Abstract. BRCA1 acts as a tumour suppressor and germ-line mutations within this gene are found in a large proportion of families with breast cancer. The aim of our study was to unravel the mechanism of action of genistein, the major soy phytoestrogen, in BRCA1-mutant human breast cancer cell lines. Four breast cancer cell lines were studied for their response to genistein, three of them harbouring different mutations within the BRCA1 gene (HCC1937, SUM149 and SUM1315 cells) and the MDA-MB-231 cell line, which expresses a functional BRCA1 protein. We showed that genistein inhibits proliferation and induces apoptosis more efficiently in BRCA1-mutant cells than in cells expressing wild-type BRCA1 protein. Increased AKT and decreased p21WAF1/CIP1 protein levels could explain the relative resistance to genistein elicited by cells with wild-type BRCA1. BRCA1-mutant breast cancer cells are highly sensitive to genistein treatment and p21WAF1/CIP1 and AKT could be genistein targets in these cells.

Worldwide, the distribution of breast cancer is heterogeneous (1), with European and North American populations being more affected than those in African and Asian countries. The study of migrant populations showed that the relative protection of Asian populations is probably linked to lifestyle rather than their genetic background. The typical soy-rich Asian diet was correlated to low breast cancer risk in epidemiological studies (2, 3).

Genistein is the major phytoestrogen found in soy seeds. Its structure, which is similar to oestradiol, allows it to bind to both alpha and beta oestrogen receptors. It has been reported that genistein can inhibit cell growth and induce cell cycle arrest and apoptosis in breast cancer cells (4). Genistein has also been shown to inhibit the growth of carcinogen-induced tumours in rats. Many studies have also shown an inhibitory effect of soy or genistein on breast cancer cell growth in xenografts and metastases in rodent models (5).

A large panel of signalling pathways has been reported to be activated by genistein. This isoflavone has especially been shown to increase BRCA1 expression in breast cancer cells (6, 7). BRCA1 is a tumour suppressor gene known to be mutated in 20-50% of familial breast cancer cases. Seventy percent of women carrying mutations within the BRCA1 gene develop breast cancer before the age of 70 (8), therefore breast cancer prevention strategies are paramount for this particular population.

The objective of our study was to determine the impact of genistein exposure in BRCA1-mutant breast cancer cells.

Materials and Methods

Cell lines and culture. MDA-MB-231 and HCC1937 human breast cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA) and were grown in RPMI medium supplemented with 10% foetal calf serum, 2 mM L-glutamine and 20 μg/ml gentamicin. SUM149 and SUM1315 human breast cancer cell lines were obtained from Asterand (Hertfordshire, UK) and grown in Ham's F12 medium according to the manufacturer's instructions. All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO₂.

Chemicals. Genistein was purchased from Sigma Aldrich (St. Quentin Fallavier, France) and 100 mM stock solutions were prepared with dimethylsulfoxide (DMSO), stored at –20°C; final working solutions did not exceed 0.1% DMSO.

Exposure to genistein. Cells were plated in tissue culture dishes on day 1 at 10,000 cells/cm². On day 2, they were exposed for 24 hours to genistein (1, 10 and 50 μM) while control cells were exposed to...
the corresponding DMSO dilution. Each experiment was performed at least three times.

**Cytotoxicity assay.** Studies were performed using the sulforhodamine B (SRB) assay. Briefly, 48 hours after exposure to different concentrations of genistein (1-100 μM), 10% trichloroacetic acid was added to the culture medium in order to fix cells for one hour at 4°C. Cells were then stained with 0.4% SRB in 1% acetic acid and washed with 1% acetic acid. SRB was then solubilized in Tris base (10 mM; pH 10.5) and optical density at 540 nm was determined using a microplate reader.

**Analysis of apoptosis.** The percentage of apoptotic cells was determined using AnnexinV-phycoceryrin (PE) apoptosis detection kit (BD Pharmingen, Le Pont de Claix, France). Briefly, cells were washed in phosphate-buffered saline, harvested by trypsinization and incubated for 15 min with AnnexinV-PE and 7 amino-actinomycin D as recommended by the manufacturer. Analysis of at least 10,000 events was performed using a Coulter Epic XL flow cytometer (Beckman Coulter, Villepinte, France).

**RNA extraction and quantitative RT-PCR.** Total RNA was extracted from cell cultures using RNA PLUS reagent (QBiogene, France) according to the manufacturer’s protocol. The quality of RNA was assessed using a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Five micrograms of RNA were then reverse-transcribed using First-strand cDNA Synthesis Kit (GE Healthcare, Velizy Villacoublay, France). Multiplex quantitative RT-PCR was performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Predesigned and validated gene-specific probe-based TaqMan Gene Expression Assays (PE Biosystems, Villebon sur Yvette, France) were performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Five micrograms of RNA were then reverse-transcribed using First-strand cDNA Synthesis Kit (GE Healthcare, Velizy Villacoublay, France). Multiplex quantitative RT-PCR was performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Predesigned and validated gene-specific probe-based TaqMan Gene Expression Assays (PE Biosystems, Villebon sur Yvette, France) were performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Five micrograms of RNA were then reverse-transcribed using First-strand cDNA Synthesis Kit (GE Healthcare, Velizy Villacoublay, France). Multiplex quantitative RT-PCR was performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Predesigned and validated gene-specific probe-based TaqMan Gene Expression Assays (PE Biosystems, Villebon sur Yvette, France) were performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Five micrograms of RNA were then reverse-transcribed using First-strand cDNA Synthesis Kit (GE Healthcare, Velizy Villacoublay, France). Multiplex quantitative RT-PCR was performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Predesigned and validated gene-specific probe-based TaqMan Gene Expression Assays (PE Biosystems, Villebon sur Yvette, France) were performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Five micrograms of RNA were then reverse-transcribed using First-strand cDNA Synthesis Kit (GE Healthcare, Velizy Villacoublay, France). Multiplex quantitative RT-PCR was performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Predesigned and validated gene-specific probe-based TaqMan Gene Expression Assays (PE Biosystems, Villebon sur Yvette, France) were performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France). Five micrograms of RNA were then reverse-transcribed using First-strand cDNA Synthesis Kit (GE Healthcare, Velizy Villacoublay, France). Multiplex quantitative RT-PCR was performed using an ABI Prism 7700 thermal cycler (Applied Biosystems, Villebon sur Yvette, France).

**Western blotting.** Proteins were extracted from cells with a lysis buffer containing 50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.25% Triton X-100 and 1 mM dithiothreitol (DTT). Protease inhibitors (Complete® protease inhibitor cocktail; Roche, Rosny-sous-bois, France) and phosphatase inhibitors (50 mM NaF, 1 mM Na3VO4) were added to the basic buffer. Fifty micrograms of protein were electrophoresed on a 6% or 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. After one-hour blocking in Tris-buffered saline-Tween 0.1% (TBST) containing 5% milk, membranes were incubated overnight at 4°C with primary antibody 2922 anti-AKT from Cell Signaling Technology, Danvers, MA, USA, and sc-817 anti-p21WAF1/CIP1 from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then washed 3 times in TBST and incubated for one hour with horseradish peroxidase-conjugated secondary antibody (1:2000). Immunodetection was performed with the ECL detection system (GE Healthcare, Velizy Villacoublay, France).

**Immunofluorescence assay.** Cells were seeded in tissue culture chambers (Becton-Dickinson, Le Pont de Claix, France). They were then fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100 and incubated overnight in a blocking solution (2% bovine serum albumin, 5% foetal calf serum) at 4°C. Incubations with a mouse anti-BRCA1 primary antibody (Ab-1 1/100; Merck, Nottingham, UK) and with a fluorescein isothiocyanate (FITC)-conjugated Alexa 488 anti-mouse IgG secondary antibody (Invitrogen, Carlsbad, NM, USA) were carried out for 1 h at room temperature in the dark. Slides were mounted using 4'-6'-diamidino-2-phenylindole (DAPI)-containing Vectashield medium (Vector Laboratories, Burlingame, CA, USA) and examined using a LSM510 Meta (Carl Zeiss, Le Pecq, France) confocal microscope.

**Statistical tests.** Statistical analyses were performed using two-sided Student’s t-test. A p-value <0.05 was considered as significant.

**Results**

Four human breast cancer cell lines were used in order to study the role of BRCA1 in cell response to genistein (Figure 1A). MDA-MB-231 cells express wild-type BRCA1 protein consisting of 1863 amino acids. In HCC1937, SUM149 and SUM1315 cell lines, BRCA1 is mutated at distinct spots, which results in truncated BRCA1 proteins of different sizes (10). In the Breast Cancer Information Core database (http://research.nhgri.nih.gov/bic/), 185delAG (or c.68_69delAG which is detected in SUM1315) and 5382insC (or c.5266dupC) mutations (present in HCC1937 cells) are the two most frequent mutations found in BRCA1-mutant breast cancer families. In order to localize the BRCA1 protein expressed in these cell lines, immunofluorescence analyses were performed using an anti-BRCA1 antibody that recognises the N-terminal part of the protein (Figure 1B). MDA-MB-231 cells express a nuclear BRCA1 protein, whereas BRCA1 is cytoplasmic in the HCC1937 cell line, which suggests an impaired protein function. BRCA1 was not detected in SUM149, nor in SUM1315 cells.

**BRCA1-mutant breast cancer cells are more sensitive to genistein than are wild-type cells.** We addressed the possibility that BRCA1 mutations may modulate the effect of genistein on cell proliferation. The SRB assay (Figure 2A) was thus performed for the four cell lines exposed to increasing concentrations of genistein. Physiological genistein concentrations (≤10 μM) (11-13) had no effect on the growth of MDA-MB-231 cells, which express wild-type BRCA1 protein. Genistein inhibited cell proliferation when concentrations exceeding 10 μM were used and the 50% inhibitory concentration (IC50) was in excess of 100 μM.

In breast cancer cells harbouring a mutated BRCA1 gene, exposure to genistein resulted in a stronger inhibition of cell growth, with the SUM149 cell line being the most sensitive (IC50=13 μM). SUM1315 cell proliferation was also greatly reduced, by even lower genistein concentrations (IC50=29 μM). Exposure to genistein had a lesser impact in HCC1937 cells, the third cell line carrying a BRCA1 mutation, although growth inhibition in these cells was significantly higher than in MDA-MB-231 cells (IC50=86 μM, p=0.02).
Genistein exerts different effects on apoptosis depending on the cell line used. The percentage of apoptotic cells was then determined in the four breast cancer cell lines using the Annexin V test (Figure 2B). The apoptotic cell population was significantly larger in MDA-MB-231 controls (7.1%) than in the three untreated cell lines expressing mutant BRCA1 (1.2, 1.6 and 1.9% for HCC1937, SUM149 and SUM1315 respectively).

In response to genistein treatment, a significant increase in the apoptotic population was observed in all three BRCA1-mutant cell lines. In HCC1937 and SUM149 cells, apoptosis increased upon exposure to a pharmacological concentration (50 μM) and a significant increase was observed at both physiological and pharmacological concentrations of genistein in SUM1315 cells. In contrast, genistein did not induce apoptosis in MDA-MB-231 cells with wild-type BRCA1.

Genistein target genes are differentially regulated depending on BRCA1 status. We then addressed whether gene signaling known to be regulated by genistein was altered in BRCA1-mutant cells. Real-time PCR experiments were performed to evaluate the effect of genistein exposure on the transcription of some phytoestrogen target genes. We chose a panel of 11 genes for which transcript levels have been demonstrated to be regulated upon genistein exposure in cells with wild-type

BRCA1. BARD1, GADD45 and p21WAF1/CIP1 transcript levels were previously shown to be increased by genistein in MDA-MB-231 cells (14, 15). Ise and co-workers showed an increase in PDZK1, CTIP and TPD52L1 mRNA levels in MCF-7 cells exposed to 10 μM genistein (16). CHK2 expression was also down-regulated by genistein in neuroblastoma cells (17) and AKT expression was reduced in Panc1 cell line (18). ERA, ERB and PS2 gene expressions were also measured in order to monitor the oestrogen receptor-dependent response to genistein.
In response to genistein, transcript levels of 9 of these 11 genes were regulated, as expected from the literature, without any differences between BRCA1-mutant and BRCA1 wild-type cell lines (data not shown). More interestingly, AKT and p21WAF1/CIP1 transcript levels were differentially regulated depending on BRCA1 status (Figure 3).

p21WAF1/CIP1 transcript levels were higher in BRCA1 wild-type cells than in the three BRCA1-mutant cell lines (Figure 3A). Furthermore, exposure to 50 μM genistein only resulted in an increase in p21WAF1/CIP1 mRNA levels (two-fold) in MDA-MB-231 cells (Figure 3A).

AKT mRNA levels were slightly more prominent in BRCA1 wild-type MDA-MB-231 cells when compared to the three BRCA1-mutant cell lines (Figure 3B, p<0.05 for SUM149 and SUM1315 cells). Moreover, these levels were only increased in response to 50 μM genistein in MDA-MB-231 cells (Figure 3B).

As mRNA levels were often shown to poorly correlate with protein levels (19), we used the latter endpoint to assess the regulatory effect of genistein in MDA-MB-231 cells expressing wild-type BRCA1 and in SUM1315 cells expressing the shorter theoretical BRCA1 protein (Figure 1A). MDA-MB-231 and SUM1315 cell response to 50 μM genistein was thus evaluated by Western blotting (Figure 4). In the absence of genistein, MDA-MB-231 cells expressed three times as much p21 protein as did SUM1315 cells (Figure 4A), which was consistent with RT-PCR results. However, the p21WAF1/CIP1 mRNA level increase in response to 50 μM genistein did not result in the expected up-regulation at the protein level in MDA-MB-231 cell line: p21WAF1/CIP1 protein levels in fact slightly, but significantly, decreased (p=0.002) following exposure to genistein. A very small increase in p21WAF1/CIP1 protein levels was detected in the SUM1315 cell line.

In the absence of genistein, AKT protein levels were not consistent with RT-PCR results: SUM1315 control cells expressed about twice as much AKT protein than did MDA-MB-231 cells (Figure 4B). In contrast, Western blotting experiments confirmed RT-PCR results in response to genistein exposure: a high concentration of genistein induced a two-fold increase in AKT protein levels in MDA-MB-231 cells, while there was a slight decrease (p=0.039) in the SUM1315 cell line (Figure 4B).

Discussion

Genistein was shown to inhibit cell proliferation more efficiently in BRCA1-mutant breast cancer cell lines than in BRCA1 wild-type cells. This result confirmed our previous study in another experimental model showing that BRCA1-mutant cells were highly sensitive to genistein (20). P21 and AKT were differentially expressed depending on BRCA1 status. p21WAF1/CIP1, also known as cyclin-dependant kinase 1A (CDKN1A) is a major cell cycle regulator, mainly at the G1/S checkpoint. It can also induce apoptosis via a p53-independent mechanism (15, 21, 22). AKT is an oncogene which is overexpressed in many carcinoma cell lines. This protein is involved in cell survival pathways by inhibiting apoptotic processes (23). AKT acts downstream of the PI3-kinase and can promote cell survival through phosphorylation and inactivation of pro-apoptotic factors such as BCL2-associated agonist of cell death protein (BAD). AKT can also activate nuclear factor kappa B (NF-kB) through the regulation of IκB kinase (IKK) thus resulting in pro-survival gene transcription.

In the absence of genistein, we showed that p21WAF1/CIP1 mRNA and protein levels were lower in BRCA1-mutant cell lines as compared to MDA-MB-231 wild-type cells. Our results confirmed that BRCA1 protein may play a role in the regulation of p21WAF1/CIP1 expression (24, 25). Moreover,
AKT protein expression in SUM1315 cells was shown to be about twice as high as that observed for MDA-MB-231 cells. This apparent BRCA1-dependent expression of p21\textsuperscript{WAF1/CIP1} and AKT shed light on our analysis of apoptosis. High p21\textsuperscript{WAF1/CIP1} and low AKT expression observed in MDA-MB-231 cells may indeed contribute to the higher rate of apoptosis in cells with wild-type BRCA1 as compared to those with mutant BRCA1. This suggests that BRCA1 may be involved, at least in part, in apoptosis induction by increasing p21\textsuperscript{WAF1/CIP1} levels and modulating AKT signaling.

In response to genistein, AKT mRNA and protein levels were strongly increased in MDA-MB-231 cells. This effect of genistein could result in apoptosis inhibition and therefore in the higher cell survival observed in genistein-treated MDA-MB-231 cells. Furthermore, p21\textsuperscript{WAF1/CIP1} protein levels slightly decreased in MDA-MB-231 cells following genistein treatment, allowing cells to complete their cycle. An increase in AKT signaling combined with a decrease in p21\textsuperscript{WAF1/CIP1} could thus explain why MDA-MB-231 cells were more resistant to genistein than were BRCA1-mutant cell lines. In contrast, the SUM1315 cell line exhibited a decrease in AKT protein and a weak increase in p21\textsuperscript{WAF1/CIP1} protein in response to genistein treatment, which could have an impact on the induction of apoptosis. This phenomenon may therefore contribute to the higher sensitivity of BRCA1-mutant SUM1315 cells to genistein.

In conclusion, our study showed that genistein inhibited cell proliferation of BRCA1-mutant cell lines more efficiently than BRCA1 wild-type MDA-MB-231 cell line. Changes in AKT and p21\textsuperscript{WAF1/CIP1} expression upon genistein treatment in MDA-MB-231 cells may contribute to the relative resistance of this cell line to genistein treatment. Cells with mutant BRCA1 may have lost the capacity to modulate AKT and p21\textsuperscript{WAF1/CIP1} signaling, which would result in apoptosis induction and cell growth inhibition following their exposure to genistein.

Acknowledgements

This study was funded by grants from the M.E.N.R.T. (Ministère de l’Education Nationale, de la Recherche et de la Technologie), the Ligue contre le Cancer d’Auvergne, the Conseil Régional d’Auvergne, Clermont-Communauté, FNADT en Auvergne, the FEDER Massif Central, Conseil général du Puy de Dôme and the Institut du Cancer.

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Anticancer Research 30: 2049-2054 (2010)


