Mol. Nutr. Food Res. 2008, *52*, 439 – 446 DOI 10.1002/mnfr.200700218

Research Article

Puma is a novel target of soy isoflavone genistein but is dispensable for genistein-induced cell fate determination

Moe Tategu, Takako Arauchi, Rena Tanaka, Hiroki Nakagawa and Kenichi Yoshida

Department of Life Sciences, Meiji University School of Agriculture, Kanagawa, Japan

Here, we attempted to identify novel target genes of genistein in human A549 cells. Using analysis of proteins related to cell cycle and apoptotic pathways, we confirmed an elevated level of p53 accompanying p21 Waf1/Cip1 protein in genistein-treated or genistin-treated A549 and WI-38 cells, but not in HeLa cells. In addition, a p53-upregulated modulator of apoptosis (Puma) protein accumulated significantly in genistein-treated A549 and WI-38 cells, but not in genistin-treated or β-estradiol-treated cells, though the growth of any ingredient-treated cells was severely inhibited. Intriguingly, the caspase-3 activity of genistein-treated A549 cells, in which Puma or p53 expression was knocked-down by RNA interference (RNAi), remained unaltered compared to that in cells transfected with irrelevant RNAi. These results raise a concern that molecular targets identified by powerful omic approaches may not necessarily represent key molecules responsible for given cellular phenotypes and thus must be verified by conclusive assays.

Keywords: Apoptosis / Genistein / Puma / p53 / RNA interference

Received: June 17, 2007; revised: August 15, 2007; accepted: August 24, 2007

1 Introduction

Soy isoflavones are regarded as beneficial healthy foods in Asian countries [1]. Recent concerns about possible adverse effects from the over-consumption of isoflavones have been raised, however, because isoflavones share structural homologies with estrogens and modulate the activity of estrogen receptors. Especially in Japan, the increased use of dietary supplements containing isoflavones in women has raised concern about the well being of fetuses. Soy isoflavone exists in the form of aglycones (genistein, daidzein, and glycitein) and glucosides (genistin, daidzin, and glycitin) [2]. Basically, isoflavone naturally exists in the form of glucosides. Absorption of isoflavones is likely to occur in the small intestine where gut microflora hydrolyzes conjugated isoflavones, and releasing the bioactive aglycones for absorption or further metabolism and reconjugation [3]. Among aglycones, genistein, a major isoflavone aglycon component of soybean (*Glycine max*), possesses the most potent action on estrogen receptor binding [4]. Besides estrogen receptor-mediated biological activities, isoflavones have other unique effects on specific target molecules and signaling pathways. Genistein is known to inhibit cell growth in breast and prostate cancer cell lines [5 – 7]; this anti-cancer effect may arise through the inhibition of protein tyrosine kinases, such as epidermal growth factor receptor and insulin-like growth factor-I receptor, and topoisomerase II, which participates in DNA replication, transcription and repair [8 – 14]. Intensive molecular target searches have shown that genistein inhibits many cell cycle-related genes, including cyclin B, CDC25A, Ki67, and TGF-β [7, 15 – 17]. On the other hand, genistein induces p21 Waf1/Cip1 expression [18 – 22]. Genistein also regulates apoptotic pathways by down-regulating Bcl-2, Bcl-X_L, HER-2/neu and up-regulating Bax protein in cancer cells [19, 23 – 25]. These findings strongly suggest that genistein inhibits cancer cell growth by inducing apoptotic pathway genes as well as arresting cell growth and proliferation [24, 26]. However, genistein can promote cell proliferation in breast cancer cells [27].

A previous study demonstrated that genistein induces increased ATM protein kinase activity, the ATM-dependent phosphorylation of p53 on serine 15, the activation of the

Correspondence: Dr. Kenichi Yoshida, Department of Life Sciences, Meiji University School of Agriculture, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan
E-mail: yoshida@isc.meiji.ac.jp
Fax: +81-44-934-7107

Abbreviations: BrdU, 5-bromo-2′-deoxy-uridine; **Puma,** p53-upregulated modulator of apoptosis; **RNAi,** RNA interference

DNA-binding properties of p53, and the phosphorylation of p53 at serines 6, 9, 15, 20, 46, and 392 [20, 28 – 30]. In the human mammary epithelial cell line MCF-10F, genistein increased p21 Waf1/Cip1 and the expression of p53 [22]. The induction of p21 Waf1/Cip1 in A549 cells has been reported to occur in a p53-dependent manner [21]. In contrast, the genistein-induced increase in p21Waf1/Cip1 in a number of breast carcinoma cell lines is mediated through estrogen receptor and p53-independent mechanisms [31]. A previous study reported that genistein induces apoptosis in non-small-cell lung cancer cells through a p53-independent pathway, whereas p21 Waf1/Cip1 expression was elevated [32, 33]. In human LNCaP prostate cancer cells, genistein arrested the cell cycle at the G2/M phase and was accompanied by the suppression of cyclin B expression and the induction of p21 Waf1/Cip1 in a manner that was independent of p53 [34]. The same phenomenon was also observed in MCF7 cells [35]. Genistin as well as genistein exhibited dose-dependent growth inhibition of bladder cancer cell lines by inducing a G2/M cell cycle arrest [36]. Genistein and genistin markedly reduced motility of breast cancer MDA-MB-231 cells; however, genistein and genistin inhibit motility of breast cancer cells by distinct signaling pathways [37]. Moreover, in human ovarian SK-OV-3 cells, genistein caused cell cycle arrest at G2/M phase, and genistin caused cell cycle arrest not only at G2/M phase but also at G1 phase [38].

In this study, we aimed to identify novel biomarkers related to cell cycle and apoptotic signaling that were induced by soy isoflavones, genistein and genistin, in A549 cells. We found that, a p53-upregulated modulator of apoptosis (Puma), appeared to be a novel target of genistein in A549 cells. However, the RNA interference (RNAi)-mediated suppression of Puma as well as p53 protein resulted in unaltered genistein-induced cell growth and proliferation inhibition and apoptosis, compared to irrelevant RNAi-treated A549 cells. Taken together, our results suggest that Puma, as well as the p53 pathway, is dispensable for the genistein-induced inhibition of cancer cell proliferation and the induction of apoptosis. Genistein may utilize distinct signaling pathways, such as a p53-independent apoptotic pathway, to suppress aberrant cell growth and proliferation.

2 Materials and methods

2.1 Reagents

Genistein was purchased from Wako Chemicals (Richmond, VA). Genistin was purchased from Fujicco (Kobe, Japan). β-estradiol was obtained from Sigma (St. Louis, MO). Phospho-Chk1 (Ser345), Phospho-Chk2 (Thr68), p53, Phospho-p53 (Ser15), Phospho-p53 (Ser20), Phospho-p53 (Ser46), p21 Waf1/Cip1, Puma, Bmf, Bim, Bik, Bax, Bok, Bad, Phospho-Bad (Ser112), Cleaved Caspase-7 (Asp198), Cleaved PARP (Asp214), Phospho-pRb (Ser795), Phospho-pRb (Ser807/811), p15 Ink4B, p16 Ink4A, p27 Kip1, Phospho-Cdc2 (Tyr15), Cdk4, Cdk6, Cyclin D1, Cyclin D3, and c-Jun antibodies were purchased from Cell Signaling Technology (Danvers, MA). Chk1, Chk2, E2F1, Cdk2, Cyclin A, Cyclin B1, Cyclin E, and Geminin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-E2F1 (Ser364) antibody was purchased from Abcam (Cambridge, UK). TopBP1 antibody was purchased from BD Biosciences (San Diego, CA). GAPDH antibody was purchased from Applied Biosystems (Foster City, CA). Alkaline phosphatase (AP)-labeled secondary antibodies were purchased from Promega (Madison, WI). Cell Proliferation Reagent WST-1 was purchased from Roche Diagnostics (Indianapolis, IN). The CaspACE Assay System, Colorimetric, was purchased from Promega.

2.2 Cell lines and cell cultures

A549, HeLa, and WI-38 cells were obtained from public institutions and were cultured in modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum in a 5% CO_2 humidified atmosphere at 37°C. To prepare for protein lysis, the cells were plated into a 60 -mm dish at a concentration of $0.5\sim1 \times 10^6$/dish 24 h before chemical treatment and sample collection. The chemical treatment control cells were treated with 0.1% DMSO (the solvent for the chemicals) at the indicated time.

2.3 WST-1 assay

The cells were grown into a 24-well plate at a concentration of $1\sim2 \times 10^4$ cells/well 24 h before treatment. Genistein and genistin (0 – 200 μM for each compound) was added to A549 and WI-38 cells, respectively, or 24 and 48 h. β-estradiol (0 – 100 μM) was added to A549 cells or 24 and 48 hours. WST-1 assay was then performed, according to the manufacturer's protocol. Briefly, WST-1 solution was added to each well, followed by 1 h of incubation at 37°C, and the absorbance was measured at 450 nm. The reference wavelength was 600 nm. Cell viability was calculated as the absorbance in the treated cells over the control (treated with 0.1% DMSO).

2.4 5-bromo-2′-deoxy-uridine (BrdU) incorporation assay

To evaluate DNA synthesis, BrdU labeling was performed, according to the manufacturer's protocol (BrdU labeling and detection kit III, Roche). Briefly, 8×10^3 cells were plated into a 96-well plate. Two days later, the cells were treated with DMSO or genistein for 24 h. Then, the cells were prelabeled with 10 μM of BrdU for 16 h before treatment. After incubation with a peroxidase-conjugated anti-BrdU monoclonal antibody for 30 min at 37°C, BrdU label-

ing was visualized using the peroxidase substrate and was measured at 405 nm with a reference wavelength of 490 nm.

2.5 Caspase-3 assay

The induction of apoptosis was assessed using a CaspACE Assay System, colorimetirc (Promega) according to the manufacturer's instructions. This assay is based on the quantitative determination of free chromophore p-nitroaniline, which is released from the colorimetric substrates upon cleavage by caspase-3. Free p-nitroaniline produces a yellow color that can be monitored using spectrophotometry at 405 nm. In brief, cells in a six-well plate were treated with genistein at the indicated concentrations and times. Both floating and adherent cells were collected for lysis preparation.

2.6 Western blot

Control and isoflavonoid-treated cells were lyzed using a lysis buffer [39] on ice for 30 min. The protein concentration was then determined using the Quick Start Bradford Dye Reagent kit (Bio-Rad Laboratories Inc., Hercules, CA). Twenty micrograms (or 40 μg for the WI-38 cells) of protein lysate were loaded onto each lane of a gel. The proteins were then separated using 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with a specific primary antibody, followed by washing and probing with a corresponding secondary antibody. The specific protein band was visualized using a Western blue stabilized substrate (Promega).

2.7 Stealth RNA transfection

To silence the expression of Puma (GenBank Accession Number NM_014417) in A549 cells, Puma-specific Stealth Select RNAi (BBC3-HSS146893, BBC3-HSS 146894, and BBC3-HSS 146895) and the Stealth RNAi Negative Control Duplex (Invitrogen) was transfected into the cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The Validated Stealth RNAi DuoPak was also used to eliminate p53 (Invitrogen). The sequences of the siRNAs used in this work are as follows: BBC3-HSS 146893; ACGGCUGAUGGACUCAGCAUCGGAA and UUCCGAUGCUGAGUCCAUCAGCCGU, BBC3-HSS 146894; GCAAAUGAGCCAAACGUGACCACUA and UAGUGGUCACGUUUGGCUCAUUUGC, BBC3-HSS 146895; CCCAUCAAUCCCAUUGCAUAGGUUU and AAACCUAUGCAAUGGGAUUGAUGGG. For the preparation of the cell lysate, 2×10^5 cells were plated into each well of a six6-well plate 24 h before transfection. The cells in each well were transfected with 125 pmol of control, Puma, or p53-specific Stealth RNA on consecutive days. Genistein was added 24 h after transfection.

2.8 Statistical analysis

Statistical analysis was performed using Student's *t* test between treated and untreated samples. *P* values less than 0.05 indicate statistical significance. Treatment was repeated at least in three wells and the experiment was repeated twice. All numerical data were expressed as the average of the values obtained for each group per time point. Data are shown as means ± SD.

3 Results

3.1 Soy isoflavone, genistin, and genistein suppress the growth of A549 and WI-38 cells

To compare the cellular responses regulated by genistin or genistein in human lung carcinoma A549 cells and primary fibroblast WI-38 cells, both of which contain wild-type p53, we first examined cell growth/proliferation inhibition of the two cell lines treated with genistin or genistein. Exponentially growing cells were exposed to increasing concentrations of genistin or genistein for 24 and 48 h, and cell viability was assessed using a WST-1 assay. As shown in Fig. 1(A), genistin showed a little effect on the growth of A549 cells. In contrast, 50 μM of genistein severely suppressed the growth of A549 cells. Cell viability decreased by 60%, when compared with that in DMSO-treated cells (100% growth). Genistin suppressed the viability of WI-38 cells, whereas the response pattern suggested that genistein inhibited WI-38 cells in a concentration dependent manner, resembling the situation observed for A549 cells. Longer exposures produced an even more potent effect in all cells except for genistin-treated A549 cells (Fig. 1(A)). Taken together, suppressive effect of genistin or genistein on the cell growth/proliferation was almost the same between A549 cells and WI-38 cells. Therefore, we have determined to select A549 cancer cells and aimed to identify molecular markers highly induced by genistein. Before conducting Western blot analyses, we checked whether the cell growth/proliferation suppression observed in A549 cells was caused by cell cycle arrest or apoptosis. We conducted BrdU incorporation and caspase-3 activity assays in 100 or 200 μM of genistein-treated A549 cells. A549 cells were treated with genistein for 24 h, and BrdU was added to the culture medium for 16 h. DNA replication activity based on BrdU incorporation was equally suppressed both in 100 or 200 μM of genistein-treated A549 cells (Fig. 1(B)). On the other hand, apoptosis based on caspase-3 activity showed that the genistein-induced apoptosis in the A549 cells was time and dose dependent (Fig. 1(C)). Regardless of incubation time (24 or 48 h), 200 μM of genistein was enough for inducing caspase-3 activity in A549 cells. In contrast, 100 μM of genistein for 48 h was enough for inducing caspase-3 activity in A549 cells, whereas 100 μM of genistein for 24 h was not. Taken together, these data suggest that cell

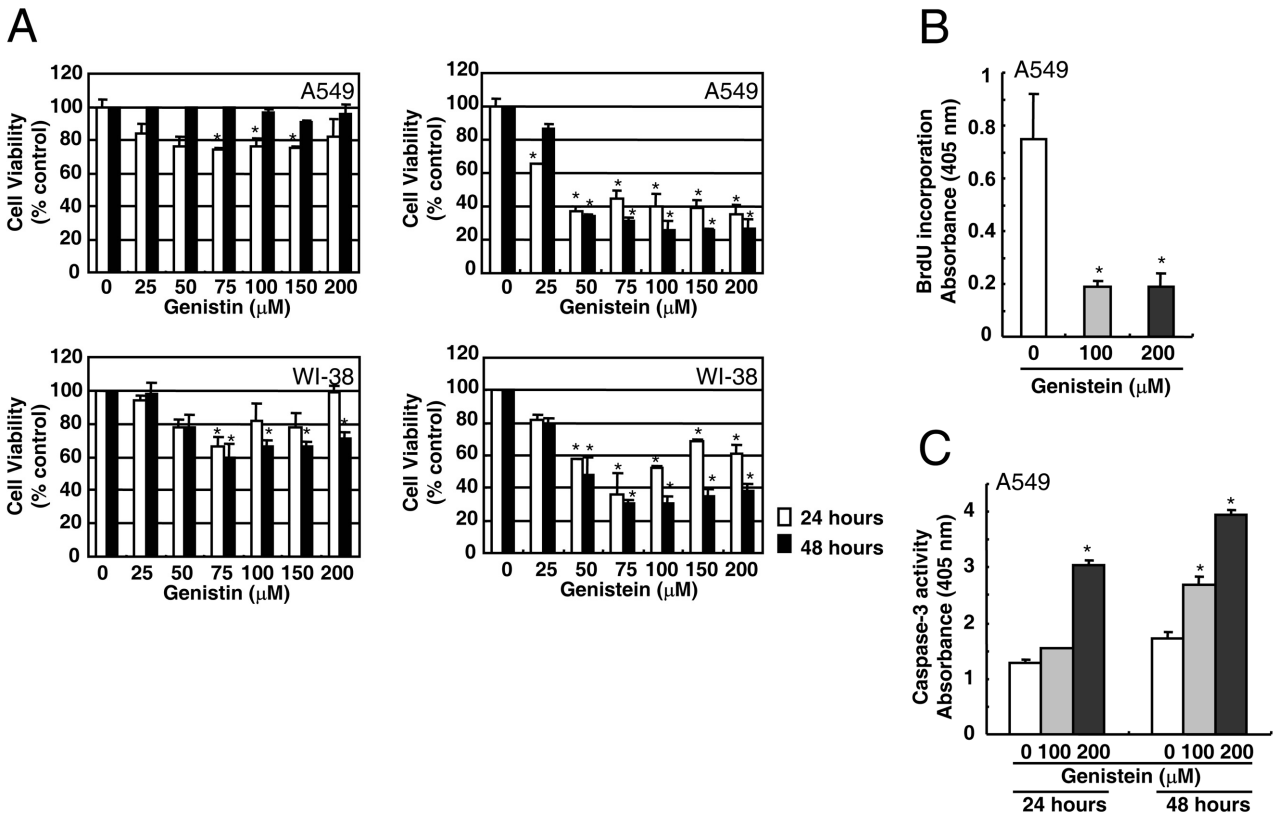

Figure 1. Responses in A549 and WI-38 cells treated with soy isoflavones, genistin and genistein. (A) Cells from each line were plated into 24-well plates at 1~2 $\times$ 10^4 cells/well. Chemicals were added for 24 (indicated by white box) or 48 h (indicated by black box) at the indicated concentrations. Cell viability (% control) was measured using a WST-1 assay, as detailed in Section 2. Values are expressed as means $\pm$ SD (n=6 from two independent experiments). Asterisk, $p < 0.05$ (compared with DMSO-treated cells), based on a Student's t-test. (B) BrdU incorporation after genistein treatment in A549 cells. Genistein was treated for the indicated concentrations. Values are expressed as means $\pm$ SD (n=6). Asterisk, $p < 0.05$ (compared with DMSO-treated cells), based on a Student's t-test. (C) Genistein-induced apoptosis in A549 cells. Cells from each line were plated into six well plates at 1 $\times$ 10^6/well for 24 h, followed by genistein treatment for the indicated concentrations and periods. Apoptosis in the treated cells was detected by a spectrophotometer at 405 nm, based on caspase-3 activity. An average of two experiments is shown ($\pm$ SD). Asterisk, $p < 0.05$ (compared with DMSO-treated cells), based on a Student's t-test.

cycle arrest and apoptosis are important mechanisms for the genistein-induced inhibition of cell growth/proliferation in A549 cells.

3.2 Differential regulation of cell cycle and apoptotic factors in human cells exposed to genistin and genistein

We examined the protein levels or phosphorylation status of various cell cycle and apoptotic factors in A549 cells together with WI-38 cells and p53-negative HeLa cells. Although 100 μM of genistein for 24 h was not enough for inducing caspase-3 activity in A549 cells, genistin or genistein (each 100 μM) was added to the culture medium of the cells for 24 h. This is because we aimed to identify effectors rather highly expressed in an early stage of genistein treatment to the A549 cells. The recovered cell lysates were then subjected to immunoblot analyses. DNA damaging/checkpoint pathways, like Chk1/Chk2, p53, and p21 Waf1/Cip1, were examined first. An immunoblot analysis indicated that the Chk1/Chk2 proteins and the phosphorylation of Chk1 Ser345 were unaffected in all of the cells (Fig. 2). An increased level of p53 accompanying the phosphorylation of Ser15 was detected in both A549 and WI-38 cells, but not in HeLa cells-though the phosphorylation of Chk2 Thr68 was observed in HeLa and A549 cells when they were treated with genistin and genistein (Fig. 2). The phosphorylation of p53 Ser20 was only seen in genistein-treated A549 cells, whereas the Ser46 status was unaffected in all of the cells examined (Fig. 2). As a novel finding, Puma was strongly up-regulated in genistein-treated A549 cells and weakly observed in genistein-treated WI-38 cells, but was not seen in HeLa cells. Interestingly, cleaved caspase-7 (Asp198) and cleaved PARP (Asp214) were detected exclusively in genistein-treated A549 cells, but not in WI-38 or HeLa cells, suggesting that Puma may be a plausible molec-

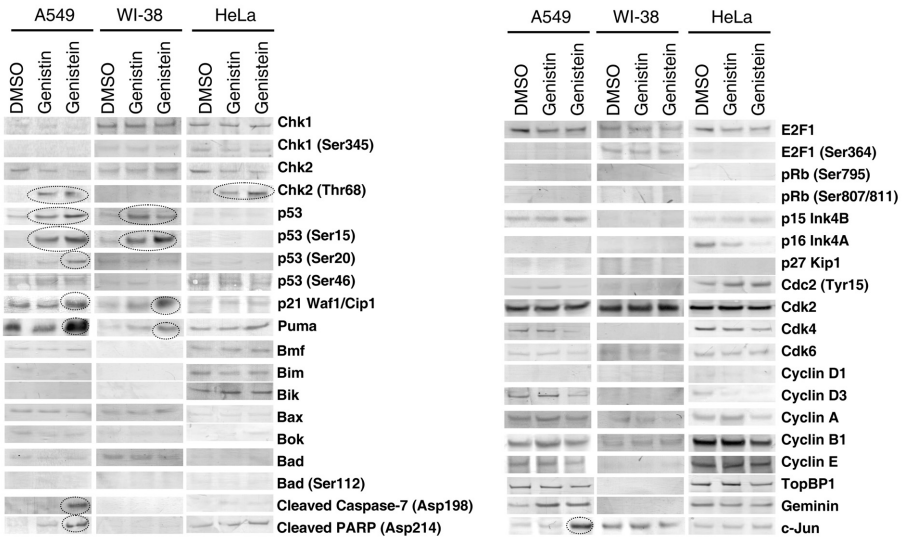

Figure. 2. Protein levels of cell cycle and apoptotic factors among A549, WI-38, and HeLa cells. Protein lysates were prepared from non-synchronized cells of the indicated cell lines that had been pretreated with DMSO, genistin (100 μM), or genistein (100 μM) for 24 h. The indicated proteins were probed using corresponding specific antibodies and examined using a Western blot analysis. Up-regulated proteins were marked with a dotted circle. Data from single experiment.

ular target that specifically suppresses the growth/proliferation of cancer cells by inducing apoptosis (Fig. 2). Genistin failed to induce Puma protein in A549 and WI-38 cells (Fig. 2). Other apoptotic factors, including Bmf, Bim, Bik, Bax, Bok, and Bad, and the phosphorylation of Bad Ser112 were unchanged in any of the cells that were examined. E2F1 can induce apoptosis-like p53, and its protein level increases when DNA is damaged by various anticancer drugs [40]. Both genistin and genistein, however, failed to induce E2F1 expression and the phosphorylation of E2F1 Ser364 (Fig. 2). Other cell cycle regulators, including pRb Ser795, pRb Ser807/811, p15 Ink4B, p16 Ink4A, p27 Kip1, Cdc2 Tyr15, Cdk2, Cdk4, Cdk6, Cyclin D1, Cyclin D3, Cyclin A, Cyclin E, TopBP1, and Geminin, were not dramatically changed except for genistein-treated A549 cells where Cdk4 and Cyclin D3 were decreased (Fig. 2). The expression level of c-Jun increased in genistein-treated A549 cells, suggesting that the upstream signaling of c-Jun is activated (Fig. 2). These results suggest that Puma could be a promising biomarker involved in the regulation of cell fate by genistein in A549 cells.

3.3 Induction of Puma is not seen after β-estradiol treatment

To test whether Puma induction is specific for genistein, we studied the effect of β-estradiol on A549 cells. As shown in Fig. 3(A), the cell viability of β-estradiol-treated A549 cells was inhibited when 100 μM of β-estradiol was added. At this concentration, the phosphorylation of p53 Ser15 was detected; however, the Puma and p21 Waf1/Cip1 levels were unaltered (Fig. 3(B)). In contrast, in A549 and WI-38 cells treated with 50 μM of genistein, Puma and p21 Waf1/Cip1 were induced and the phosphorylation of p53 Ser15 increased (Fig. 3(C)). Collectively, these data suggest that the induction of Puma is specifically induced by genistein, but not by β-estradiol.

3.4 Puma as well as p53 knockdown in A549 cells has no effect on genistein-induced apoptosis

To demonstrate the specific role of Puma in the genistein-mediated inhibition of cell growth/proliferation and apoptosis in A549 cells, we examined the effect of Puma as well as p53 knockdown in genistein-treated A549 cells. The cell lysates were collected 24 h after the genistein treatment (100 μM), because this condition was sufficient to induce Puma in A549 cells. As shown in Fig. 4(A), the transfection of three different Puma-specific stealth RNAis resulted in the significant down-regulation of Genistein-induced Puma protein (compare lane 5 vs. lanes 6, 7, and 8). As well, p53 stealth RNAi reduced the genistein-induced Ser 15 phosphorylation status of p53 in A549 cells (Fig. 4(B)). As expected, two different p53-specific stealth RNAis resulted in down-regulation of genistein-induced Puma protein (compare lane 4 vs. lanes 5 and 6). GAPDH was equally detected in all lanes (Fig. 4(B), bottom panel).

Surprisingly, Puma as well as p53 knockdown failed to reduce apoptosis induced by 100 μM genistein treatment in A549 cells for 48 h, as compared to that in cells transfected with control oligonucleotides (Fig. 4(C)). We selected a condition as 100 μM genistein for 48 h, because, as we have shown above, 100 μM genistein for 24 h was insufficient to induce caspase-3 activity in A549 cells. Consistently, Puma as well as p53 knockdown in A549 cells led to no changes in the WST-1 assay (data not shown). Taken together, these

results demonstrate that Puma, as well as p53, are not functionally involved in genistein-induced apoptosis, though Puma and p53 protein do accumulate in A549 cells after genistein treatment.

4 Discussion

In this report, we showed for the first time that Puma, a p53-upregulated modulator of apoptosis, is highly up-regulated in A549 cells; however, a functional analysis revealed that Puma, as well as p53, were not involved in genistein-induced apoptosis in A549 cells. Puma binds to Bcl-2, is localized to the mitochondria and induces cytochrome c release, and activates the rapid induction of programmed cell death [41]. Because Puma and Noxa are critical mediators of the apoptotic responses induced by p53 and other agents [42], this molecular pathway may be more prominent when genistein is coupled with other chemotherapeutic agents, such as etoposide. Indeed, the accumulation of Puma is also seen by etoposide treatment, but not by 5-fluorouracil treatment, in A549 cells (data not shown). Both etoposide and genistein are known topoisomerase II inhibitors [29].

First of all, we compared the effects of genistin and genistein on cell growth/proliferation. From the WST-1 assay, genistein, but not genistin, severely suppressed the cell viability. The differences between genistein and genistin on the WST-1 assay were probably caused by differences in the efficiency at which they were absorbed into the cell membrane. Interestingly, genistin, but not genistein, failed to induce Puma expression in A549 cells. Based on cell viability assessed by WST-1 assay, suppressive effect of genistein on the cell growth/proliferation was almost the same between A549 cells and WI-38 cells. These phenomena suggest that high concentrations of genistein tend to suppress the cell viability regardless of cell types.

Genistein-induced inhibition of cell growth/proliferation in A549 cells could be explained by cell cycle arrest or apoptosis. Therefore we aimed to identify protein markers specifically expressed in A549 cells after genistein treatment. By conducting a series of Western blotting using various antibodies against proteins related to cell cycle and apoptosis, we identified Puma as a plausible marker. Considerable work has focused on the identification of key molecules regarding antiproliferative activities exhibited by genistein. p53 is one of the most intensively examined protein whether it could be involved in genistein-induced antiproliferative activities. It has been shown that genistein induced phosphorylation of p53 at serines 6, 9, 15, 20, 46, and 392 in human lymphoblastoid cell lines [28]. Genistein is also known to induce the p21 Waf1/Cip1 expression in a p53-dependent or p53-independent manner [20–22, 31–34]. Regardless of p53 status, genistein could induce apoptosis and inhibit proliferation in a variety of human cancer

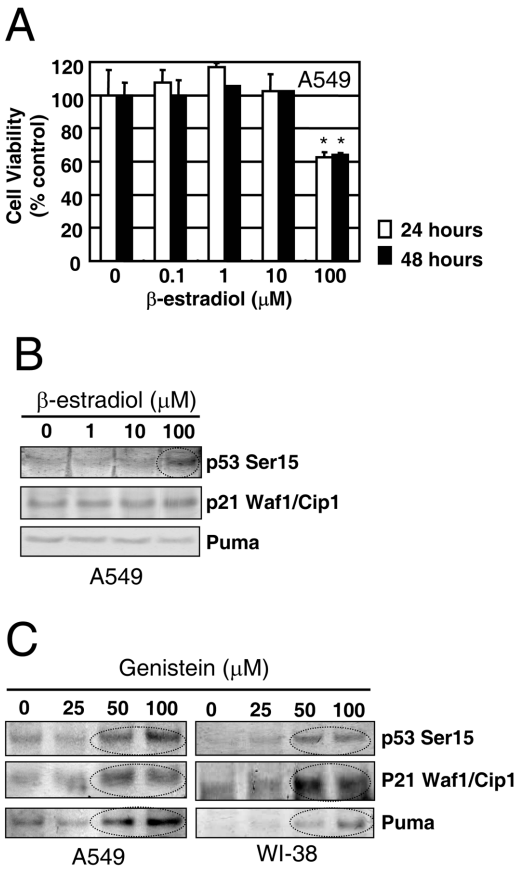

Figure 3. Responses in A549 cells treated with β-estradiol. (A) The cell viability of β-estradiol-treated A549 cells was determined as described in Fig. 1. Values are expressed as means ± SD (n=6 from two independent experiments). Asterisk, $p < 0.05$ (compared with DMSO-treated cells), based on a Student's t-test. (B) The status of phosphorylation of Ser15 in p53, and protein levels of p21 Waf1/Cip1 and Puma in A549 cells after incubation with β-estradiol (0, 1, 10, and 100 μM) for 24 h. Data from single experiment. (C) The status of phosphorylation of Ser15 in p53, and protein levels of p21 Waf1/Cip1 and Puma in A549 and WI-38 cells after incubation with Genistein (0, 25, 50, and 100 μM) for 24 h. The up-regulated proteins were marked with a dotted circle. Data from single experiment.

cell lines [43]. From our results, the phosphorylation of p53 Ser15 was detected in both A549 and WI-38 cells, whereas the Ser20 status was only affected in genistein-treated A549 cells. An increased level of p21 Waf1/Cip1 was manifested in both A549 and WI-38 cells after genistein incubation. In contrast, high expression of Puma was solely detected in genistein-treated A549 cells but not in WI-38 cells. Remarkably, 100 μM of β-estradiol induced the phosphorylation of p53 Ser15 but not the Puma in A549 cells, whereas 50 μM of genistein was enough to induce Puma expression as well as the phosphorylation of p53 Ser15.

p53 knockdown failed to reduce apoptosis, suggesting that p53 is not functionally involved in genistein-induced

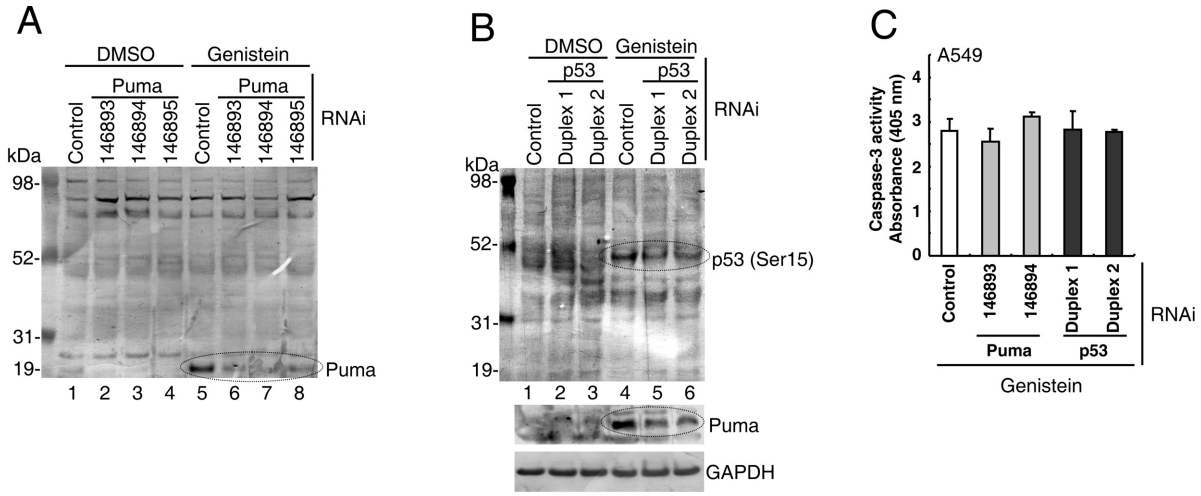

Figure 4. Genistein-induced caspase-3 activity in Puma or p53 specific knocked down A549 cells. Puma (A) and p53 (B) protein levels in control and gene-specific RNAi transfected cells. A549 cells were transfected with control or gene-specific stealth RNAs, followed by 100 μM of genistein for 24 h. The protein lysates were examined using Western blot analysis and anti-Puma and anti-phospho p53 (Ser15) antibodies. Up-regulated and then knocked down proteins are marked with a dotted circle. Data from single experiment. (C) Genistein was added to A549 cells at a concentration of 200 μM for 24 h. An average of two experiments is shown (± SD).

apoptosis, though p53 protein does accumulate in A549 cells after genistein treatment. Unexpectedly, Puma knock-down also failed to reduce apoptosis induced by genistein in A549 cells. We evaluated apoptosis by examining caspase-3 activity, since caspase-3 is a key member of the caspase family and its involvement in genistein-induced apoptosis has been reported elsewhere [19]. Other than cell cycle and apoptotic pathways, genistein is thought to regulate nuclear factor-kappaB, Akt, and mitogen-activated protein kinase pathways in cancer cells [44]. Therefore, the elucidation of which molecules or which pathways are important as crucial downstream effectors of genistein in certain cell types is needed.

In summary, our data demonstrate that Puma protein is a notable biomarker in genistein-treated cells; however, genistein-treated A549 cells in which Puma or p53 expression had been knocked-down by RNAi showed no changes in caspase-3 activity, compared with irrelevant RNAi-treated cells. Thus, our findings strongly indicate that the molecular targets induced by genistein do not necessarily represent the key molecules responsible for given cellular phenotypes.

This study was partly supported by grant from the Kampou Science Foundation.

The authors have declared no conflict of interest.

5 References

[1] Adlercreutz, H., Epidemiology of phytoestrogens, *Baillieres Clin. Endocrinol. Metab.* 1998, *12*, 605 – 623.

[2] Rowland, I., Faughnan, M., Hoey, L., Wahala, K., *et al*., Bioavailability of phyto-oestrogens, *Br. J. Nutr.* 2003, *89*, S45 – S58.

[3] Turner, N. J., Thomson, B. M., Shaw, I. C., Bioactive isoflavones in functional foods: the importance of gut microflora on bioavailability, *Nutr. Rev.* 2003*, 61*, 204 – 213.

[4] Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., *et al.,* Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, *Endocrinology* 1998, *139*, 4252 – 4263.

[5] Bektic, J., Guggenberger, R., Eder, I. E., Pelzer, A. E., *et al.,* Molecular effects of the isoflavonoid genistein in prostate cancer, *Clin. Prostate Cancer* 2005, *4*, 124 – 129.

[6] Ying, C., Hsu, J. T., Hung, H. C., Lin, D. H., *et al.,* Growth and cell cycle regulation by isoflavones in human breast carcinoma cells, *Reprod. Nutr. Dev.* 2002, *42,* 55 – 64.

[7] Agarwal, R., Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents, *Biochem. Pharmacol.* 2000, *60*, 1051 – 1059.

[8] Strick, R., Strissel, P. L., Borgers, S., Smith, S. L., Rowley, J. D., Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukemia, *Proc. Natl. Acad. Sci. USA* 2000, *97*, 4790 – 4795.

[9] Ravindranath, M. H., Muthugounder, S., Presser, N., Viswanathan, S., Anticancer therapeutic potential of soy isoflavone, genistein, *Adv. Exp. Med. Biol.* 2004, *546*, 121 – 165.

[10] Valachovicova, T., Slivova, V., Sliva, D., Cellular and physiological effects of soy flavonoids, *Mini Rev. Med. Chem.* 2004, *4*, 881 – 887.

[11] Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., *et al.,* Genistein, a specific inhibitor of tyrosine-specific protein kinases, *J. Biol. Chem.* 1987, *262,* 5592 – 5595.

[12] Kim, E. J., Shin, H. K., Park, J. H., Genistein inhibits insulin-like growth factor-I receptor signaling in HT-29 human colon cancer cells: a possible mechanism of the growth inhibitory effect of Genistein, *J. Med. Food* 2005, *8*, 431 – 438.

[13] Koroma, B. M., de Juan, E., Inhibition of protein tyrosine phosphorylation in endothelial cells: relationship to antiproliferative action of genistein, *Biochem. Soc. Trans.* 1997, *25*, 35 – 40.

[14] Kaufmann, W. K., Human topoisomerase II function, tyrosine phosphorylation and cell cycle checkpoints, *Proc. Soc. Exp. Biol. Med*. 1998, *217,* 327 – 334.

[15] Sarkar, F. H., Adsule, S., Padhye, S., Kulkarni, S., Li, Y., The role of genistein and synthetic derivatives of isoflavone in cancer prevention and therapy, *Mini Rev. Med. Chem.* 2006, *6*, 401 – 407.

[16] Kim, H., Peterson, T. G., Barnes, S., Mechanisms of action of the soy isoflavone genistein: emerging role for its effects via transforming growth factor beta signaling pathways, *Am. J. Clin. Nutr.* 1998, *68*, 1418S – 1425S.

[17] McMichael-Phillips, D. F., Harding, C., Morton, M., Roberts, S. A. *et al.,* Effects of soy-protein supplementation on epithelial proliferation in the histologically normal human breast, *Am. J. Clin. Nutr.* 1998, *68*, 1431S – 1435S.

[18] Kuzumaki, T., Kobayashi, T., Ishikawa, K., Genistein induces p21(Cip1/WAF1) expression and blocks the G1 to S phase transition in mouse fibroblast and melanoma cells, *Biochem. Biophys. Res. Commun.* 1998, *251*, 291 – 295.

[19] Li, Y., Upadhyay, S., Bhuiyan, M., Sarkar, F. H., Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein, *Oncogene* 1999, *18,* 3166 – 3172.

[20] Chang, K. L., Kung, M. L., Chow, N. H., Su, S. J., Genistein arrests hepatoma cells at G2/M phase: involvement of ATM activation and upregulation of p21waf1/cip1 and Wee1, *Biochem. Pharmacol.* 2004, *67*, 717 – 726.

[21] Ding, H., Duan, W., Zhu, W. G., Ju, R., *et al.,* P21 response to DNA damage induced by genistein and etoposide in human lung cancer cells, *Biochem. Biophys. Res. Commun.* 2003, *305*, 950 – 956.

[22] Frey, R. S., Li, J., Singletary, K. W., Effects of genistein on cell proliferation and cell cycle arrest in nonneoplastic human mammary epithelial cells: involvement of Cdc2, p21(waf/cip1), p27(kip1), and Cdc25C expression, *Biochem. Pharmacol.* 2001, *61,* 979 – 989.

[23] Su, S. J., Chow, N. H., Kung, M. L., Hung, T. C., Chang, K. L., Effects of soy isoflavones on apoptosis induction and G2-M arrest in human hepatoma cells involvement of caspase-3 activation, Bcl-2 and Bcl-XL downregulation, and Cdc2 kinase activity, *Nutr. Cancer* 2003, *45,* 113 – 123.

[24] Katdare, M., Osborne, M., Telang, N. T., Soy isoflavone genistein modulates cell cycle progression and induces apoptosis in HER-2/neu oncogene expressing human breast epithelial cells, *Int. J. Oncol.* 2002, *21*, 809 – 815.

[25] Constantinou, A. I., Kamath, N., Murley, J. S., Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle, and induces apoptosis of human breast adenocarcinoma MCF-7 cells, *Eur. J. Cancer* 1998, *34,* 1927 – 1934.

[26] Xiang, H., Schevzov, G., Gunning, P., Williams, H. M., Silink, M., A comparative study of growth-inhibitory effects of isoflavones and their metabolites on human breast and prostate cancer cell lines, *Nutr. Cancer* 2002, *42*, 224 – 232.

[27] Murata, M., Midorikawa, K., Koh, M., Umezawa, K., Kawanishi, S., Genistein and daidzein induce cell proliferation and their metabolites cause oxidative DNA damage in relation to isoflavone-induced cancer of estrogen-sensitive organs, *Biochemistry* 2004, *43,* 2569 – 2577.

[28] Ye, R., Goodarzi, A. A., Kurz, E. U., Saito, S., *et al.,* The isoflavonoids genistein and quercetin activate different stress signaling pathways as shown by analysis of site-specific phosphorylation of ATM, p53 and histone H2AX, *DNA Repair (Amst.)* 2004, *3*, 235 – 244.

[29] Ye, R., Bodero, A., Zhou, B. B., Khanna, K. K., *et al.,* The plant isoflavenoid genistein activates p53 and Chk2 in an ATM-dependent manner, *J. Biol. Chem.* 2001, *276,* 4828 – 4833.

[30] Shackelford, R. E., Pharmacologic manipulation of the ataxia-telangiectasia mutated gene product as an intervention in age-related disease, *Med. Hypotheses* 2005, *65,* 363 – 369.

[31] Shao, Z. M., Alpaugh, M. L., Fontana, J. A., Barsky, S. H., Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by P21WAF1/CIP1 induction, G2/M arrest, and apoptosis, *J. Cell. Biochem.* 1998, *69*, 44 – 54.

[32] Lian, F., Li, Y., Bhuiyan, M., Sarkar, F. H., p53-independent apoptosis induced by genistein in lung cancer cells, *Nutr. Cancer* 1999, *33,* 125 – 131.

[33] Lian, F., Bhuiyan, M., Li, Y. W., Wall, N., *et al.,* Genistein-induced G2-M arrest, p21WAF1 upregulation, and apoptosis in a non-small-cell lung cancer cell line, *Nutr. Cancer* 1998, *31*, 184 – 191.

[34] Kobayashi, T., Nakata, T., Kuzumaki, T., Effect of flavonoids on cell cycle progression in prostate cancer cells, *Cancer Lett.* 2002, *176*, 17 – 23.

[35] Xu, J., Loo, G., Different effects of genistein on molecular markers related to apoptosis in two phenotypically dissimilar breast cancer cell lines, *J. Cell. Biochem*. 2001, *82*, 78 – 88.

[36] Zhou, J. R., Mukherjee, P., Gugger, E. T., Tanaka, T., *et al.,* Inhibition of murine bladder tumorigenesis by soy isoflavones via alterations in the cell cycle, apoptosis, and angiogenesis, *Cancer Res*. 1998, *58*, 5231 – 5238.

[37] Valachovicova, T., Slivova, V., Bergman, H., Shuherk, J., Sliva, D., Soy isoflavones suppress invasiveness of breast cancer cells by the inhibition of NF-kappaB/AP-1-dependent and -independent pathways, *Int. J. Oncol.* 2004, *25,* 1389 – 1395.

[38] Choi, E. J., Kim, T., Lee, M. S., Pro-apoptotic effect and cytotoxicity of genistein and genistin in human ovarian cancer SK-OV-3 cells, *Life Sci.* 2007, *80,* 1403 – 1408.

[39] Hayashi, R., Goto, Y., Ikeda, R., Yokoyama, K. K., Yoshida, K., CDCA4 is an E2F transcription factor family-induced nuclear factor that regulates E2F-dependent transcriptional activation and cell proliferation, *J. Biol. Chem.* 2006, *281*, 35633 – 35648.

[40] Stevens, C., La Thangue, N. B., The emerging role of E2F-1 in the DNA damage response and checkpoint control, *DNA Repair (Amst.)* 2004, *3,* 1071 – 1079.

[41] Nakano, K., Vousden, K. H., PUMA, a novel proapoptotic gene, is induced by p53, *Mol. Cell* 2001, *7,* 683 – 694.

[42] Villunger, A., Michalak, E. M., Coultas, L., Mullauer, F., *et al.,* p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa, *Science* 2003, *302*, 1036 – 1038.

[43] Li, Y., Ahmed, F., Ali, S., Philip, P. A., *et al.,* Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells, *Cancer Res*. 2005, *65*, 6934 – 6942.

[44] Aggarwal, B. B., Shishodia, S., Molecular targets of dietary agents for prevention and therapy of cancer, *Biochem. Pharmacol.* 2006, *71*, 1397 – 1421.

