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ORIGINAL PAPER

Genistein affects *HER2* protein concentration, activation, and promoter regulation in BT-474 human breast cancer cells

Mary S. Sakla · Nader S. Shenouda · Pete J. Ansell · Ruth S. MacDonald · Dennis B. Lubahn

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Abstract The *HER2* proto-oncogene, a member of the epidermal growth factor receptor family, is overexpressed in 20–30% of breast cancers. Genistein, the main soy isoflavone, interacts with estrogen receptors (ER) and it is also a potent tyrosine kinase inhibitor. Previously, our laboratory found that genistein delayed mammary tumor onset in transgenic mice that overexpress *HER2* gene. Our goal was to define the mechanism through which genistein affects mammary tumorigenesis in *HER2* overexpressing mice. We hypothesized that genistein inhibits *HER2* activation and expression through ER-dependent and ER-independent mechanisms. Genistein inhibited total *HER2* protein expression and tyrosine phosphorylation in BT-474, an ERα (–) and ERβ (+) human breast cancer cell line, however, E2 had no effect. Taken together, these data suggest that genistein has an ER-independent inhibitory effect, presumably, through tyrosine kinase inhibition activity. Genistein at 1.0 μM mimicked E2 and down-regulated *HER2* protein phosphorylation when BT-474 was co-transfected with ERα, but not ERβ. Although E2 and overexpression of *HER2* can promote mammary tumorigenesis, an inverse relationship between ER expression and *HER2* overexpression has been found in human breast cancer. We cloned a 500-bp promoter region upstream of the *HER2* transcription initiation site. Co-transfection with ERα, but not with ERβ, down-regulated *HER2* promoter reporter in BT-474. At concentrations ≥1 μM, genistein inhibited *HER2* promoter reporter in the absence of ERα. In conclusion, genistein at ≥1 μM inhibited *HER2* protein expression, phosphorylation, and promoter activity through an ER-independent mechanism. In the presence of ERα, genistein mimicked E2 and inhibited *HER2* protein phosphorylation. These data support genistein's chemoprevention and potential chemo-therapeutic roles in breast cancer.

Keywords Genistein · *HER2* · ER · Breast cancer

M. S. Sakla · D. B. Lubahn
Genetics Area Program, University of Missouri, Columbia, MO, USA

M. S. Sakla · N. S. Shenouda · D. B. Lubahn
Department of Biochemistry, University of Missouri, 920 E campus Dr., ASRC 110A, Columbia, MO, USA

N. S. Shenouda · D. B. Lubahn
Missouri University Center for Phytonutrient and Phytochemical Studies (MUCPPS), University of Missouri, Columbia, MO, USA

P. J. Ansell
Abbott Laboratories, Abbott Park, IL, USA

R. S. MacDonald (✉)
Department of Food Science and Human Nutrition, Iowa State University, 2312 Food Science Building, Ames, IA, USA
e-mail: ruthmacd@iastate.edu

D. B. Lubahn
Department of Child Health, University of Missouri, Columbia, MO, USA

D. B. Lubahn
Department of Animal Sciences, University of Missouri, Columbia, MO, USA

Introduction

Breast cancer is the most common cancer and is the second leading cause of cancer death among women in the United States. There are two important receptors whose expression are used as predictive factors in breast cancer: estrogen receptor alpha (ERα) and *HER2* (*ErbB2*, *neu*) [1]. The

American Society of Clinical Oncology has recommended the assessment of *HER2* in all breast tumors, either at the time of diagnosis or recurrence [2].

HER2 is the second member of the Human Epidermal Growth Factor Receptor Family (EGFR) which is composed of 4 members: *HER1*, *HER2*, *HER3*, and *HER4*. The *HER2* receptor gene maps to chromosome 17q21 and is a 1,255 amino acid, 185-kDa trans-membrane glycoprotein [3]. All four *HER* receptors are composed of an extracellular ligand-binding domain, transmembrane lipophillic domain, and an intracellular domain with tyrosine kinase catalytic activity [3]. Although *HER2* does not have known ligands, *HER2* protein has an important role in normal cell growth and differentiation and its overexpression is linked to the development of many human cancers [4].

HER2 receptor is overexpressed in 20–30% of primary breast cancer cases, and its presence can impede the antiproliferative effect of hormonal therapy [5]. Moreover, a high level of *HER2* expression is associated with lymph node-positive, negative hormone receptor status, and high proliferative activity [4]. Because *HER2* has an important role in tumorigenesis, it has been investigated as a target for cancer therapy. One of these approaches is the use of tyrosine kinase inhibitors [6]. It has been found that blockade of EGFR function with the EGFR-specific tyrosine kinase inhibitor ZD1839 (Iressa) inhibits phosphorylation of the *HER2* receptor and growth of *HER2* overexpressing breast carcinoma cells. Moreover, a combined molecular approach using a combination of ZD1839 and the *HER2* antibody, herceptin, had more anti-tumor effectiveness than either approach alone [7]. Although both *HER2* and ligand-activated ERα are growth promoting [8], an inverse relationship was found between overexpression of *HER2* and expression of ERα in human breast cancer specimens [9]. Natural alternatives, like phytoestrogens, that target tumorigenesis at multiple molecular sites such as *HER2* and ER may be effective in breast cancer prevention and/or treatment.

The phytoestrogen genistein, the main isoflavone in soy, has been proposed to be the agent responsible for lowering the rate of breast cancer in Asian women [10]. Genistein has been proposed to work through many mechanisms [11, 12]. It can interact with both estrogen receptors (ERα and ERβ), with preference for ERβ, demonstrating both agonist and antagonist effects [13, 14]. Genistein is also a potent inhibitor of tyrosine kinase [15], and may reduce in vivo tyrosine kinase activity, specifically epidermal growth factor (EGF) receptor phosphorylation in rat prostate [16].

Several lines of evidence indicate that soy is protective in breast cancer. First, epidemiological studies have shown that the age-adjusted death rates from breast cancer are 2- to 8-fold lower in Asia compared to Western countries, suggesting that dietary factors play an important role in reducing breast cancer risk in Asian countries [17]. In addition, immigration studies showed that first generation Asian immigrants have low rates of breast cancer compared to the second and the subsequent generations [18]. Secondly, the average daily intake of soy in Asian culture is about 50 g per day compared to 1 g per day in Western culture [18]. Thirdly, many cell culture and experimental models provide evidence for genistein's role in preventing breast cancer (reviewed in [19]). Finally, in previous work, we observed a significant delay in mammary tumor development in genistein-treated transgenic mice expressing the activated *HER2/neu* oncogene under the control of mouse mammary tumor virus (MMTV) promoter [20].

The overall goal is to define the mechanism by which genistein affects *HER2* activation and gene expression. Our working hypothesis is that genistein regulates *HER2* through estrogen receptor-dependent and estrogen receptor-independent mechanisms.

Results

Characterization of BT-474 human breast cancer cell line

Previously, we have shown that genistein represses cancer development in MMTV/*neu* mice that overexpress *HER2* [20]. To further understand the mechanism of these results, we studied in vitro the effect of genistein on *HER2* expression, activation/phosphorylation, and promoter regulation. BT-474 human breast cancer cells have been chosen as our model because they overexpress *HER2* protein [21], which was confirmed by Western blot (Fig. 1A). We also characterized BT-474 for ER expression [22]. Although BT-474 cells have been reported as ERα-positive cells [23], others found weak ERα expression [24], a third group reported BT-474 to be an ER-negative cell line [25]. Our characterization confirmed that the BT-474 cells we used had minimal or negative ERα expression compared to the well-known ERα(+), *HER2*(+) ZR75.1 cells, and ER(+), *HER2* (–) MCF-7 cells (Fig. 1B). Characterization of ERβ expression revealed that BT-474 cells express similar amount of ERβ protein as MCF-7 cells (Fig. 1C).

Genistein, but not 17β-estradiol, inhibited BT-474 cell line growth

To further characterize the BT-474 cell line response to genistein treatment and to determine the IC_{50}, growth experiments were done using the thymidine incorporation assay. As a control experiment, cells were treated with 10 nM 17β-estradiol. Genistein demonstrated biphasic effect on BT-474 cells growth. At low doses <10 μM,

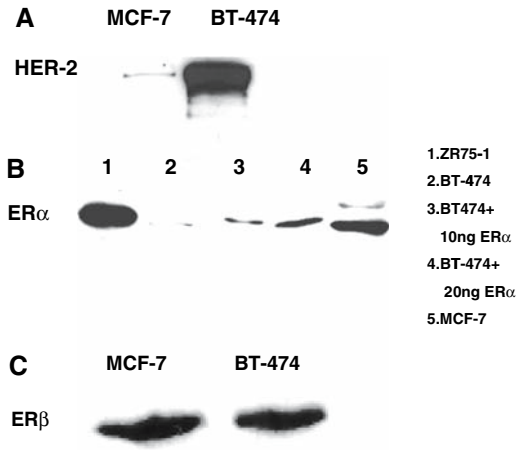

Fig. 1 Characterization of the BT-474 human breast cancer cell line by Western blot analysis of *HER2* (**A**), ERα (**B**), and ERβ (**C**) protein expression. Cell extracts were resolved by gel electrophoresis, electro-blotted onto nitrocellulose membrane, and immuno-detected. Each lane was loaded with 10 µg protein. (**A**) Extracts of MCF-7 and BT-474 cells. (**B**) Lane 1, ZR75-1 cell extract as *HER2* (+), ERα (+) positive control; lane 2, BT-474 cells extract; lane 3, BT-474 transfected with 10 ng ERα expression vector; lane 4, BT-474 cells transfected with 20 ng ERα expression; lane 5, MCF-7 cells as positive control. (**C**) Extracts of BT-474 cells and MCF-7 cells as positive control

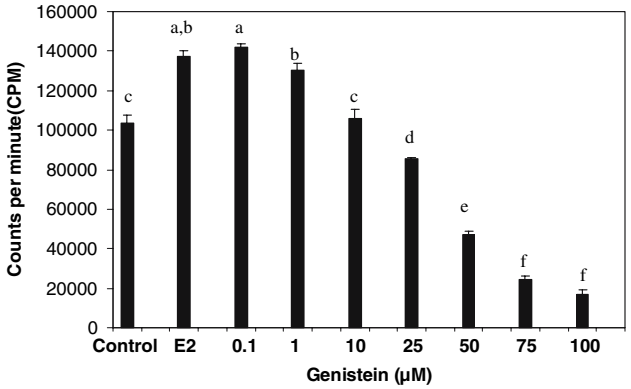

Fig. 2 Effect of genistein concentrations on BT-474 cells proliferation. Cells were plated at 10×10^4 cells per ml in 6-well dishes with complete culture medium. The next day, the medium was replaced with phenol red-free medium and charcoal-stripped serum containing the assigned treatment for 5 days. The media containing the treatment were replaced once after 2 days. 10 nM 17β-estradiol was used as a control. Cellular proliferation was determined by tritiated thymidine incorporation assay. Values are means ± SEM of two independent experiments each done in triplicate

genistein increased BT-474 cells growth, presumably as a part of its estrogenic effect. At higher doses, genistein inhibited BT-474 cells growth with 50 µM IC_{50} (Fig. 2). On the other hand, 10 nM 17β-estradiol stimulated BT-474 cells growth. These results were confirmed under the same experimental conditions using a total cellular protein assay as a method for determining cellular growth and proliferation [13] (data not shown).

Genistein, but not 17β-estradiol, inhibited *HER2* protein expression and phosphorylation/activation

The ability of genistein to regulate proliferation of the overexpressing *HER2*, BT-474 cells suggests that genistein could regulate *HER2* expression and/or activation. The effect of genistein on *HER2* protein activation/phosphorylation was determined in BT-474 cells. Treatment of BT-474 cells for 5 days with 0, 1.0, 10, and 25 µM genistein resulted in a significant inhibition of *HER2* protein expression (Fig. 3A, B) and phosphorylation/activation (Fig. 4A, B). However, treatment with 0, 0.01, 0.1, 1.0, or 10 nM 17β-estradiol for 5 days did not affect *HER2* protein expression (Fig. 3C, D) or phosphorylation/activation (Fig. 4C, D).

The binding affinity (Kd) for 17β-estradiol to ERα and ERβ is 0.13 nM and 0.12 nM, respectively, whereas, the Kd for genistein is 2.6 nM and 0.3 nM, respectively [26]. Although genistein binds to ERβ with higher affinity than to ERα, estradiol-binding affinity is more than two times higher to ERβ than genistein. Because genistein, but not E2 treatment, repressed *HER2* protein expression and activation in this ERα-negative cell line, we conclude that genistein's inhibitory action is likely through an ER-independent pathway, presumably through its ability to inhibit tyrosine kinase activity.

In summary, after 5 days of incubation (Figs. 3 and 4), genistein inhibited both total and phosphorylated *HER2* in BT474 cells. These results could be due to inhibition of *HER2* phosphorylation by genistein (via tyrosine kinase inhibition) which in turn resulted in inhibition of total *HER2* through inhibiting the MAPK pathway or inhibition of *HER2* total protein first (by an unknown mechanism) and as a result reduced phosphorylated *HER2*. In a short-term study with 20 h of incubation, phosphorylated *HER2* was inhibited by 1 µM genistein but total *HER2* protein concentration was not affected (data not shown). This supports that inhibition of *HER2* phosphorylation by genistein can occur independently of a decrease in total *HER2* protein concentration.

Role of ERα and ERβ in the effect of genistein and 17β-estradiol on HER2 protein phosphorylation/activation

To examine the role of ERα in mediating the inhibitory effect of genistein on *HER2* protein activation, phosphorylated *HER2* concentration was determined by Western

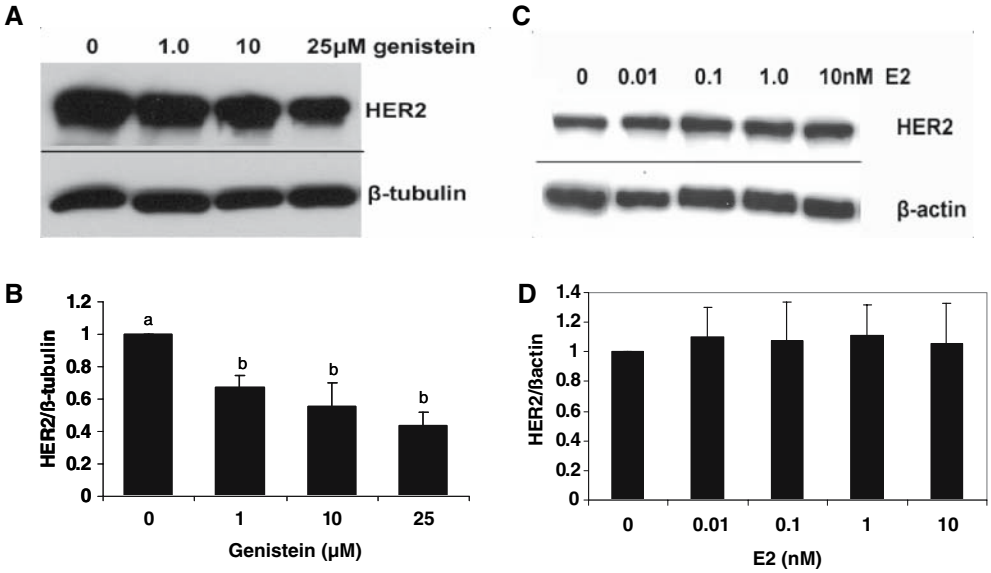

Fig. 3 Effect of genistein and 17β-estradiol on *HER2* protein expression in BT-474 cells. Cells were plated in phenol red-free and charcoal-stripped serum, treated the next day with 0, 1, 10, 25 μM genistein (**A**) or with 0, 0.01, 0.1, 1.0, 10 nM E2 (**C**) for 5 days. The medium with treatment was replaced every 3 days. In (**B**) and (**D**), individual band from (**A**), (**C**) respectively, were quantitated, and the ratio of *HER2* protein to the internal control protein. Normalized to no treatment group was done within the same experiment. Bars represent mean ± SEM for three independent experiments. Data were analyzed by one-way ANOVA. Means were considered significantly different at $P < 0.05$. Bars with same letters are not significantly different

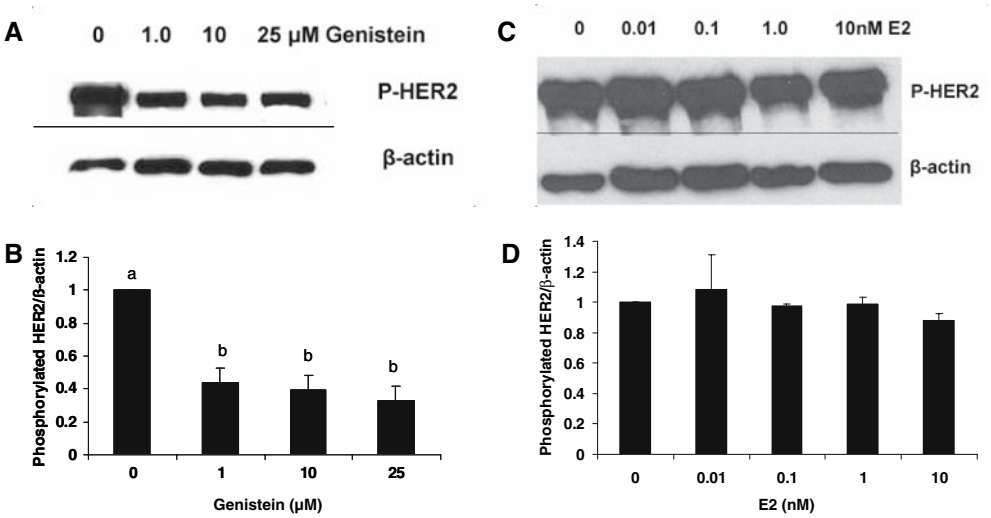

Fig. 4 Effect of 17β-estradiol and genistein on *HER2* protein activation/phosphorylation in BT-474 cells. Cells were plated in phenol red-free and charcoal-stripped medium, treated the next day with 0, 1, 10, or 25 μM genistein (**A**) or with 0, 0.01, 0.1, 1.0, or 10 nM E2 (**C**) for 5 days. The media with treatment were replaced every 3 days. In (**B**), and (**D**), individual band from (**A**), (**C**), respectively, were quantitated, and a ratio of *HER2* phosphorylated protein to the internal control protein. Normalized to no treatment group was done within the same experiment. Bars represent mean ± SEM for three independent experiments. Data were analyzed by one-way ANOVA. Means were considered significantly different at $P < 0.05$. Bars with same letters are not significantly different

blot in BT-474 cells transfected with 5 ng ERα or 5 ng empty vector. Genistein at 1.0 μM mimicked E2 inhibition of *HER2* phosphorylation/activation when BT-474 cells were co-transfected with ERα and treated for 20 h (Fig. 5 A, B).

To define the role of ERβ in *HER2* protein phosphorylation/activation, a similar transfection with 5 ng ERβ or 5 ng empty vector was made. Western blot analysis showed that the concentration of phosphorylated *HER2* was not affected by ERβ co-transfection following

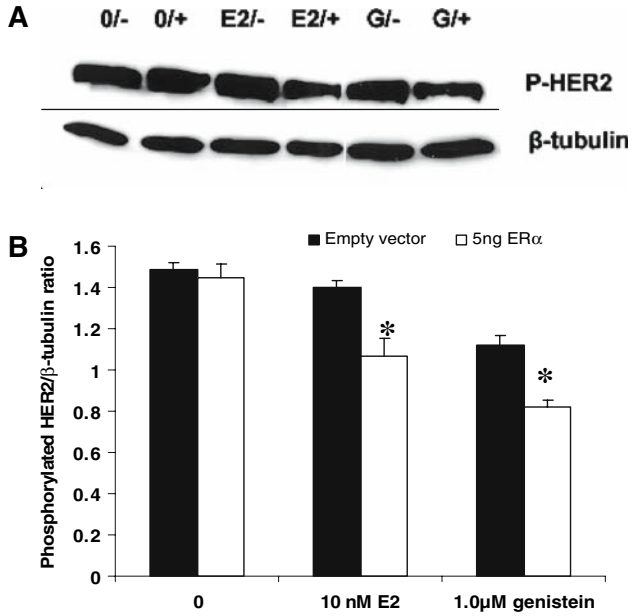

Fig. 5 The effect of ERα co-transfection on *HER2* protein activation/phosphorylation in BT-474 cells. (**A**) Western blot analysis of phosphorylated *HER2*. Lysate from transfected BT-474 and treated with control (0), 10 nM 17*β*-estradiol (E2), 1.0 μM genistein (G) with (+) or without (–) 5 ng ERα expression vector co-transfection for 20 h. (**B**) Individual bands were quantitated, and a ratio of *HER2* phosphorylated protein to the internal control protein in three independent experiments is expressed as mean ± SEM. Bar pairs with * are significantly different from its ERα-negative control using Student's *t*-test, $P < 0.05$

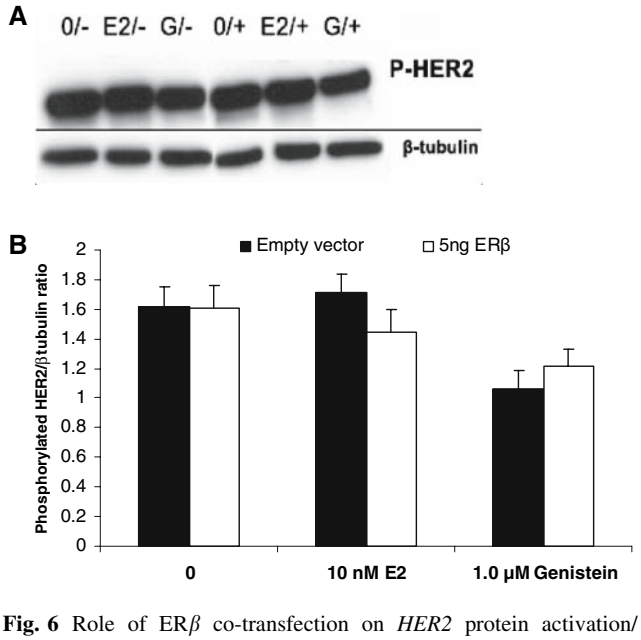

Fig. 6 Role of ER*β* co-transfection on *HER2* protein activation/phosphorylation in BT-474 cells. (**A**) Western blot analysis of phosphorylated *HER2*. Lysate from transfected BT-474 and treated with control (0), 10 nM 17*β*-estradiol (E2), or 1.0 μM genistein (G) with (+) or without (–) 5 ng ER*β* expression vector co-transfection for 20 h. (**B**) Individual bands from (**A**) were quantitated, and a ratio of *HER2* phosphorylated protein to the internal control protein in two experiments done in duplicate depicted graphically as mean ± SEM

incubation with either 1 μM genistein or 10 nM E2 treatment for 20 h (Fig. 6A, B).

The effect of 17*β*-estradiol and genistein on HER2 promoter reporter and the role of ERα and ER*β*

To determine if genistein can regulate *HER2* expression transcriptionally, we cloned 525 bp from the *HER2* promoter (–495/+30) [27] into the luciferase reporter vector PGL-3 basic. This reporter assay system was used to determine the effect of genistein on *HER2* transcriptional regulation in the absence or presence of co-expressed ERα (Fig. 7). BT-474 cells were transfected with the *HER2* promoter luciferase construct with either ERα expression vector or an empty vector to correct for the transfection efficiency. Treatments were added for 20 h.

In the absence of ERα, a dose-dependent inhibition of *HER2* promoter activity by genistein was observed starting at 1.0 μM concentration (Fig. 7A). The *HER2* promoter activity was inhibited by 60% at 50 μM concentration. However, 17*β*-estradiol and 0.1 μM genistein treatments did not significantly inhibit *HER2* promoter in absence of ERα. Our transcriptional study suggests a possible ERα ligand-independent inhibition of the human *HER2* promoter. In the 10 nM E2 and low concentrations of genistein (0.1 and 1.0 μM) groups, the *HER2* promoter reporter was repressed significantly upon ERα co-transfection in BT-474 cells (Fig. 7B). However, at high doses (10, 50, 75, and 100 μM), genistein generated similar repression on *HER2* promoter reporter in both the presence and absence of ERα. To our knowledge, this is the first evidence that genistein inhibits *HER2* promoter expression and reveals the role of ERα in its action.

To define the role of ER*β* in *HER2* promoter regulation, BT-474 cells were transfected with *HER2* promoter luciferase construct with ER*β* expression vector (0, 5, 10, or 20 ng). Assigned treatments (0, 10 nM E2, or 1.0 μM genistein) were added for 20 h. ER*β* had no effect on *HER2* promoter activation as determined by luciferase reporter assays (Fig. 8). As a control experiment, 10 nM E2 increased the expression of 3× ERE luciferase reporter construct 15-fold in Ishikawa cells, co-transfected with ER*β* expression vector (data not shown). Surprisingly, E2 did induce the ERE luciferase 6-fold in BT-474 cells by activation of the endogenous ER*β*, but no more activation was observed upon exogenous ER*β* co-transfection (data not shown). This suggests to us, not surprisingly from Fig. 1, that ER*β* is present and active in BT-474 cells but that the cell responses are saturated with the endogenous ER*β* and thus it is fully active.

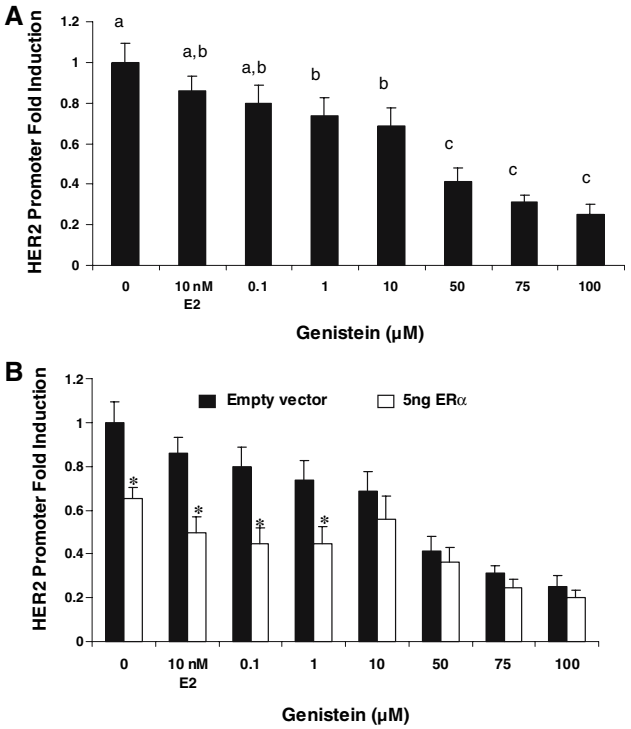

Fig. 7 Effect of genistein concentrations on *HER2* promoter/luciferase reporter in the presence and absence of ERα in BT-474 cells. BT-474 cells were transiently transfected with 400 ng of *HER2* promoter luciferase reporter and 5 ng of empty vector (**A**) or 5 ng of ERα expression vector (**B**). Results are expressed as mean ± SEM from three independent experiments run in duplicate. ERα-negative/no treatment control was normalized to 1. Treatments in the same experiment are reported as the ratio to its control. In (**A**), data were analyzed by one-way ANOVA and pair comparison of means was done by multiple *t*-test comparison. Means were considered significantly different at $P < 0.05$. Bars with same letters are not significantly different. In (**B**), bar pairs with * are significantly different from its ERα-negative control using Student's *t*-test, $P < 0.05$

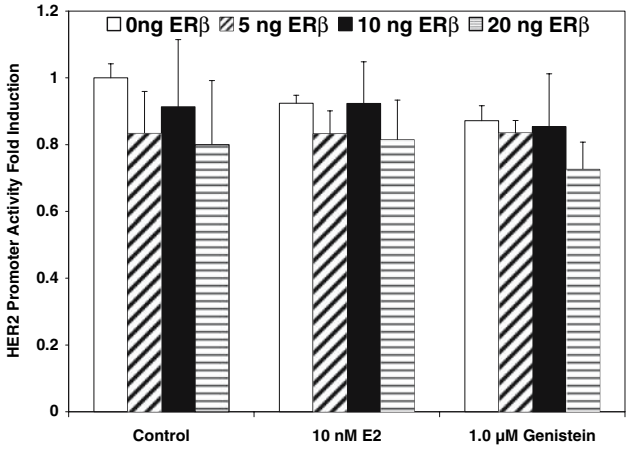

Fig. 8 Role of ERβ co-transfection on *HER2* promoter/luciferase reporter in BT-474 cells. BT-474 cells were transiently transfected with 400 ng *HER2* promoter luciferase construct and 0, 5, 10, or 20 ng ERβ expression vector. Transfection efficiency was corrected by ERβ empty vector co-transfection to bring the total DNA concentration to 20 ng. Cells were incubated with 0, 10 nM E2, or 1.0 μM genistein for 20 h. Results are expressed as mean ± SEM from three independent experiments each with duplicate samples. No statistically significant differences were observed

Discussion

Genistein is an effective treatment for inhibiting ER-negative breast cancer cell growth [28, 29]. This is of clinical importance because most *HER2* overexpressing breast tumors are ER-negative and resistant to hormonal treatment. Most studies of phytoestrogen effects on breast cancer cell growth have been performed using genistein in estrogen-dependent MCF-7 cells. These reports suggested biphasic effects, with stimulation of growth at low concentrations (<10 μM), and inhibition at higher concentrations [29–34]. In our model, we found that genistein produced biphasic effects on BT-474 cell growth with stimulation at low doses followed by inhibition at higher doses (50 μM IC_{50}). The initial stimulation of breast cancer cell proliferation by genistein was attributed to its estrogenic activity through ERβ because this stimulation could not be demonstrated in ER-negative cells [34].

We further found that 25 μM genistein inhibited total *HER2* protein expression in ERα-negative BT-474 cells by about 50% (Fig. 3A, B). Although different cell lines were used, Li et al. [28] showed that 30 μM genistein treatment for 3 days inhibited *HER2* protein expression in MDA-MB-435 ER-negative human breast cancer cells by 50%. The focus of that study was on the effect of genistein on cell apoptosis in ER-negative cells. In addition, their aim was to determine the effect of genistein on *HER2* protein dynamics as they used CMV-driven *HER2* expression vectors. In another study, Katdare et al. [35] showed by immuno-histochemistry that 10 μM genistein inhibited *HER2* protein and tyrosine phosphorylation in 184-B5/HER cells, an ER-negative model for carcinoma in situ. Their focus was to show the effect of genistein on cell cycle and cell growth. We used a different research approach to examine the transcriptional inhibition of human *HER2* at the promoter level by genistein. Additionally, we demonstrated the role of ERα and ERβ in *HER2* protein activation and promoter expression in the BT-474 cell line which expresses a high level of endogenous *HER2* protein.

Genistein is a specific inhibitor of tyrosine protein kinase [15] and thereby, could inhibit the tyrosine kinase domain in *HER2* protein. We found that genistein starting at 1.0 μM significantly inhibited *HER2* phosphorylation after 5 days of treatment (Fig. 4A, B), however, 10 nM E2 did not change *HER2* phosphorylation in BT-474 breast cancer cells (Fig. 4C, D). This low and physiologically achievable serum concentration by regular consumption of

a high soy diet has been found to inhibit tyrosine kinase activity [36]. In vivo, Dalu et al., found that 0.25 mg/g dietary genistein down-regulated EGFR expression and phosphorylation in the dorsolateral prostate of Lobund-Wistar rats despite free genistein concentrations in serum and prostate tissue of only 18.4 nmol/l and 17.5 pmol/g, respectively [16]. The authors inferred that genistein may be more potent in vivo than in vitro and they concluded that free genistein is more available to tissues because they found the concentration of free genistein was higher in prostate tissues than in serum. Moreover, genistein has been shown to alter protein tyrosine phosphorylation in human peripheral blood mononuclear cells after an oral dose of dietary amounts of genistein [37, 38]. Thus, genistein's ER-independent effects reported here and by others suggest that genistein could potentially be used in drug therapy at high doses to target *HER2* overexpressing tumors that do not express ERα.

At the *HER2* protein activation level, after 20 h of incubation, genistein at 1.0 μM mimicked E2 and inhibited *HER2* phosphorylation when BT-474 cells were co-transfected with ERα (Fig. 5). This supports speculation for a role of ERα in inhibiting *HER2* total protein expression as demonstrated by Russell and Hung [25] in that ER might be a negative regulatory factor for *HER2* gene expression. The effect of 17β-estradiol on *HER2* phosphorylation is controversial. Estradiol was proposed to directly bind and activate *HER2* in ER-negative fibroblasts transfected with human *HER2* cDNA [39]. These results, however, could not be reproduced in mammary cells overexpressing *HER2* [23]. Induction of *HER2* phosphorylation by E2 was not supported by Antoniotti et al. who found very slight induction of tyrosine phosphorylation in T47D ER(+) breast cancer cells by estrogen [40]. Estradiol was also found to induce *c-myc* expression (a downstream target for estrogen) but not *ras p21* (a downstream target for *HER2* activation) in T47D and MCF-7 cells [9]. These observations suggest that the presence of ERα modulates the effect of E2 on *HER2* activation.

At the transcriptional level, AP-2 [41] and Ets-binding sites [27] located in the *HER2* promoter may play an important role in regulation of *HER2* gene expression. Several Ets transcription factors are nuclear targets of signaling pathways including MAP kinase which is a downstream target of *HER2* activation. MAP kinase phosphorylates Ets proteins at particular serine or threonine residues [42]. We demonstrated that genistein, presumably as a tyrosine kinase inhibitor, inhibited *HER2* phosphorylation (Fig. 4A, B). Therefore, we propose that genistein at doses high enough to inhibit the tyrosine kinase could inhibit *HER2* promoter in the absence of ERα. Because most *HER2* overexpressing tumors are ERα-negative, genistein could be a useful treatment. The rationale of inhibiting *HER2* transcriptionally is that it will be more efficient to inhibit *HER2* concentration by reducing transcription than attempting to neutralize about 10^6 receptor molecules found in *HER2* overexpressing cells [43].

To determine if genistein can regulate *HER2* expression transcriptionally, we cloned 525 bp from the human *HER2* promoter (–495/+30) into the luciferase reporter vector PGL-3 basic [27]. In the absence of ERα, a dose-dependent inhibition of *HER2* promoter activity by genistein was observed starting at 1.0 μM (Fig. 7). The *HER2* promoter activity was inhibited by 60% at 50 μM. However, 10 nM E2 and 0.1 μM genistein treatments did not inhibit the *HER2* promoter in the absence of ERα. In the control, 10 nM E2 and genistein (0.1 and 1.0 μM) treatment groups, the *HER2* promoter reporter was repressed significantly upon ERα co-transfection in BT-474 cells; however, we could not show significant ligand-dependent inhibition. On the other hand, at higher doses (10, 50, 75, and 100 μM), genistein generated similar repression on the *HER2* promoter reporter in both the presence and absence of ERα.

Surprisingly, our transcriptional study suggests a possible ligand-independent inhibition of the human *HER2* promoter when ERα expression vector was co-transfected in BT-474 cells. The ligand-independent inhibition has been reported with other promoters. Ligand-independent ERα inhibition of cell migration and metastasis occurred in MDA-MB-231 breast cancer cells suggesting a protective role of the unliganded ERα [44]. Interestingly, *HER2* overexpression has been co-related to cell migration and metastasis [28] suggesting a ligand-independent inhibitory role of ERα on *HER2* gene expression. In addition, ERα ligand-independent inhibition has been shown with the ERE luciferase reporter in MCF-7 cells when co-transfected with steroid co-repressor MRF-1 [45]. Unlike our study, an E2-dependent inhibition of a 218-bp fragment of human *HER2* promoter has been shown previously in ZR75.1 and SKBR.3 cells which was reported to be through inhibition of the AP-2 transcription factor [46]. In that study, the proximal 218-bp promoter region was used, whereas, we used the –495/+30 region. In the same study, overexpression of different AP-2 isoforms resulted in different estrogenic responses. Constitutive expression of AP-2β or AP-2γ, but not AP-2α, abrogated the ligand-dependent repression. Moreover, co-transfection of AP-2β resulted in about 50% reduction of the promoter reporter activity in the control and the estradiol treatment groups [46]. Taken together, there is a possibility of cell-specific *HER2* promoter regulation potentially dependent on the relative amounts of AP-2 isoforms expression in different cell lines.

The biological interactions between ERs and *HER2* are complex. Although activation of *HER2* down-regulated ER

expression in the short-term and gave rise to an ER-negative phenotype in the long-term [23], *HER2* activation also promotes E2-independent transcription activity of ER [47]. Conversely, E2 down-regulated *HER2* in human breast cancer cells [46]. Bai and Giguere [5] have shown differential effects of *HER2* overexpression on ERα and ERβ. They showed that expression of *HER2* results in recruitment of SRCs (steroid receptor co-activators) to ERα, but not ERβ. The differential effect of *HER2* on ERα versus ERβ activation could be involved in turn, in the different effects of the two ERs on *HER2* expression.

Our recommendation from this work is that high dose of genistein (>10 μM) may be a useful therapeutic treatment for breast cancer. There remains the question of whether such a concentration could be achieved in humans through oral administration of genistein. To our knowledge, the highest total genistein C_{max} serum concentration reported in humans was 16.34 μM which occurred in a clinical trial to evaluate genistein's toxicity in patients with cancer [38]. However, studies suggest that phytoestrogens may be concentrated in specific tissues. For example, genistein concentration was over twofold higher in prostate than in plasma of prostate cancer patients assigned for radical prostatectomy after consuming 240 mg of clover phytoestrogens for 2 weeks [48]. At the body fluids level, local accumulation of genistein is possible within breast tissues, similar to what occurs with estrogens [49]. One study showed that estradiol was 10 times more concentrated in NAF than in serum of pre-menopausal women and 50 times in post-menopausal women [50]. Although these findings suggest that it may be possible to reach high genistein levels in the mammary gland and NAF, more studies need to be done to determine delivery methods and the potential maximum concentration achievable in serum and peripheral tissues.

In conclusion, our data support our hypothesis that genistein works through ERα at low doses and as a tyrosine kinase inhibitor at high doses to regulate *HER2*. Through these pathways, genistein regulates *HER2* protein expression, tyrosine phosphorylation, and *HER2* promoter activity. Although extensive pharmacokinetics study are needed, the demonstration of non-ER-dependent inhibition of *HER2* at higher doses, raise the possibility of using this inexpensive natural compound, genistein, as single or adjuvant therapy in ERα (–) and *HER2* (+) breast cancer.

Materials and methods

Chemicals

Genistein was obtained from LC Laboratories (Woborn, MA), dissolved in DMSO and stored as 100 mM stocks at –20°C. 17β Estradiol and β-tubulin antibody (cat #T7816) were obtained from Sigma Chemical Co. (St. Louis, MO). The human reactive polyclonal antibodies for ERα (PA1-308) and ERβ (PA1-312) were purchased from Affinity Bio Reagents (Golden, CO). The human reactive rabbit polyclonal for *HER-2* antibodies (cat # sc-284-R) and the human reactive rabbit polyclonal for phosphorylated Tyr 1248 *HER2* (cat # sc-12352-R) were purchased from Santa Cruz Biotechnology, INC (Santa Cruz, CA).

Plasmids

Human *HER2* promoter construct was prepared by cloning a 525-bp region (–495/+30) in relation to the transcription initiation site [27]. *Xho*I and *Hin*dIII restriction sites were inserted by using the following primers: forward primer sequence: 5′-ttccagaagatactcgaggggggtcctgga-3′ and reverse primer sequence: 5′-gctgcccgggggaagcttctggtttctc cg-3′. Amplified DNA products were ligated into the basic PGL-3 luciferase vector (Promega, Madison, WI). After transformation, sequences from independent colonies were verified at the DNA core facility of the University of Missouri, Columbia.

ERα and ERβ expression vectors cloned in pcDNA Zeo 3.1(+) expression vector were prepared as described previously [51].

Cell culture and transfection experiments

BT-474 human breast cancer cells from ATCC (Manassas, VA) were maintained in Hybri-Care Medium (from ATCC, cat #46-x) supplemented with 10% fetal bovine serum. For transient transfection experiments, BT-474 cells were plated in 24-well plates in phenol red-free medium [DMEM (Gibco, Carlsbad, CA), 5 mM L-glutamine, 1% sodium pyruvate (v/v), and 10 mM HEPES buffer] and charcoal-stripped serum for 24 h and transiently transfected using Fugene 6 (Roche). Cells were transfected with 5 ng ERα, ERβ, or empty vector and 400 ng *HER2* promoter reporter vector. After 24 h, transfected cells were treated with the assigned treatment for 20 h. Transfection experiments were normalized to co-transfected *pRL-SV40 Renilla* vector (Promega, Madison, WI). Luciferase assays were done using the Dual Luciferase Assay kit (Promega).

Thymidine incorporation assay

BT-474 cells were plated at 10 $\times 10^4$ cells per ml in 6-well dishes complete culture medium. The next day, the media were replaced with phenol red-free medium and

charcoal-stripped serum containing the assigned treatment for 5 days. The media containing the treatment were replaced once after 2 days. The day before harvest, 0.5 μCi/well of [methyl-3H]-thymidine was added. The cells were rinsed twice with ice-cold PBS, twice with ice-cold 5% (v/v) trichloroacetic acid, and twice with ice-cold 95% ethanol. The cells were lysed in 1.0 ml of 1 N NaOH and counted in a liquid scintillation.

Western blotting

BT-474 cells were washed with cold PBS then lysed in ice-cold buffer (50 mM Tris–HCL, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, and 100 mM PMSF (pH 7.4)). The cells were scraped, collected in microfuge tubes and sonicated for 30 s. The cell lysate was cleared by centrifugation at $14{,}000 \times g$ for 15 min at 4°C and the supernatant (total cell lysate) was used or immediately stored at –80°C. The total cellular protein concentration was determined by the DC Bio-Rad assay (Bio Rad laboratories, Hercules, CA). For Western blotting, 10 μg protein was resolved over 8–12% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were transversely cut according to the molecular weight marker to use the upper part to show protein expression for the target protein and the lower part was used for analysis of internal control expression (β-actin or β-tubulin). Membranes were incubated in blocking buffer (5% (w/v) nonfat dry milk/1% (v/v) Tween 20; in PBS, pH 7.6) for 2 h at room temperature, then with appropriate primary antibody overnight at 4°C, followed by incubation with secondary antibody horseradish peroxidase conjugate (Amersham Life Science Inc., Arlington Height, IL). Bands were detected by chemiluminescence and autoradiography using XAR-5 film (Eastman Kodak CO., Rochester, NY).

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