Genistein induces enhanced growth promotion in ER-positive/erbB-2-overexpressing breast cancers by ER–erbB-2 cross talk and p27kip1 downregulation

Xiaohe Yang1, Shihe Yang, Christine McKimmey, Bolin Liu1, Susan M.Edgerton1, Wesley Bales2, Linda T.Archer2 and Ann D.Thor1

Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA, 1Department of Pathology, University of Colorado Health Sciences Center, Aurora, CO 80045, USA and 2Department of Pathology, Oklahoma City Veterans Affairs Medical Center, Oklahoma City, OK 73104, USA

Abbreviations: EGFR, epidermal growth factor receptor; ER, estrogen receptor; City, OK 73104, USA of Pathology, Oklahoma City Veterans Affairs Medical Center, Oklahoma City, OK 73104, USA

Genistein is a major isoflavone with known hormonal and tyrosine kinase-modulating activities. Genistein has been shown to promote the growth of estrogen receptor positive (ER+) MCF-7 cells. In ER-negative (ER–)erbB-2-overexpressing (erbB-2+) cells, genistein has been shown to inhibit cell growth through its tyrosine kinase inhibitor activity. The effects of genistein on cell growth and tamoxifen response in ER+/erbB-2-altered breast cancers (known as luminal type B and noted in ~10 to 20% of breast cancers) have not been well explored. Using erbB-2-transfected ER+ MCF-7 cells, we found that genistein induced enhanced cellular proliferation and tamoxifen resistance when compared with control MCF-7 cells. These responses were accompanied by increased phosphorylation of ERK and ER signaling, without increase in ER protein levels. Genistein-treated MCF-7/erbB-2 cells also showed enhanced activation/phosphorylation of erbB-2, Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase. Blockade of the phosphatidylinositol 3-kinase and/or MAPK pathways abrogated genistein-induced growth promotion, suggesting that genistein effects involve both critical signaling pathways. We also found that p27kip1 was markedly downregulated in genistein-treated MCF-7/erbB-2 cells. Overexpression of p27kip1 attenuated genistein-mediated growth promotion. In aggregate, our data suggest that the concomitant coexpression of ER and erbB-2 makes breast cancers particularly susceptible to the growth-promoting effects of genistein across a wide range of doses. The underlying mechanisms involve enhanced ER–erbB-2 cross talk and p27kip1 downregulation.

Materials and methods

Cell lines and cell culture

MCF-7/Neo and MCF-7/erbB-2 (MCF-7/THER2-18, transfected with human erbB-2) cells (23) were a gift from Dr Christopher Benz (Buck Institute for...
Age Research, Novato, CA). MDA-MB-435/Neo and MDA-MB-435-erbB-2 (MDA-MB-435-EB1) cells (29) were a gift from Dr Dihua Yu (MD Anderson Cancer Center, Houston, TX), ZR-75-1 and SK-BR-3 cells were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal bovine serum and penicillin-streptomycin. For cell starvation experiments in Figures 3 and 4, 2 × 10^6 cells were inoculated into each 100 mm dish 24 h before starvation. They were starved in phenol red-free Dulbecco’s modified Eagle’s medium/F-12 medium containing 0.5% of charcoal-stripped fetal bovine serum for 48 h. The cells were then treated with control or genistein-containing phenol red-free Dulbecco’s modified Eagle’s medium/F-12 medium for the time indicated in each experiment.

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay

As described previously (30), 800 cells were inoculated into each well of a 96-well plate 24 h before treatment. The cells were then treated with drugs at the indicated concentrations for 6 days. After removal of the medium, 50 μl of 3-(4,5-dimethylthiazole-2-yl)-2,5-bisphospho tetrazolium bromide (MTT) solution (1500 μg/ml) was added to each well, followed by a 4 h incubation. The cell culture medium was replaced by dimethyl sulfoxide. The absorbance was measured at 562 nm using an enzyme-linked immunosorbent assay reader. The data based on eight parallel samples were analyzed using Student’s t-test.

Clonogenic assay

Five hundred cells were inoculated into each well of a six-well plate and grown in media containing genistein at indicated concentrations for 14 days. The media were carefully changed every 5 days. At the end point, the media were replaced with the staining buffer (50% ethanol and 0.25% dimethyl-methylene blue) for a 45 min stain. After washing with phosphate-buffered saline, the colonies were counted with the PUV imager analysis system. The survival fractions were analyzed with Student’s t-test based on three parallel samples.

Western blotting

Cell lysates were prepared as described previously (30). Fifty micrograms of protein lysate was loaded onto each lane of a gel. Proteins were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed with specific primary antibodies at dilutions of 1:300–1:2000. Antibodies against pErk1/2, p21, p27/kip1, cyclin D1, E2F-1, cyclin B1 and Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against ERα was purchased from Upstate Signaling Solutions (Charlottesville, VA). An anti-phospho-Akt (Ser473) rabbit monoclonal antibody, a phospho-ERα (Ser118) mouse monoclonal antibody and a phospho-erbB-2 (Tyr1248) were purchased from Cell Signaling Technology (Beverly, MA). After washing, the membranes were probed with horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibodies (GE Healthcare, Piscataway, NJ). The specific protein bands were visualized by autoradiography using an enhanced chemiluminescence kit (Pierce Biotechnologies, Rockford, IL).

Cell cycle analysis

Treated cells were trypsinized and washed with phosphate-buffered saline, followed by fixation in 80% of ethanol. After being washed three times with phosphate-buffered saline, the cells were counted with the PUV image analysis system. The survival fractions were analyzed with Student’s t-test based on three parallel samples.

Luciferase assay

Cells were inoculated into 12-well plates at 1 × 10^5 per well 24 h before the transfection. Four hundred nanograms of pERE-luciferase construct (a gift from Dr Donald McDonnell, Duke University) and 100 ng of pSVβ-gal DNA were transfected into each well using FuGENE 6 Transfection Reagent (Roche, IN). Genistein or 17-β-estradiol was added 16 h after transfection. The cells were harvested 24 h posttransfection, followed by luciferase assay using a kit from Promega (Madison, WI). The chemiluminescence was read using a TD-20/20 Luminometer. β-Galactosidase activity was detected using an Invitrogen kit according to the manufacturer’s protocol. The estrogen-responsive element (ERE)-luciferase activity of each sample was normalized with β-galactosidase activity. Results are expressed as fold increase over control luciferase activity normalized by β-galactosidase activity. All experiments were performed in triplicates.

Reverse transcription–polymerase chain reaction

Total RNA was isolated from treated cells using the RNeasy mini kit (Qiagen, Valencia, CA). First-strand complementary DNA synthesis was performed using SuperscriptIII™ First Strand synthesis system (Invitrogen, Carlsbad, CA). The sequences of the primers were as follows: p27 forward: 5'-TGGAGAAGCAGTGCAGAGAC-3' and p27 reverse: 5'-GGCGTGCTTCCAGAGTTGACC-3'; actin forward: 5'-GCACACATCTTCTACAATGAGC-3' and actin reverse: 5'-GAGGTCGACAGGCTTCCTCTAATG-3'.

Transfection

Cells from each line were inoculated into six-well plates at 3 × 10^4 per well 24 h before transfection. pcDNA3/p27 (a gift from Dr Zhen Fan, MD Anderson Cancer Center) or a control vector were transfected into MCF-7/Neo and MCF-7/erbB-2 cells using FuGENE 6 Transfection Reagent.

Results

Genistein induces enhanced growth promotion of ER+ but not ER– breast cancer cells that overexpress erbB-2

To study the impact of genistein on breast cancer cells by ER and erbB-2 status, we examined the effect of genistein on two pairs of cancer cell lines with different ER and erbB-2 status. These included the ERα-negative MCF-7/Neo cells and erbB-2-transfected MCF-7/erbB-2 cells, as well as the ERα-negative MDA-MB-435/Neo and MDA-MB-435/erbB-2 cells (Figure 1D). Although MDA-MB-435 cells are breast cancer cells or melanoma cells remains controversial (31,32), these ER+ cells have been tested for genistein response in previous studies and may serve as control for ER+ MCF-7 cells (28). When MDA-MB-435/Neo and MDA-MB-435/erbB-2 cells were treated with genistein at concentrations ranging from 0.02 to 50 μM, results from MTT assays show that the responses of both cell lines were in a similar pattern (Figure 1A). There was no growth promotion at lower concentrations, and the cells were significantly inhibited at 17 and 50 μM. In contrast, when the ER+ MCF-7/Neo and MCF-7/erbB-2 cells were treated with genistein, the two sublines responded differently across a wide range of doses (Figure 1B). Genistein promoted the growth of MCF-7/Neo cells at lower concentrations (<5 μM) and inhibited their growth at higher concentrations. This is consistent with the biphasic effect of genistein reported by others for ER+ cells (27). In contrast, the survival fractions of MCF-7/erbB-2 cells treated with 0.02–17 μM of genistein were higher than MCF-7/Neo cells under the same conditions. The difference between the two cell lines was more evident in 6 and 17 μg of M. These results suggest that genistein may induce enhanced growth promotion at lower concentrations and reduced inhibition at higher concentrations in ER+ and erbB-2+ breast cancer cells. In support of this notion, our results also showed that spontaneous erbB-2-overexpressing breast cancer cell lines with different ER statuses responded differently to genistein, as indicated by more genistein resistance in ER+/erbB-2+ ZR-75-1 cells than ER-/erbB-2+ SK-BR-3 cells (Figure 1C). To confirm the MTT results, we examined the effect of genistein on MCF-7/Neo and MCF-7/erbB-2 cells using clonogenic assays. As shown in Figure 1E, survival fractions of the two cell lines based on colony numbers also indicate that MCF-7/erbB-2 cells had more proliferative advantages over MCF-7/Neo cells. Of note, the colonies of genistein-treated MCF-7/erbB-2 cells were also larger than the control cells under the same conditions, suggesting a faster proliferation.

The induction of cells into S phase by genistein is enhanced by erbB-2

To further characterize the genistein-mediated growth promotion of ER+ MCF-7 cells, we compared the cell cycle distribution of MCF-7/Neo and MCF-7/erbB-2 cells by flow cytometry (Figure 1F). After 48 h of genistein treatment, the percentage of MCF-7/Neo cells in the S phase decreased modestly in response to 5–10 μM of genistein. In contrast, treatment of the MCF-7/erbB-2 cells with 5–10 μM of genistein resulted in more cells in the S and G2/M phases. These data are consistent with our MTT results, which showed growth promotion by genistein in the erbB-2-transfected MCF-7 cells.

Genistein induces enhanced tamoxifen resistance in MCF-7/erbB-2 cells

erbB-2 alterations have been associated with tamoxifen resistance in breast cancer patients (23). We have previously reported that...
genistein interferes with tamoxifen-mediated growth inhibition of MCF-7 cells (7) and that this deleterious interaction can be reduced by increasing the concentrations of either tamoxifen or genistein. To investigate whether coexpression of erbB-2 modifies this response, we studied the cell growth/inhibition of MCF-7/erbB-2 and MCF-7/Neo cells treated with genistein or tamoxifen alone or in combination, as compared with untreated control. As shown in Figure 2, genistein alone induced enhanced growth promotion of both cell lines at concentrations of 2–10 μM, but it was more evident in MCF-7/erbB-2 cells. In the absence of genistein, 0.4 μM of tamoxifen inhibited the growth of MCF-7/Neo and MCF-7/erbB-2 cells to 71 and 85% of the untreated control, respectively. The combination of tamoxifen with 2 or 10 μM of genistein, however, abrogated tamoxifen’s inhibitory effect in both cell lines. In particular, the increases in the proliferation rate between tamoxifen alone and combined treatment in MCF-7/erbB-2 cells were significantly greater than MCF-7/Neo cells under the same conditions. These results suggest that genistein may be a critical risk factor that undermines tamoxifen efficacy in ER⁺/erbB-2⁺ breast cancers.

Genistein induces enhanced ER activity in MCF-7/erbB-2 cells
The enhanced growth promotion and tamoxifen resistance in genistein-treated control and erbB-2-overexpressing MCF-7 cells suggest that the estrogenic effects of genistein may override its inhibitory effects on tyrosine kinase activity. To evaluate the role of the ER pathway activation in genistein-treated cells by erbB-2, we performed an ERE-luciferase assay on both the MCF-7/Neo and the MCF-7/erbB-2 cells under a variety of treatment conditions. As
shown in Figure 3A, 10–20 μM genistein induced greater ERE activity than 10 nM of E2 in both cell lines. ERE induction by genistein was significantly greater in MCF-7/erbB-2 cells as compared with MCF-7/Neo cells (50-fold in MCF-7/Neo; >150-fold in MCF-7/erbB-2).

We next studied whether the modulation of tamoxifen resistance by genistein involved activation of ER-dependent signaling. Genistein alone (15 μM) induced a 50- to 100-fold increase in ERE activity in the MCF-7/Neo and MCF-7/erbB-2 cells, whereas tamoxifen (3 μM) alone induced no significant estrogenic activity in either cell line (≤3-fold increase of ERE-luciferase activity; Figure 3B). When genistein and tamoxifen were administered simultaneously, the activation of the ERE was remarkably profound in both cell lines, particularly in ER+/erbB-2-expressing cells. These data suggest that the interaction between genistein and tamoxifen is more deleterious in these cells.

Because increased ER-dependent activity (ERE activation) could result from an increase in ER expression and/or functional modulation of the receptor, we compared the protein levels of ERα in non-synchronized and serum-starved MCF-7/Neo and MCF-7/erbB-2 cells, in the presence or absence of genistein (Figure 3C). In general, ERα protein levels were lower in MCF-7/erbB-2 cells as compared with control MCF-7/Neo cells regardless of the culture conditions. Starvation downregulated ER levels in both cell lines. We next examined ERα phosphorylation, using a phospho-Ser118-specific antibody. The data show that phosphorylated ERα levels were higher in MCF-7/erbB-2 cells, as compared with MCF-7/neo cells under both starved and non-synchronized conditions (Figure 3C). Genistein treatment, especially after starvation, induced enhanced ERα phosphorylation in MCF-7/erbB-2 cells. Given that the total protein levels of ERα were lower in genistein-treated MCF-7/erbB-2, as compared with control cells, the phosphorylation status of ERα may be pivotal to the regulation of genistein-mediated ER signaling.

**Figure 2.** Genistein induces enhanced tamoxifen resistance in MCF-7/erbB-2 cells. MCF-7/Neo and MCF-7/erbB-2 cells were inoculated into 96-well plates at 800 per well. The cells were then treated with genistein (0.2, 10 or 50 μM G), tamoxifen (0 or 0.4 μM) alone or in combination for 6 days. Cell survival/proliferation was measured using MTT assay as detailed in the Materials and Methods. The increases in the proliferation rate between tamoxifen alone and combined treatment of each cell line were compared and analyzed using Student’s t-test; *P < 0.01.

**Figure 3.** Modulation of ER signaling in genistein-treated MCF-7/Neo and MCF-7/erbB-2 cells. (A) Genistein induces enhanced ERE-luciferase activity in MCF-7/erbB-2 cells. The cells were cotransfected with pERE-luciferase and pSV-β-galactosidase, followed by genistein (10 or 20 μM), 0.1% of dimethyl sulfoxide (Con) or E2 (10 nM) treatments for 24 h. ERE-luciferase activity in E2 or genistein-treated cells was normalized and analyzed as described in the Materials and Methods. (B) Interaction between genistein- and tamoxifen-induced synergetic activation of ERE-luciferase. After the transfection of the reporter plasmids, the cells were treated with 0.1% of dimethyl sulfoxide (Con), 3 μM of tamoxifen (T or Tam), 15 μM of genistein (G) alone or in combination (T + G), followed by ERE-luciferase activity analysis. (C) Total ERα, erbB-2, pERα, and perbB-2 levels in control and genistein-treated MCF-7/Neo and MCF-7/erbB-2 cells. The cells were either non-synchronized or starved in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 0.5% of charcoal-stripped fetal bovine serum for 48 h followed by 24 h treatment with 10 μM of genistein or 0.1% of dimethyl sulfoxide. Total ERα, erbB-2, pERα/Ser118 and perbB-2/Tyr1248 were detected using western blot.

**Activation of erbB-2, PI3K and MAPK pathways in genistein-treated MCF-7/erbB-2 cells**

Increased phosphorylation of ERα in genistein-treated MCF-7/erbB-2 cells suggests elevated tyrosine kinase activity in these cells. However, previous studies have shown that genistein is a potent inhibitor of tyrosine kinase activity, especially for the related receptor EGFR (33), and that erbB-2 can be activated by heterodimer formation with EGFR (22). To investigate the effect of genistein on receptor tyrosine kinase activity in our cell lines, we examined erbB-2 activation in genistein-treated cells. As shown in Figure 3C, genistein induced erbB-2 phosphorylation in the ER+ MCF-7/erbB-2 cells with or...
without starvation. These results suggest that genistein may act as a receptor tyrosine kinase agonist, rather than a tyrosine kinase inhibitor, in cells with coexpression of ER and erbB-2.

Since MAPK/ERK and the PI3K/Akt signaling are major pathways downstream of erbB-2 activation, we examined the activation of the two pathways in genistein-treated cells. To this end, the paired cell lines were starved for 48 h followed by treatment with 0, 2 or 10 μM of genistein in phenol red-free medium for 45 min. As shown in Figure 4A, genistein treatment induced the activation/phosphorylation of Akt and ERK1/2 in both cell lines. More importantly, activation of the PI3K/Akt and MAPK/ERK pathways, as indicated by pAkt1 and p-Erk2 levels, was significantly enhanced in MCF-7/erbB-2 cells. Consistent with Akt and ERK1/2 activation, phosphorylation of ERα was also enhanced in MCF-7/erbB-2 cells. These results correlate ERα phosphorylation/activation with the activation of Akt and MAPK pathways and suggest that ER-erbB-2 cross talk may be the mechanism that abrogates genistein’s tyrosine kinase inhibitor activity in these cells.

To assess the functional role of MAPK and PI3K pathways in genistein-mediated growth promotion in these cells, we next examined the effects of genistein on the proliferation of MCF-7/Neo and MCF-7/erbB-2 cells in the presence or absence of LY294002 and PD098059 (specific inhibitors of the PI3K and MAPK pathways, respectively). As shown in Figure 4B, both LY294002 and PD098059 abrogated the genistein-mediated growth promotion of the MCF-7/erbB-2 cells. These data show that genistein-associated cellular proliferation involves the activation of both the PI3K and the MAPK pathways in erbB-2-overexpressing ER⁺ MCF-7 cells.

**Fig. 4.** Activation of MAPK and PI3K pathways in genistein-treated MCF-7/Neo and MCF-7/erbB-2 cells. (A) Genistein induces activation of ERK, Akt and ERα in synchronized cells. MCF-7/Neo and MCF-7/erbB-2 cells were starved as in Figure 3C, followed by treatment with 0 (0.1% dimethyl sulfoxide), 2 or 10 μM of genistein for 45 min. Protein levels of pErk1/2/Tyr202/Tyr204, tErk2, pAkt1/Ser473, tAkt1, tERα and pERα/Ser118 were probed with antibodies against each specific form of protein. (B) Blocking PI3K and/or MAPK pathways abrogates genistein-induced growth promotion. The cells in 24-well plates at 4000 per well were treated with 0.1% dimethyl sulfoxide (Con), 2 or 10 μM of genistein (G) for 45 min. The protein levels of pErk1/2/Tyr202/Tyr204, tErk2, pAkt1/Ser473, tAkt1, tERα and pERα/Ser118 were probed with antibodies against each specific form of protein.

**Fig. 5.** Modulation of cell cycle regulators in genistein-treated MCF-7/Neo and MCF-7/erbB-2 cells. (A) Effect of genistein concentration on cell cycle regulators in MCF-7/Neo and MCF-7/erbB-2 cells. Cells were treated with genistein at the indicated concentrations for 24 h. The protein levels of p21, cdc-2, E2F-1, cyclin B1, cyclin D1 and p27/kip1 were detected using western blot. (B) Effect of genistein on p27/kip1 protein and messenger RNA levels in MCF-7/Neo and MCF-7/erbB-2 cells. (B1) Western blot detection of p27/kip1 protein levels in both cell lines treated with genistein for 24 h. (B2) Reverse transcription–polymerase chain reaction detection of p27/kip1 messenger RNA levels in cells with the same treatment as in B1. (C) Herceptin (anti-erbB-2) inhibits p27 downregulation in genistein-treated ER⁺/erbB-2⁺ BT-474 cells. The cells were treated with Herceptin (10 μg/ml) and genistein (12.5 μM) alone or in combination for 48 h, followed by western blot detection of p27.
To explore the mechanism by which genistein mediates down-regulation of p27 in MCF-7/erbB-2 cells, we examined p27 messenger RNA and protein levels at various concentrations of genistein. We found that genistein induced downregulation of p27/kip1 protein without significant alteration of its messenger RNA levels in MCF-7/erbB-2 cells. In contrast, neither p27/kip1 protein nor messenger RNA levels were modified by genistein in control MCF-7/Neo cells (Figure 5B1 and B2). These data indicate that modulation of p27/kip1 expression in genistein-treated ER+/erbB-2 cells is primarily at the protein level and suggest that altered regulation of p27/kip1 protein stability may be the major mechanism. To demonstrate that genistein also induces p27/kip1 downregulation in other ER+/erbB-2+ cells, we generated a genistein-induced downregulation can be blocked by anti-erbB-2 agent Herceptin, we detected p27 levels in BT-474 cells, a known ER+/erbB-2 breast cancer cell line, treated with genistein or Herceptin alone or in combination. Genistein induced striking downregulation of p27/kip1 in BT-474 cells (Figure 5C). However, genistein-mediated p27/kip1 downregulation was only partially rescued by Herceptin. While confirming our findings in MCF-7 cells, these results also suggest that other pathways may also be involved in genistein-mediated downregulation of p27/kip1 in ER+/erbB-2+ breast cancer cells.

**Overexpression of p27/kip1 attenuates genistein-mediated growth promotion in MCF-7/erbB-2 cells**

To test whether genistein-induced downregulation of p27/kip1 in MCF-7/erbB-2 cells contributes to the enhanced proliferation of these cells, we transfected p27 complementary DNA and control vector into MCF-7/Neo and MCF-7/erbB-2 cells, respectively, followed by genistein treatment and cell cycle analysis (Figure 6A). Overexpression of p27 decreased the percentage of cells in S phase in each cell line treated with genistein, as compared with the cells transfected with control vector (Figure 6B). However, the decrease was more significant in MCF-7/erbB-2 cells. These results support a functional correlation between altered p27/kip1 regulation and genistein-mediated growth promotion in MCF-7/erbB-2 cells.

**Discussion**

In this study, we focused on the biologic and molecular effects of genistein on ER+/erbB-2 breast cancer cells. This phenotype (known in the literature as luminal type B) comprises 10–20% of breast cancers (19). Previous studies have shown that genistein may stimulate the growth of ER+ MCF-7 cells and xenograft tumors, but it induces persistent inhibition of ER−/erbB-2+ cancer cells (5,28). The responses of cells coexpressing ER and erbB-2 to genistein, tamoxifen or genistein plus tamoxifen, however, have not been explored but probably have significant translational value.

Results from this study demonstrate that genistein may induce enhanced growth promotion and tamoxifen resistance in ER+/erbB-2 breast cancer cells. We further demonstrate that genistein induces enhanced signaling in both ER and erbB-2 pathways, as indicated by increased activation of ERE activity and enhanced phosphorylation of ERα, erbB-2, Akt1 and ERK1/2 in MCF-7/erbB-2 cells. Since ER activation may upregulate growth factors/growth factor receptors (34,35) and activation of erbB-2/EGFR may induce ERα activation/phosphorylation (36,37), our data suggest that genistein-associated growth promotion and tamoxifen resistance in ER+/erbB-2+ breast cancer cells involve ER–erbB-2 cross talk. This is consistent with the studies showing that ER–erbB-2 cross talk plays a critical role in tamoxifen resistance in breast cancer treatment (38). Although genistein is also known to be a tyrosine kinase inhibitor and erbB-2-overexpression-associated tyrosine kinase activity could be a target of genistein, our data demonstrate that genistein at lower doses induces estrogenic activity that overrides its tyrosine kinase inhibitor activity in ER+/erbB-2+ cells. This suggests that ER status is a critical determinant of genistein-associated tyrosine kinase inhibitor activity and both ERα and erbB-2 are important factors that can modify the effects of genistein on breast cancer cells.

Genistein-induced downregulation of p27 in MCF-7/erbB-2 cells is another important finding in this study. It is known that p27/kip1 is a critical inhibitor of cyclin-dependent kinases that binds to and inhibits the activities of cdk2–cyclin E and cdk2–cyclin A complexes to block cell cycle progression at the G1–S transition (39). Downregulation of p27/kip1 would allow more cells entry into the S phase. We found that genistein induces striking downregulation of p27/kip1 in MCF-7/erbB-2 cells but not in MCF-7/Neo cells, suggesting a specific interaction between genistein and erbB-2 and its contribution to growth promotion in MCF-7/erbB-2 cells. This is supported by the results showing that overexpression of p27/kip1 in these cells inhibits genistein-mediated growth promotion in both MCF-7/Neo and MCF-7/erbB-2 cells (Figure 6). Moreover, we found that downregulation of p27/kip1 in genistein-treated MCF-7/erbB-2 cells was primarily at the protein level. This is consistent with the report that downregulation of p27/kip1 in erbB-2-altered cells can result from increased Akt activity via phosphorylation of p27/kip1 at threonine 157 (40), which causes its translocation from the nucleus to the cytoplasm and results in changes in p27/kip1 stability and degradation through the ubiquitin pathway (40,41). However, since genistein-induced p27 downregulation in BT-474 cells was only partially rescued by Herceptin (Figure 5C) and p27/kip1 can also be regulated through other signaling pathways (42), p27/kip1 downregulation in genistein-treated cells may involve the modulation of other factors/pathways, which warrants further investigation.

![Fig. 6](https://academic.oup.com/carcin/article-abstract/31/4/695/2477154/1485047715a)
Taken together, our results suggest that genistein at lower concentrations may induce growth promotion and tamoxifen resistance in ER+/erbB-2+ cells, as compared with ER− cells and ER+/erbB-2 normal cells. The molecular mechanisms involve the activation of ER–erbB-2 cross talk, including increased activation of ER-associated transcription, MAPK/ERK1/2 pathway, PI3K/Akt pathway and the downregulation of p27kip1 in these cells. Signaling downstream of these pathways would lead to enhanced proliferation. Moreover, marked higher survival fractions in MCF-7/erbB-2 cells treated with 6–17 μM of genistein, as compared with the MCF-7/Neo cells (Figure 1B and E), suggest that the reduced inhibition in these cells may also involve inhibition of apoptosis in these cells, which will be examined in future studies.

Given the widespread use of soy/genistein products and the possible deleterious effects, our data have translational relevance to patient care. Although soy might be beneficial to tumor prevention under certain conditions, it may be a potent risk factor in some cases. Our data suggest that ER+/erbB-2+ invasive breast cancers in particular may be most vulnerable to soy/genistein-associated risk (25). In addition, since ER and erbB-2 are overexpressed in the majority of ductal carcinomas in situ (43), interaction in these patients may also occur. Therefore, our study alerts patients with ER+/erbB-2+ breast cancers and tamoxifen taking or at-risk women exposed to genistein, either by supplement or by involuntarily.

Results from this in vitro study have provided proof of principle of the impact of soy/genistein on ER+/erbB-2+ breast cancer cells. For the interpretation of these in vitro data, it should be noted that the threshold for low and high dosages varies depending on treatment conditions and in vitro or in vivo settings. Although in vivo genistein concentrations are rarely >4 μM (44), the threshold for in vitro studies usually refers to 5–10 μM (45). In this study, genistein treatment in most tests was in the presence of 10% fetal bovine serum, and genistein-mediated inhibition was mainly observed at concentrations >10 μM in this study (up to 20 μM for MCF-7/erbB-2 cells). Low doses here are referred to the concentrations in the stimulating phase. To solve the discrepancy between in vitro and in vivo concentrations issue, we have recently performed animal studies to examine the effect of soy/genistein on mammary tumor development in mouse mammary tumor virus-erbB-2 transgenic mice. We found that exposure to soy-rich diet Purina-5001 after 20 weeks promoted mammary tumor development in these animals, and this was preceded by increased activation of ER and erbB-2 pathways in the mammary tissues (Xiaohe Yang, Zhikun M and Shihe Yang, manuscript in preparation).

Since non-transformed mammary tissues in these mice are ER+/erbB-2+ (46), the data are consistent with the in vitro results. These in vivo data support the notion that physiological concentration of soy/genistein may stimulate erbB-2-overexpressing mammary tumor cells, which underscores the possible impact of soy/genistein on breast cancer survivors or women at risk.

In summary, we have found that genistein induces enhanced growth promotion and tamoxifen resistance in ER+/erbB-2 cells. ER+/erbB-2 breast cancers might be most vulnerable to soy/genistein-associated risk. The underlying mechanisms involve enhanced activation of ER signaling and MAPK/PI3K pathways, indicating a cross talk between ERα and erbB-2 pathways. We also found that genistein induces striking downregulation of p27kip1 protein in MCF-7/erbB-2 cells and that overexpression of p27kip1 attenuated genistein-mediated growth promotion, suggesting that p27 may be a critical target and marker for genistein-induced growth promotion. Given that ER+/erbB-2-overexpressing tumors count for a substantial group of breast cancers and the widespread exposure of women to soy/genistein, our findings are valuable in breast cancer risk control and management.

Funding

Research Scholar Grant from American Cancer Society (RSG-08-138-01-CNE), Health Research Grant from Oklahoma Center for the Advancement of Science (HRP07-108) to X.Y.

Acknowledgements

We thank Dr Christopher Benz for MCF-7/Neo and MCF-7/erbB-2 cells, Dr Dihua Yu for MDA-MB-435/Neo and MDA-MB-435/erbB-2 cells, Dr Zhen Fan for pcDNA3/p27, Dr Donald P. McDonnell for pERE/Luciferase plasmids and Mr James Collins for manuscript preparation.

Conflict of Interest Statement: None declared.

References


Received June 7, 2009; revised November 23, 2009; accepted December 20, 2009.