200 ng of total RNA per sample was used as a template for random primed cDNA synthesis with a recombinant Moloney murine leukemia virus reverse transcriptase (TaqMan MultiScribe Reverse Transcriptase and RT-PCR Reagents, Applied Biosystems), according to manufacturer’s instructions. A reverse transcriptase enzyme minus control reaction was also included. The cDNA samples were then used as templates for quantitative real-time PCR (qRT-PCR) analysis with previously described specific primers for the target genes Brca1 (40), amphiregulin (30), Rankl (41), and CK5 and CK18 (42) using QuantiTect SYBR green PCR kit (Qiagen Inc.) and an ABI Prism 7900 Sequence Detection System. Each sample was run in triplicate, and qPCR run was repeated 1 to 2 times. Absolute gene expression levels were determined using SDS2.3 software from Applied Biosystems and the standard curve method. Concentration of each sample was normalized to the reference gene 18S rRNA (42).
Statistical analyses
Data obtained on (i) mammary gland morphology (total number of TEBs and ductal elongation), (ii) mRNA levels, (iii) mammary gland and tumor HER-2 and ER-α protein levels, (iv) PgR staining, and (v) number of HANs were analyzed by 2-way ANOVA using genotype (WT or knockout) and treatment (control or genistein) as variables. Kaplan–Meier curves were used to compare differences in tumor incidence, followed by the log-rank test. Normalized qRT-PCR results were analyzed with Kruskal–Wallis 1-way ANOVA on Ranks and 1-way ANOVA with Holm–Sidak method for pairwise multiple comparison procedures. All tests were carried out using the SPSS SigmaStat Software, and differences were considered significant if the value of P was less than 0.05. All probabilities are 2 tailed.

Results
Mammary tumorigenesis
Prepubertal genistein exposure significantly reduced mammary tumor incidence in the WT mice (P = 0.029; Fig. 1A). Among the Brca1+/– mice, however, the difference in mammary tumor incidence failed to reach statistical significance (P = 0.26; Fig. 1B). The number of HANs (Fig. 1C), tumor multiplicity (the total number of tumors per animal; ranged between 1.2 and 1.6 in the WT and Brca1+/– mice), or tumor latency (time to tumor appearance; ranged between 10.1 and 11.9 weeks) were not affected by prepubertal genistein diet in either the WT or Brca1+/– mice (Table 1).

There was no evidence that the incidence of mammary tumors was higher in the Brca1+/– mice; in fact, a slightly
In the WT mice, the incidence of high-grade malignant tumors was similar in the WT and genistein-containing diet before puberty. The malignant tumors consisted of adenocarcinomas and other carcinomas. These malignant carcinomas were divided into low- and high-grade tumors, and the ratio of low- to high-grade tumors was similar in the WT and Brca1+/− mice (Table 2). In the WT mice, the incidence of high-grade malignant tumors dropped from 60% in the control diet group to 20% in the genistein group (P < 0.001) but only from 67% to 50% in the Brca1+/− mice (nonsignificant). The tumor mitotic index, another indicator of tumor growth and aggressiveness, was reduced by prepubertal exposure to genistein in the WT mice (P < 0.011), but genistein had no effect on the Brca1+/− mice (P = 0.93; Table 2).

### Mammary gland Brca1 mRNA levels

The expression levels of Brca1 mRNA in the mammary gland were determined on PND50 (Fig. 1C). As expected, qRT-PCR showed lower levels of Brca1 mRNA in Brca1+/− mice compared with WT mice (P < 0.001), regardless of the diet. Higher levels of Brca1 mRNA were observed in the mammary glands of WT mice fed genistein-containing diet compared with those fed AIN93G control diet (P = 0.04). No effects by genistein diet on Brca1 levels were observed in Brca1+/− mice (Fig. 2).

### Mammary gland morphology

Mammary gland morphology and the expression of CK5 and CK18 were assessed at PND50. CK5 levels were significantly higher in the Brca1+/− mice fed the control diet than in the WT controls (P < 0.016; Fig. 2A), but CK18 levels were not altered (Fig. 2B). Regardless of the genotype, the mice fed genistein during prepuberty exhibited significantly reduced levels of both cytokeratines compared with the mice fed the control diet (CK5: P < 0.002; CK18: P < 0.003). The ratio between CK5 and CK18 was higher in the Brca1+/− mice than in the WT mice (P < 0.022). The difference was particularly apparent in mice fed genistein during prepuberty because genistein reduced it in the WT mice and significantly increased the ratio in the Brca1+/− mice (P < 0.04; Pinteraction = 0.079; Fig. 2C). The reductions in both CK5 and CK18 by genistein reflected changes in the mammary gland morphology. As seen in Figure 3A, mice fed genistein during prepuberty exhibited significantly smaller mammary epithelium than mice fed the control diet. No significant differences in the number of TEBs or ductal elongation were seen between the WT and Brca1+/− mice, when determined at PND50. However, prepubertal genistein exposure reduced the

### Table 1. Mammary tumor multiplicity and latency in DMBA-exposed WT and Brca1+/− mice fed a control or genistein-supplemented AIN93G diet during prepuberty

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of tumors</th>
<th>Tumor multiplicity (number of tumors/tumor-bearing mice)</th>
<th>Tumor latency, wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT control</td>
<td>10</td>
<td>1.2 ± 0.4</td>
<td>10.1 ± 0.7</td>
</tr>
<tr>
<td>WT genistein</td>
<td>5</td>
<td>1.6 ± 0.3</td>
<td>11.6 ± 1.2</td>
</tr>
<tr>
<td>Brca1+/− control</td>
<td>12</td>
<td>1.2 ± 0.3</td>
<td>11.9 ± 1.0</td>
</tr>
<tr>
<td>Brca1+/− genistein</td>
<td>10</td>
<td>1.2 ± 0.2</td>
<td>10.8 ± 1.1</td>
</tr>
</tbody>
</table>

**NOTE:** Mean ± SEM are shown.

The lower number of these mice (14 of 25, 56%) developed mammary tumors compared with WT mice (15 of 22, 68%). The number of premalignant lesions, that is, HANs, was significantly higher in the Brca1+/− mice than in the WT mice (P = 0.047; Fig. 1C).

Histopathologic analysis indicated that most mammary tumors were malignant carcinomas (Table 2) in all 4 groups. The incidence of benign adenomas was 10% and 8% in the WT and Brca1+/− mice fed the control diet, respectively, and 20% in both groups which were fed genistein-containing diet before puberty. The malignant tumors consisted of adenocarcinomas and other carcinomas. These malignant carcinomas were divided into low- and high-grade tumors, and the ratio of low- to high-grade tumors was similar in the WT and Brca1+/− mice (Table 2). In the WT mice, the incidence of high-grade malignant tumors dropped from 60% in the control diet group to 20% in the genistein group (P < 0.001) but only from 67% to 50% in the Brca1+/− mice (nonsignificant). The tumor mitotic index, another indicator of tumor growth and aggressiveness, was reduced by prepubertal exposure to genistein in the WT mice (P < 0.011), but genistein had no effect on the Brca1+/− mice (P = 0.93; Table 2).

### Table 2. Mammary tumor histopathology in DMBA-exposed WT and Brca1+/− mice fed a control or genistein-supplemented AIN93G diet during prepuberty

<table>
<thead>
<tr>
<th>Group</th>
<th>Benign</th>
<th>Malignant</th>
<th>Tumor grade Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenoma (10%)</td>
<td>Adeno carcinoma (60%)</td>
<td>Low (33%)</td>
</tr>
<tr>
<td>WT control</td>
<td>1</td>
<td>6</td>
<td>3 (33%)</td>
</tr>
<tr>
<td>WT genistein</td>
<td>1</td>
<td>1</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Brca1+/− control</td>
<td>1 (8%)</td>
<td>9</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>Brca1+/− genistein</td>
<td>2 (20%)</td>
<td>4</td>
<td>3 (37%)</td>
</tr>
</tbody>
</table>

**NOTE:** Values within a column which are marked with a different letter are significantly different from each other, P < 0.05.
number of TEBs ($P < 0.001$) and inhibited ductal elongation ($P = 0.007$) in both the WT and $Brca1^{+/–}$ mice (Fig. 3B).

**Mammary gland and mammary tumor protein expression levels of ER-α, ER-β, PgR, and HER-2, and their downstream targets amphiregulin and RANKL**

Because genistein acts by binding to ER-α and ER-β (8, 9) and upregulation of PgR is indicative of ER-α activation, we determined the expression of these receptors. We also tested pathways downstream of ER-α and PgR, that is, the expression of amphiregulin and RANKL. In addition, genistein is a tyrosine kinase inhibitor (17) and therefore HER-2 levels were assessed. ER-α and HER-2 protein levels were detected by Western blotting; ER-β, amphiregulin, and RANKL mRNA levels by RT-qPCR; and PgR by qRT-PCR and immunohistochemistry.

**Changes in mammary glands**

The assays revealed that mammary glands and tumors were positive for ER-α, ER-β, PgR, and HER-2 expression. Protein levels of ER-α (Fig. 4A, mammary gland) and HER-2
(Fig. 4B) in mammary gland tissues were not different between the genotypes or dietary groups. PgR protein levels were higher in the mammary glands of Brca1+/− mice than in the WT mice (P < 0.033; Fig. 4C). Genistein decreased the PgR protein levels in the WT mice (P < 0.030). ER-β protein levels were not different among the groups (data not shown).

**Changes in mammary tumors**

Assessment of expression of the different receptors in the mammary tumors revealed that ER-α protein levels were not different in the mammary tumors between WT and Brca1+/− mice fed the control diet. The expression of ER-α was reduced in the tumors by prepubertal genistein...
exposure in the WT but not in the Brca1<sup>+/−</sup> mice (<i>P</i> < 0.04; Fig. 5A, mammary tumor). In contrast to normal mammary gland tissue, the levels of PgR were significantly lower in the mammary tumors of the Brca1<sup>+/−</sup> mice than in the WT mice (<i>P</i> = 0.007; Fig. 5C). Furthermore, although the WT mice exposed to genistein at prepuberty exhibited a significant reduction in PgR expression, no reduction was seen in the mammary tumors of the Brca1<sup>+/−</sup> mice (<i>P</i> < 0.034).

Mammary tumors of Brca1<sup>+/−</sup> mice expressed higher levels of HER-2 than the tumors in the WT mice, regardless of diet (<i>P</i> < 0.03; Fig. 5B). Prepubertal exposure to genistein diet decreased the expression levels of HER-2 in mammary tumors of WT and Brca1<sup>+/−</sup> mice compared with their respective controls (<i>P</i> < 0.002).

**Amphiregulin and Rankl expression in the mammary gland**

Although mammary gland ER-α levels were similar in the WT and Brca1<sup>+/−</sup> mice, amphiregulin mRNA levels were lower in the mammary glands of Brca1<sup>+/−</sup> mice than WT mice (<i>P</i> < 0.011; Fig. 6A). Prepubertal genistein exposure...
significantly reduced the levels in both groups ($P < 0.001$), and consequently, no differences in amphiregulin levels between the two genotypes were seen.

Downstream target of PgR, Rankl, was significantly higher in the control diet–fed WT mice than in the Brca1$^{+/−}$/C0 mice on PND50 ($P < 0.018$), but the difference was lost in mice fed genistein during prepuberty (Fig. 6B). This is because among the WT mice genistein reduced Rankl, but among the Brca1$^{+/−}$/C0 mice, genistein exposure increased Rankl mRNA ($P_{\text{interaction}} < 0.026$).

Discussion

Effect of prepubertal genistein diet on mammary tumorigenesis

We found that in the WT mice, prepubertal dietary exposure to 500 ppm genistein significantly reduced carcinogen-induced mammary tumor incidence, in accordance with data previously reported in rats (6). Because the dose we used generates blood genistein levels comparable to those seen in the Asians (43), our study adds to the convincing evidence obtained in human studies that childhood soy intake protects against the development of breast cancer later in life (1–5).

However, no significant reduction in mammary tumorigenesis was seen in the Brca1$^{+/−}$/mice fed the genistein diet before puberty onset. Furthermore, in contrast to the WT mice, these mice did not exhibit an increase in Brca1 expression, possibly because the remaining Brca1 allele was already maximally expressed in the heterozygous Brca1$^{+/−}$/mice. The increase in Brca1 expression in the WT mice is in agreement with the earlier findings obtained in both in vitro and in vivo studies indicating that genistein upregulates BRCA1.
(12, 20, 21). Our results thus suggest that the genistein-induced upregulation of Brca1 may be required for prepubertal genistein exposure to reduce breast cancer risk.

**Mammary tumorigenesis in the Brca1+/− mice: role of PgR signaling**

Palpable mammary tumor incidence was not different between the genotypes, although Brca1+/− mice developed more premalignant HAN lesions than WT mice did. These findings suggest that the Brca1+/− mice have a higher potential to develop mammary tumors than the WT mice do but most of these HANs do not progress to malignancy in the Brca1+/− mice. Because Brca1+/− mice expressed higher levels of PgR than those of the WT mice; a finding which is consistent with the data by Poole and colleagues (44) in conditional Brca1+/−/p53−/− mice, these genetically modified mouse models should be at least as sensitive as the wild-type mice to the MPA exposure, which is required in both wild-type and various genetically modified mouse models for DMBA to induce mammary tumors (45). This was confirmed by determining that MPA similarly increased Rankl expression and TEB number in the WT and Brca1+/− mice (data not shown).

**Differences in the response to prepubertal genistein diet between the genotypes**

Prepubertal genistein diet affected some endpoints in the mammary glands and tumors differentially, and some similarly in the WT and Brca1+/− mice. This diet had an opposite effect on the following endpoints in the 2 genotypes: Rankl expression and CK5/CK18 ratio in the mammary gland, PgR expression in the mammary gland and tumor, ER-α expression in the tumor, and histopathology of the tumor. In the WT mice, prepubertal genistein exposure reduced the expression of Rankl in the mammary gland and PgR both in the mammary gland and tumor. The effect on the PgR is in agreement with the study by Pei and colleagues (46) showing that prepubertal genistein exposure reduced PgR expression in the (rat) mammary gland. Thus, consumption of genistein during prepuberty may induce persistent downregulation of PgR. Downregulation of Rankl by genistein is consistent with previous data in bone and prostate (34, 35). Because reduced expression of PgR and Rankl may be associated with a reduction in the mammary progenitor cell proliferation (47), one of the mechanisms by which prepubertal genistein exposure reduces breast cancer risk may be through increased mammary luminal cell differentiation. Changes in these three endpoints could have occurred through upregulation of BRCA1, which is known to suppress progesterone signaling (24) and induce luminal-specific differentiation of mammary progenitor cells (25).

Prepubertal genistein diet affected Rankl and CK5/CK18 ratio differently in the Brca1+/− mice. Genistein-treated Brca1+/− mice expressed more Rankl and higher CK5/CK18 ratio than the control diet–fed mice. Progenitor cells, including committed myoepithelial and some ductal luminal progenitor cells, express CK5, and the expression is lost when the cells differentiate to mature myoepithelial and luminal cells (36). Because high CK5/CK18 ratio is indicative of increased presence of undifferentiated luminal...
cells (36) as is high \textit{Rankl} expression (47), prepubertal genistein exposure may have led to reduced luminal cell differentiation in the \textit{Brca1} \textminus \textit{C0} mice.

Prepubertal genistein diet reduced mammary tumor grade and tumor mitotic index, both of which are indicators of aggressiveness of a tumor, in the wild-type but not \textit{Brca1} \textplus \textit{C0} mice. In addition, tumors in the prepubertally \textit{Brca1}+-fed wild-type mice expressed significantly lower levels of the ER-\(\alpha\), PgR, and HER-2 than tumors in the control diet–fed wild-type mice. Again, mammary tumors in \textit{Brca1} \textplus \textit{C0} mice failed to show similar changes, with the exception of downregulation of HER-2 in the \textit{Brca1}+-fed mice. These findings indicate that genistein failed to reduce aggressiveness of the mammary tumors in the mice lacking 1 \textit{Brca1} allele.

**Similarities in the response to prepubertal genistein diet in the two genotypes**

Genistein had some effects on the \textit{Brca1} \textplus \textit{C0} mice which were similar to those seen in the WT mice; that is, it reduced the number of targets for malignant transformation (TEB), inhibited ductal elongation, downregulated amphiregulin expression in the mammary gland, and reduced HER-2 expression in the tumors. Some of these changes have been previously reported to occur by genistein (6, 17, 48), but we are not aware of any studies showing that genistein delays ductal elongation or inhibits amphiregulin expression.

Activation of ER-\(\alpha\) increases amphiregulin expression which then binds to the EGFR/ErbB1/HER-1 and induces cell proliferation (30). ErbB2/HER-2 does not have any known ligands, but its activity and possibly expression can be modified through heterodimerization with amphiregulin-bound EGFR (49, 50). Amphiregulin expression was lower in the control diet–fed \textit{Brca1} \textplus \textit{C0} mice than in the WT controls, suggesting that ER-\(\alpha\) signaling may be reduced in the \textit{Brca1} \textplus \textit{C0} mice, although no differences in the ER-\(\alpha\) expression in the normal mammary gland were seen between the genotypes. Our results also indicate that both in the \textit{Brca1} \textplus \textit{C0} and WT mice, prepubertal genistein diet induced a persistent reduction in mammary amphiregulin levels. Lamber and colleagues have reported that BRCA1 suppresses amphiregulin (31), and therefore, the reduction in the WT mice may be due to genistein-induced upregulation of Brca1. However, because amphiregulin was reduced also in the genistein-exposed \textit{Brca1} \textplus \textit{C0} mice, but genistein failed to upregulate their Brca1 expression, other mechanisms than genistein-induced upregulation of Brca1 suppressed their amphiregulin expression.

Amphiregulin was recently found to mediate self-renewal of stem/progenitor cells in the mammary duct (32); its inhibition by siRNA reduced mammosphere formation by preventing the expansion of ductal progenitor cells. Reduction in amphiregulin expression by prepubertal genistein diet could thus explain the significant delay in ductal elongation observed here in the postpubertal mammary gland in both WT and \textit{Brca1} \textplus \textit{C0} mice. Because amphiregulin also is expressed in the TEBs (51), genistein-induced reduction in TEB number could perhaps be explained by a reduction in amphiregulin expression. Mammary gland morphology on PND50 in the control diet–fed WT and \textit{Brca1} \textplus \textit{C0} mice was similar; that is, loss of one Brca1 allele did not affect gross mammary gland morphology. In humans, mammographic density also is similar in germ line \textit{BRCA1} mutation carriers and controls (52, 53). Animal studies have generated conflicting data and reported that conditional or mammary-specific \textit{BRCA1} mutations impair mammary ductal development (16, 54–56) or induce ductal elongation (57).

**Conclusions**

In this study, we showed that a prepubertal dietary exposure to genistein in mice reduces the risk of developing mammary tumors later in life and upregulates \textit{Brca1} mRNA expression in an adult mammary gland. We also showed that the protective effect was not seen in mice with a germ line \textit{Brca1} mutation and neither was the remaining \textit{Brca1} allele upregulated by genistein in these mice. WT mice fed genistein before puberty onset exhibited reduced expression of PgR and \textit{Rankl}; changes in the opposite direction were seen in the \textit{Brca1} \textplus \textit{C0} mice, and they also exhibited increased CK5/CK18 ratio. These findings are indicative of reduced progenitor cell proliferation and increased luminal differentiation in the genistein-exposed WT mice but reduced differentiation in the genistein-exposed \textit{Brca1} \textplus \textit{C0} mice (36, 47).

Genistein downregulated amphiregulin expression and inhibited ductal elongation and reduced TEBs in the mammary glands of both the WT and \textit{Brca1} \textplus \textit{C0} mice and also reduced the expression of HER-2 in the mammary tumors, suggesting that prepubertal dietary genistein exposure induces some breast cancer risk–protective effects in the mammary gland, regardless of the level of \textit{Brca1} expression. However, these effects do not offer full protection to \textit{Brca1} \textplus \textit{C0} mice, indicating that upregulation of \textit{Brca1} is required for prepubertal genistein to prevent cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

The authors thank Salim Shah for advising in establishing \textit{Brca1} \textplus \textit{C0} mouse colony. LCCC Animal Shared Resource for help in feeding mice and in tumorigenesis experiments, and Histopathology Shared Resource for tissue processing.

**Grant Support**

The work was supported by the National Cancer Institute (U54 CA100970) and American Cancer Society (postdoctoral fellowship 116602-PF-09-018-01-CNE).

Received November 23, 2010; revised April 20, 2011; accepted May 23, 2011; published OnlineFirst June 16, 2011.
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Genistein, BRCA1, and Breast Cancer
doi: 10.1158/1940-6207.CAPR-10-0346


Cancer Prevention Research

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