

Genistein inhibits the proliferation of human HER2-positive cancer cells by downregulating HER2 receptor

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ABSTRACT:

Background: It was well studied that HER2/ErbB2/p185 overexpression in human malignant cancers correlates with poor prognosis and chemo-resistance. Meanwhile, Genistein (4,5,7-trihydroxyisoflavone), a major isoflavone component of soybeans and other leguminous plants, has been shown to exhibit a potent anti-proliferative effect on some sex hormone dependent cancers.

Objective: The effects of genistein on the proliferation of human HER2-overexpressing breast and ovarian cancer cell lines were investigated, and the action mechanism was explored.

Methods: Western blotting, fluorescence-activated cell sorting (FACS) and immunofluorescence methods, cell proliferation assay kit from Promega and cell apoptosis assay kit from Biologend were used. The dose- or time-response relationship of genistein were observed on the HER2-negative breast cancer cell line MCF-7 or HER2-positive breast cancer cell lines BT-474 and MCF-7/*Her2* derived from MCF-7, and ovarian cancer cell line SKOV-3.

Results: The addition of genistein ranged from 1-10 μ g/ml in the medium for 48 hours had a marked inhibition on the proliferation of HER2-positive cancer cell lines MCF-7/*Her2*, BT-474 and SKOV-3, compared with tamoxifen and DMSO control ($P<0.01$), and a dose-dependent response was presented. However, genistein exerted a weak inhibitive effect on HER2-negative breast cancer cell line MCF-7. There was a significant apoptosis or necrosis effect of BT-474 cells induced by 10 μ g/ml genistein for 12-48 hours in comparison with DMSO control ($P<0.01$), and a time-dependent response relationship. Moreover, genistein could down-regulate HER2 receptor expression and AKT kinase activity by using immunofluorescence and western blotting methods.

Conclusion: Our findings demonstrated that genistein could effectively inhibit the proliferation of HER2-positive cancer cell lines, which should be through down-regulating HER2 receptor and downstream signaling.

Key words: genistein, HER2/ErbB2/p185, breast cancer, ovarian cancer, cancer therapy

BACKGROUND:

HER2 (also named ErbB2, p185^{her2/neu}) is a member of the transmembrane epidermal growth factor receptor (EGFR) family. HER2 gene amplification and/or overexpression are found in a number of human malignancies including breast, ovarian and gastric cancers, and associated with enhanced cancer metastasis and poor prognosis. It is shown that patients with HER2-overexpressing cancers have lower long-term survival and disease-free survival rates [1, 2]. Although there have been anti-HER2 humanized monoclonal antibody drugs trastuzumab (1998, Genentech, USA) and pertuzumab (2012, Genentech, USA) and EGFR/HER2 dual inhibitor lapatinib (2007, GSK, Britain) has been proved for therapeutic use in patients with HER2-positive metastatic breast cancer patients, many patients either do not respond to the therapy or progress for drug resistance [2-5]. In this regard, more HER2-targeted drug researches are being needed.

A number of studies suggest that intake of a soy-rich diet may have a protective effect against sex hormone-dependent cancer [6, 7]. Genistein (4,5,7-trihydroxyisoflavone), a major isoflavone constituent of soybeans and soy's products, has been shown to exhibit potent anti-proliferative effect on various cancer cell lines, both *in vitro* and *in vivo* [8–10]. Structurally, genistein resembles the biphenolic 17 β -estradiol and displays high binding affinities to the estrogen receptors (ER), and hence genistein may mimic and regulate the actions of estrogens by acting as either an ER agonist or antagonist [11]. In addition, genistein is also a protein tyrosine kinase inhibitor, which may lead to cell cycle arrest, apoptosis, invasion, metastasis, and angiogenesis of cancer cells by regulating protein tyrosine kinase mediated signaling pathways [9, 12].

Previously we have found that genistein can inhibit the growth of estrogen-dependent breast and endometrial cancer cells *in vitro* and *in vivo* (data not shown). In this study, we elucidate a novel mechanism by which genistein mediates its effect on some HER2-overexpression breast and ovarian cancer cell lines by down-regulating HER2 and in turn downstream signal protein AKT activity, which is required for the cell proliferation and metastasis.

MATERIALS AND METHODS:

Reagents and cell lines: Gensitein (C15H10O5) and tamoxifen (C26H29NO) were obtained from Sigma Chemical Co. Anti-HER2 antibodies (Ab-1 against c-terminus domain, Ab-20 against ECD, and Ab-18 against p-HER2 at Tyr1248) were ordered from NeoMarkers. AKT, p-AKT (Ser473) antibodies were from Cell Signaling. GAPDH antibody was from Santa Cruz. HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies were from Pierce. Other reagents were commercially available in China.

Human breast cancer cell lines BT-474, MCF-7 and ovarian carcinoma cell line SKOV-3 were obtained from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) in 2012. MCF-7/*Her2* was derived from MCF-7 cells through the stable transfection of *HER2* plasmids in our lab.

Cell Proliferation Assay: BT-474 (2×10^4 cells/well) and MCF-7, MCF-7/*Her2* or SKOV-3 (5×10^3 cells/well) cells were seeded in 96-well microplates (Costar) and cultured overnight in DMEM with 10% FBS. Fresh medium containing the indicated concentrations of genistein, tamoxifen or DMSO were replaced. After incubation for 72 hours, cell proliferation was assessed by using CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega). Briefly, 20 μ l MTS solution was added to each well for 1.5 h incubation and the absorbance at 490 nm was measured.

Western blot analysis: Equal amounts of proteins (30 μ g) from cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% non-fat dry milk in PBS containing 0.2% Tween-20, membranes were incubated at 4 °C overnight with primary antibody. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h. ECL reagent (Pierce) was used for protein detection.

Fluorescent-activated cell sorting and immunofluorescence: Fluorescent-activated cell sorting (FACS) was performed using indirect staining protocols. 5 million cancer cells were collected and then labeled with primary anti-HER2 antibody (Ab-20, NeoMarkers), then stained by secondary antibody with FITC (Sigma).

For immunofluorescence, cells were fixed in 4% paraformaldehyde and permeabilized for 10 min in PBS containing 0.2% Triton X-100. Cells were washed with PBS containing 0.02% Triton X-100 and 1.5% FBS, followed by incubation with antibodies against HER2 (Ab-1 against c-terminus domain, Ab-20 against ECD) for 1 hour at room temperature. Cells were incubated with FITC or Rhodamine-conjugated secondary antibody (Sigma). Nuclei were visualized by staining with 1 μ g/mL DAPI. Images were captured on a 510 LSM confocal microscope (Zeiss).

Statistical analysis: Data is presented as the mean \pm SD from at least three independent experiments. All data analyses were done by software of SPSS13.0 for Windows. $P < 0.05$ was regarded as the threshold value for statistical significance.

RESULTS:

3.1. Genistein inhibits the proliferation of HER2-overexpression cancer cell lines: Human breast cancer cell lines BT-474 and MCF-7/*Her2* derived from MCF-7, and ovarian cancer cell line SKOV-3 were confirmed to be HER2-overexpressing cells, and the parent cell line MCF-7 was of low HER2 expression using FACS (Fig. 1A) and western blotting methods (Fig. 1B). Among the HER2-overexpressing cancer cells, SKOV-3 is the most and MCF-7/*Her2* is the least of HER2 expression.

We then investigated whether genistein treatment had different effects on the proliferation of high and low HER2-expressing cancer cell lines. As shown in Fig. 2C, addition of 0.1, 1 and 10 $\mu\text{g/ml}$ genistein into the medium significantly inhibited the cell proliferation of MCF-7, MCF-7/*Her2*, BT-474 and SKOV-3 compared with DMSO control ($P < 0.01$), and showed a dose-dependent response relationship. In contrast, 10 $\mu\text{g/ml}$ of tamoxifen had only some degree of proliferation inhibition on these four cancer cell lines, and the maximum inhibition rate was not up to 8%. Moreover, genistein had a more potent inhibition efficacy on the three HER2-overexpressing cancer cell lines than MCF-7 with low HER2 expression levels ($P < 0.01$). However, tamoxifen did not show so remarkable preference on the HER2-positive MCF-7/*Her2* cells. In addition, we investigated the effect of genistein added in the culture media of human normal cell lines such as HEK293 and MCF10A, and the result demonstrated that 10 $\mu\text{g/ml}$ genistein treatment for 48 hours had no marked influence on the normal cell morphology and proliferation (data not shown).

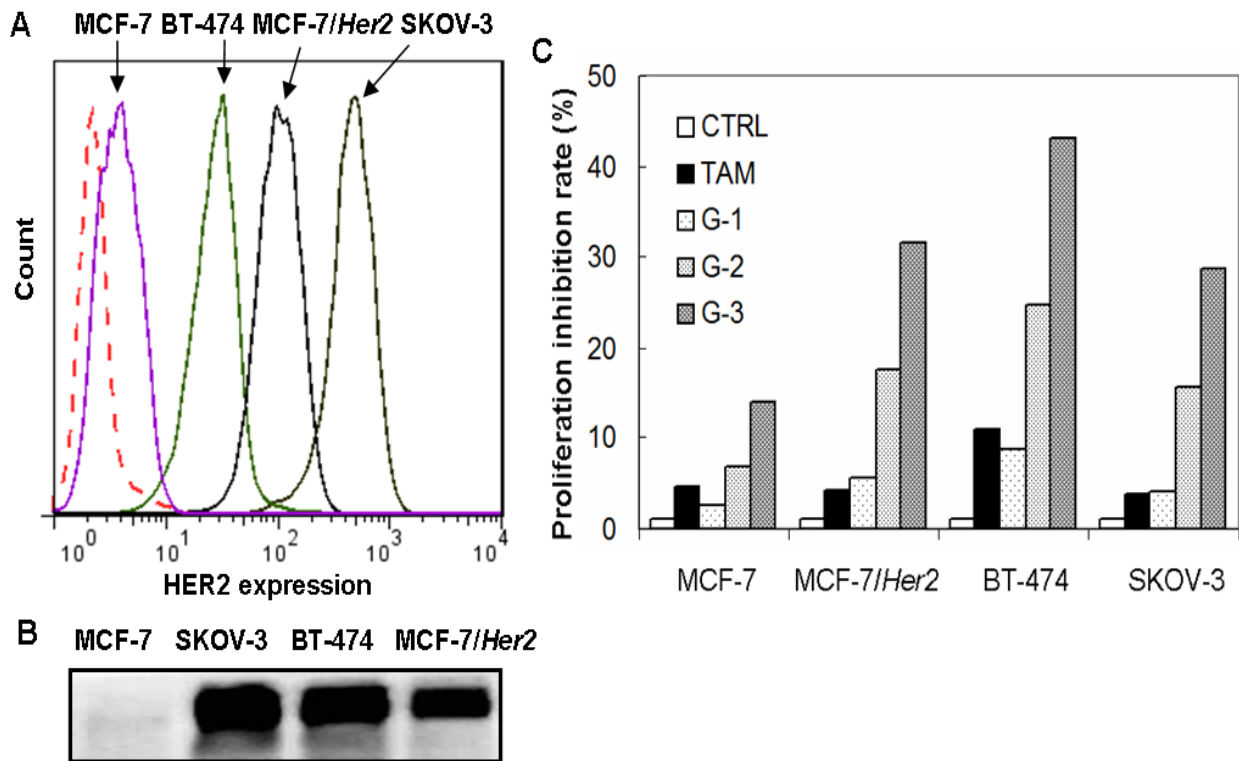


Figure 1. Genistein inhibited the proliferation of HER2-expression cancer cell lines. A, HER2 expression detection by FACS. B, HER2 expression detection by western blotting. C, The proliferation reduction of cancer cells treated with genistein for 48 hours. CTRL, DMSO control; TAM, tamoxifen with 10 $\mu\text{g/ml}$; G1, G2 and G3 mean 0.1, 1 and 10 $\mu\text{g/ml}$ genistein, representatively.

3.2. Genistein induces cell apoptosis of HER2-overexpressing cancer cell lines: Consistent with the above results, genistein treatment could induce the HER2-overexpression cell apoptosis. As shown in Fig. 3, the results showed that 10 $\mu\text{g/ml}$ of genistein for 12 hours was able to induce BT-474 cell apoptosis or necrosis and with the extension of time showed a time-dependent

response relationship. In contrast, 10 µg/ml of tamoxifen treatment BT-474 cells for 48 hours, cell death (apoptosis and necrosis ratio) is much smaller than that of genistein in the same processing conditions ($P < 0.001$).

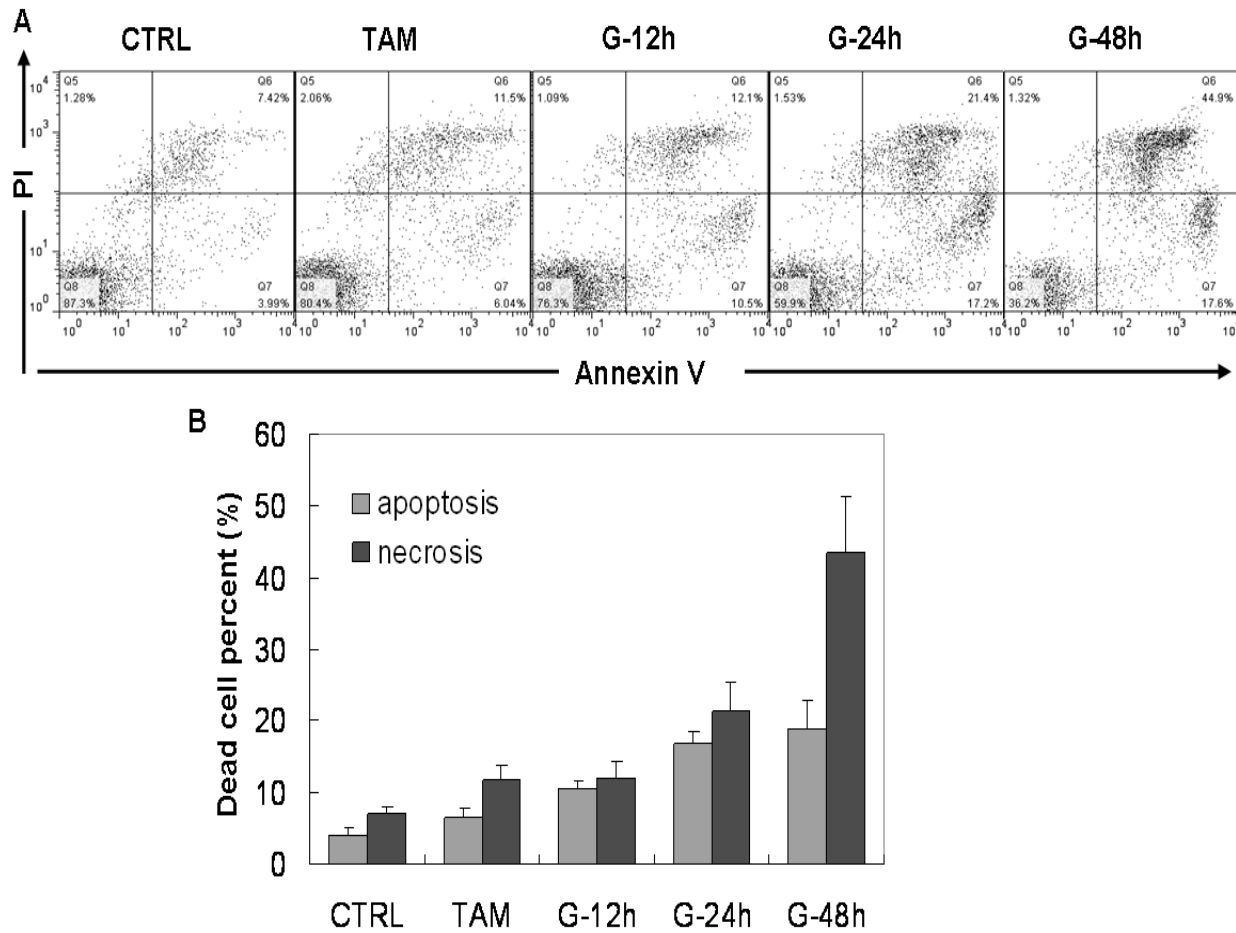


Figure 2. Genistein induced the apoptosis of BT-474 cells. A, FACS detection result using double staining of annexin V and propidium iodide (PI). B, statistical result of A figure. CTRL, DMSO control; TAM, 10 µg/ml tamoxifen for 48 hours; G-12h, G-24h and G-48h mean 12, 24 and 48 hours treated with 10 µg/ml genistein, representatively.

3.3. Genistein decreases HER2 receptor and downstream signaling of cancer cells: To investigate the underlying mechanism by which genistein targets HER2 receptor property, we tested whether genistein can affect the expression of HER2 receptor and HER2 signal transduction. Interestingly, genistein treatment resulted in significant downregulation of HER2 receptor and suppression of the downstream AKT activity of BT-474 or other HER2-positive cancer cells (Fig. 3A).

To further confirm the downregulation of HER2 receptor by genistein, we observed HER2 protein level in SKOV-3 cells treated with genistein using laser confocal microscope. It was shown that genistein was able to decrease HER2 receptor expression of SKOV-3 cells and induce the endocytosis of HER2 receptor (Fig. 3B).

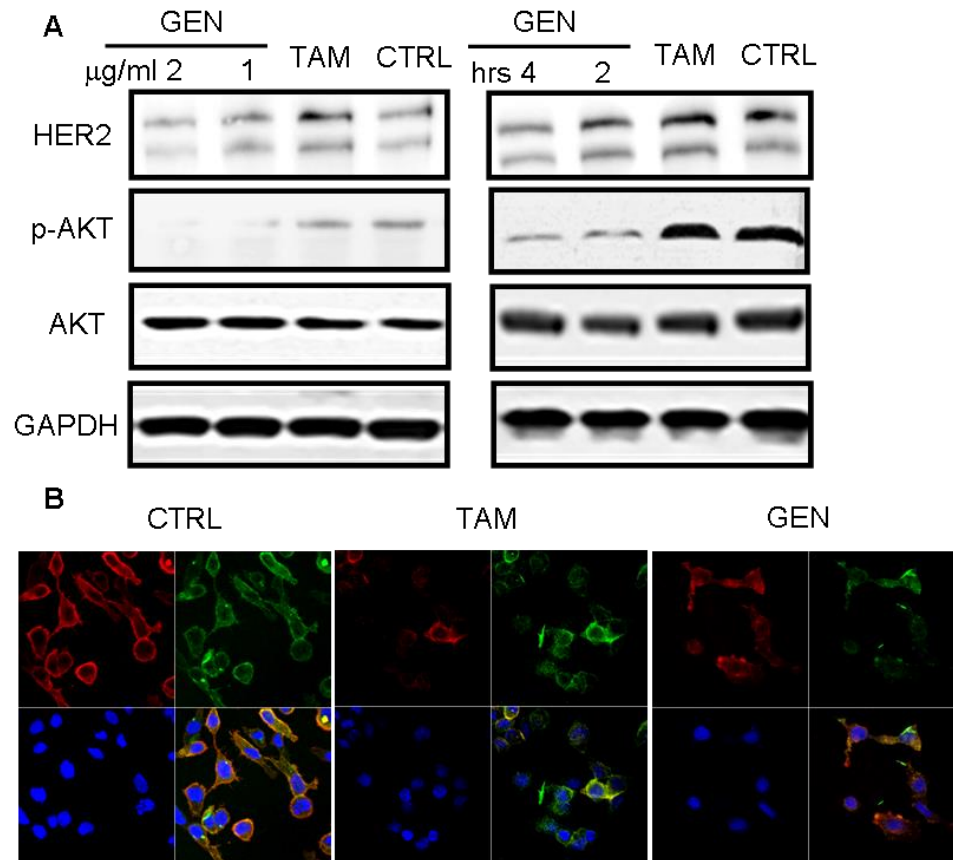


Figure 3. The decreased HER2 receptor and downstream signaling of cancer cells by genistein. A, The changes of HER2 receptor and downstream signaling of BT-474 cells treated with the indicated concentration of genistein for 4 hours or 1 $\mu\text{g/ml}$ genistein for the shown time by western blotting. B, The changes of HER2 receptor of SKOV-3 cells treated with 2 $\mu\text{g/ml}$ genistein for 4 hours by immunofluorescence. Immunofluorescence was performed using anti-HER2 antibodies (Ab-1 against c-terminus domain, Ab-20 against ECD). Cells were counterstained with DAPI to show the nuclear morphology. CTRL, DMSO control; TAM, tamoxifen with 2 $\mu\text{g/ml}$ for 4 hours; GEN, genistein.

DISCUSSION:

Many human cancers express HER2, which acts as the preferred heterodimerization partner for other members of ErbB receptors (named ErbB1/EGFR, ErbB3 and ErbB4) and stimulates several downstream signaling cascades such as MAPK and PI3K/AKT pathways [1]. Because of these properties, HER2 has currently become one of the most important cancer markers, which contribute to resistance to therapy, cancer recurrence and metastasis. Thus, targeting HER2 is crucial for curative therapies in HER2-positive cancer patients [2]. Here, we demonstrated for the first time that genistein has potent inhibition effects on HER2-positive breast and ovarian cancers *in vitro*. Furthermore, we showed that these effects were due to the downregulation of HER2 receptor and downstream signaling by genistein.

One of the key properties of cancer cells is their proliferation ability, and currently, genistein has been reported to have remarkable proliferation inhibition capability on prostate, bladder and other cancers [8-13]. Our previous result has demonstrated that genistein can inhibit

the growth of estrogen-dependent breast and endometrial cancer cells *in vitro* and *in vivo*, which is considered to be mainly through antagonizing with estrogen. In this study, we further found that genistein had a more significant proliferation inhibition and apoptosis induction capacity on HER2-positive breast cancer cell line MCF-7/*Her2* than HER2-negative parent cell line MCF-7 in a time- or dose-dependent manner. In the other regard, the effect of genistein was more remarkable on all the cancer cell lines in our study, i.e. BT-474, SKOV-3, MCF-7/*Her2* and MCF-7, than tamoxifen which is an antagonist of the estrogen receptor and has been widely used in clinic [14]. The findings implied that genistein was not only an antagonist of the estrogen receptor, and there was a new mechanism of genistein anti-cancer function. By analyzing the mechanism, it was confirmed that genistein could downregulate HER2 receptor and induce its endocytosis, and also suppress its downstream AKT activity, which is a serine/threonine-specific protein kinase and plays a key role in cell proliferation, apoptosis, transcription and cell migration [15].

A number of reports in 2006 and 2007 described that geldanamycin and its analogues had potent antiproliferative activity in many cancer cell lines and also decreased cellular levels of client proteins involved in cancer cell survival, including mutated p53, Akt and HER2, through binding to heat shock protein (Hsp)90 and inhibiting its ATPase activity [16,17]. So it is clear that the anti-cancer effects and pathways of geldanamycin and its analogues are similar with those of genistein. However, Basak Shashwati reported that genistein might induce an epigenetic change by its antiestrogenic activity to inhibit Hsp90 activity, which was distinct from geldanamycin and its analogues [18]. For this regard, the details need be further explored.

CONCLUSION:

In conclusion, our findings demonstrate for the first time that genistein, a natural compound, may be effective for HER2-positive cancer prevention and treatment, and its action may be, at least in part, via targeting HER2 receptor and its signaling pathway.

Abbreviations: ECD, extracellular domain; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hsp90, heat shock protein 90; MAPK, mitogen-activated protein kinase; p-AKT, phosphorylated AKT.

Competing Interests: The authors have no conflicts of interest.

Authors' Contributions: The first four authors contribute to this work equally. Guodong Shen: helped with design of project, supervised study progress, data analysis, and write-up; Haiying Yu: Study coordinator; Geng Bian: Study coordinator; Min Gao: Study coordinator; Linqing Liu: Statistical analysis; Min Cheng: helped with study design and data analysis; Gan Shen: helped with study design; Shilian Hu: Principal Investigator, Study design, Clinical supervision.

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