

Epigenetic modulation of *BRCA1* and *BRCA2* gene expression by equol in breast cancer cell lines

Rémy Bosviel^{1,2}, Julie Durif^{1,2}, Pierre Déchelotte^{2,3}, Yves-Jean Bignon^{1,2*} and Dominique Bernard-Gallon^{1,2}

¹Centre Jean Perrin, Département d'Oncogénétique, CBRV, 28 Place Henri Dunant, BP 38, 63001 Clermont-Ferrand, France

²Université d'Auvergne, EA 4233, Nutrition, Cancérogénèse et Thérapie anti-tumorale, 28 Place Henri Dunant, BP 38, 63001 Clermont-Ferrand, France

³CHU, Nouvel Hôpital Estaing, Service d'Anatomie Pathologique, 1 Place Lucie Aubrac, 63100 Clermont-Ferrand, France

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Abstract

S-Equol is a metabolite resulting from the conversion of daidzein, a soya phyto-oestrogen, by the gut microflora. The potential protective effects of equol in breast cancer are still under debate. Consequently, we investigated the effects of equol on DNA methylation of breast cancer susceptibility genes (*BRCA1* and *BRCA2*) and oncosuppressors in breast cancer cell lines (MDA-MB-231 and MCF-7) and in a dystrophic breast cell line (MCF-10a) following exposure to S-equol (2 µM) for 3 weeks. We demonstrated by quantitative analysis of methylated alleles a significant decrease in the methylation of the cytosine phosphate guanine (CpG) islands in the promoters of *BRCA1* and *BRCA2* after the S-equol treatment in MCF-7 and MDA-MB-231 cells and a trend in MCF-10a cells. We also showed that S-equol increases *BRCA1* and *BRCA2* protein expression in the nuclei and the cytoplasm in MCF-7, MDA-MB-231 and MCF-10a cell lines by immunohistochemistry. The increase in *BRCA1* and *BRCA2* proteins was also found after Western blotting in the studied cell lines. In summary, we demonstrated the demethylating effect of S-equol on the CpG islands inside the promoters of *BRCA1* and *BRCA2* genes, resulting in an increase in the level of expressed oncosuppressors in breast cancer cell lines.

Key words: Equol; Breast cancer susceptibility genes 1 and 2; Breast cancer

First found in equine urine⁽¹⁾, equol is a non-steroidal oestrogen. Many years after its discovery, it was found that the soya isoflavone daidzein was a precursor to equol⁽²⁾, and that soya consumption increased the excretion of equol in some, but not all, adults. Studies have shown that gut microflora was responsible for the conversion of daidzein to S-equol⁽³⁾. More recently, particular bacteria capable of this conversion were even isolated⁽⁴⁾. Multiple studies have shown that equol producers were more frequent in Asian countries than in Western countries, which led researchers to ask themselves whether particular diets would not favour equol-producing microflora⁽⁵⁾. Equol is a chiral molecule and two forms can coexist: R- and S-equol. Distinction between these two forms and purification of one of them is complex, so many studies have worked on the effects of racemic equol. Today, it has been shown that only S-equol is synthesised by gut bacteria⁽⁶⁾ and S-equol is commercially available, leading to studies on the effect of S-equol alone.

Breast cancer is the most frequent cancer in women, with 1.38 million new cases and 458 000 deaths in 2008⁽⁷⁾. The incidence of breast cancer is high in Western countries, and low in Asia. This difference has been attributed, at least in part, to the Asian traditional diet, containing larger amounts of soya than the Western diet. Particular chemicals in soya, namely phyto-oestrogens, are supposed to have protective effects on breast cancer, mainly because of their similarity of structure with 17-β-oestradiol, the natural human oestrogen, allowing them to bind and activate oestrogen receptors (ER)^(8–10), with, contrarily to 17-β-estradiol, a higher affinity for ERβ^(9,11). This is also the case for S-equol^(6,12). More recently, special attention has been paid to S-equol, as some studies have shown that equol had a greater affinity for ER than its precursor, daidzein⁽¹²⁾.

Many studies have worked on the potential protective effect of soya over breast cancer, but mixed results have been found^(1,3). Studies on the effects of S-equol on breast

Abbreviations: *BRCA1*, breast cancer susceptibility gene 1; *BRCA2*, breast cancer susceptibility gene 2; CpG, cytosine phosphate guanine; ER, oestrogen receptor; QAMA, quantitative analysis of methylated alleles.

* **Corresponding author:** Y.-J. Bignon, email yves-jean.bignon@cjp.fr

cancer risk have led to the same mixed results. As epigenetic mechanisms are implied in cancer, a growing number of studies have investigated the effect of soya phyto-oestrogens on those mechanisms, particularly DNA methylation⁽¹⁴⁾. In normal tissues, oncogenes and repeated sequences are globally methylated while oncosuppressors are hypomethylated, particularly at the level of cytosine phosphate guanine (CpG) islands found in the promoters of these genes⁽¹⁵⁾. In cancer, an inversion of this methylation profile is found, so it has been stated that soya phyto-oestrogens could have protective effects on cancer by reverting this methylation profile. Moreover, protective effects of breast cancer are observed in women consuming moderate amounts of soya since their childhood but not in women starting soya consumption after the menopause^(16,17). This observation could be the result of a protective epigenetic effect with expression changes of genes implicated in the early events of carcinogenesis. Some studies have shown a demethylating action of genistein and daidzein on oncosuppressors in cancer cells⁽¹⁴⁾. To our knowledge, only one study showed an effect of equol on DNA methylation: Lyn-Cook *et al.*⁽¹⁸⁾ showed that high doses of equol caused the hypermethylation of the *c-H-ras* proto-oncogene in the pancreas cells of neonatal rats. Here, we investigated the effects of equol on the methylation of two major breast cancer oncosuppressors: *BRCA1* and *BRCA2*. The breast cancer susceptibility gene 1 (*BRCA1*) and the breast cancer susceptibility gene 2 (*BRCA2*) are the major high-penetrance genes in which mutations increase susceptibility to breast cancer. Mutations in these genes account together for 2–3% of all breast cancers and about 30–40% of all familial breast cancers⁽¹⁹⁾. The *BRCA1* gene is located on chromosome 17q12–21. *BRCA1* is involved in many transcriptional activation or transcriptional repression processes⁽²⁰⁾. It also plays a role in apoptosis, genomic stability maintenance, and DNA recognition and repair⁽²¹⁾. The *BRCA2* gene is located on chromosome 13q12–13. The gene codes for proteins involved in DNA repair, cell-cycle control and transcription⁽²²⁾, and may have a function in the terminal differentiation of breast epithelial cells⁽²³⁾.

Although somatic mutations of these genes are rarely found in sporadic breast cancers^(22–26), methylation of the promoter of *BRCA1* coupled with a decrease in mRNA⁽²⁷⁾ or lower *BRCA1* protein^(28,29) can be found. *BRCA2* promoter methylation has also been reported in sporadic breast cancer cases⁽³⁰⁾.

As a growing number of studies have shown the effects of soya phyto-oestrogens on DNA methylation^(18,31–35), the protective effects of soya isoflavones on breast cancer could be due, at least in part, to an effect on DNA methylation.

We undertook the present study to examine changes in DNA methylation of the CpG islands in the promoters of *BRCA1* and *BRCA2* in breast cancer cells following exposure to *S*-equol at physiological doses during 3 weeks.

Materials and methods

Cell lines

MCF-7 and MDA-MB-231 breast tumour cell lines came from a pleural effusion of patients with invasive breast

carcinoma^(36,37). The MCF-10a cell line was established from the breast tissue of patients with fibrocystic breast disease⁽³⁸⁾. All three human cell lines were provided by the American Type Culture Collection. MCF-7 were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2 mM-L-glutamine (Invitrogen), gentamycin (20 µg/ml; Panpharma), 10% fetal bovine serum (Invitrogen) and insulin (1.4 µg/ml; Novo Nordisk) in a humidified atmosphere at 37°C containing 5% CO₂. This cell line has a positive ER status (ERα+/ERβ+). MCF-10a cells were maintained in Dulbecco's modified Eagle's medium F12 (Invitrogen) containing 10% horse serum (Invitrogen), 2 mM-L-glutamine, gentamycin (20 µg/ml; Panpharma), epidermal growth factor (20 ng/ml; Sigma), cholera toxin (100 ng/ml; Sigma), insulin (10 µg/ml; Novo Nordisk) and hydrocortisone (0.5 µg/ml; Sigma) held at 37°C with 5% CO₂. This cell line has a negative oestrogen receptor status (ERα−/ERβ−). MDA-MB-231 cells were grown in Leibovitz L-15 medium with 15% fetal bovine serum (Invitrogen), gentamycin (20 µg/ml; Panpharma) and 2 mM-L-glutamine in a 37°C humidified atmosphere without CO₂. This cell line has a negative ER status (ERα−/ERβ+).

The ER status of the three cell lines has previously been confirmed by immunohistochemistry⁽³⁹⁾.

Cell treatments

Cells (1 × 10⁶ per T75 flask) were seeded in the medium and treated with 2 µM-*S*-equol provided by the ENITA Unité Micronutriments-Reproduction-Santé and dissolved in dimethyl sulfoxide. As controls, the cell lines were also conditioned in the medium with the solvent dimethyl sulfoxide.

During the 3 weeks, each 48 h and just before 80% confluence, cells were trypsinised and cell number scored on a Malassez cell using Trypan blue, and then they were passed into three flasks and the treatments were added again.

DNA extraction

DNA was extracted using Millipore's non-organic DNA extraction kit as follows: after recovering the cells, 9 ml of wash buffer 1X were added to resuspend the pellet. After 15 min of incubation at room temperature, the cells were centrifuged at 1000 g for 20 min. The supernatant was discarded and the cells were resuspended in 3 ml of suspension buffer I 1X. Lysis buffer I (800 µl) and 50 µl of protein-digesting enzyme were added to the suspension. The samples were incubated for 2 h at 50°C. After adding 1 ml of a protein-precipitating agent, a 15 min centrifugation at 1000 g was carried out. The supernatant thus obtained was mixed with two volumes of absolute ethanol. The precipitated DNA was recovered using an inoculating needle, dried for 5 min at room temperature, and dipped in 5 ml of 70% ethanol. DNA was resuspended in 300 µl of suspension buffer II. After vortexing them for 5 min, the samples were left in incubation overnight at 50°C. The quantity of DNA collected as well as the quality of the extraction was then determined by spectrometry using a NanoDrop™ 8-sample spectrophotometer (ND-8000, NanoDrop Technologies®).

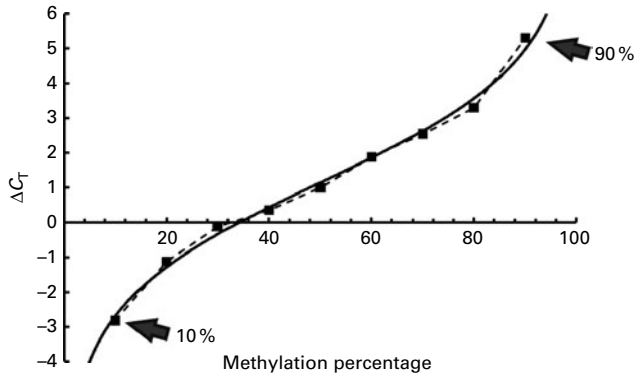


Fig. 1. Example of a standard curve for breast cancer susceptibility gene 2 (*BRCA2*) quantitative analysis of methylated alleles. ΔC_T values obtained for standard samples were plotted against their defined methylation ratio. The methylation ratio of the tested samples was found by plotting the ΔC_T values obtained onto this standard curve. In the case where only one fluorescence signal crossed the threshold, indicating a relative absence of the opposite target, the methylation percentage was set to 0 or 100%, depending on the nature of the fluorescence.

Bisulfite treatment and quantitative analysis of methylated alleles

Conversion of unmethylated cytosines to uracil⁽⁴⁰⁾, leaving methylated cytosines unaltered, was achieved using the methylSEQR™ Bisulfite Modification Kit (Applied Biosystems) following the manufacturer's instructions. We measured the methylation of oncosuppressor promoters with the real-time PCR-based quantitative analysis of methylated alleles (QAMA) assay previously described by Zeschnigk *et al.*⁽⁴¹⁾ and adapted here by Bosviel *et al.*⁽⁴²⁾. PCR was performed using a ninety-six-well optical tray with optical adhesive film at a final reaction volume of 20 μ l. Samples contained 10 μ l of TaqMan® Universal PCR Master Mix II, No AmpErase® UNG (uracil-*N*-glycosylase), 8 μ l of bisulfite-treated DNA, an additional 5U of FastStart Taq DNA Polymerase (Roche), 2.5 μ M each of the primers and 150 nM of the fluorescently labelled methylated and unmethylated *BRCA1* or methylated and unmethylated *BRCA2* probes. Initial denaturation at 95°C for 10 min to activate DNA polymerase was followed by forty cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min (7900HT, Real-Time PCR System; Applied Biosystems). Primer and probe sequences were selected with the help of Primer Express software (ABI). PCR primers were designed to amplify the bisulfite-converted sense strand of the CpG island *BRCA1* promoter sequence or the antisense strand of the CpG island *BRCA2* promoter sequence, lacking any known nucleotide polymorphisms. The software designs primers with a melting temperature (T_m) of 58–60°C and probes with a T_m value of 68–69°C. The T_m of both primers should be equal. The amplicon sizes were 79 bp for *BRCA1* (located at chromosome 17: 41278096–41278175 on the Ensembl GRCh37/hg19 assembly) and 87 bp for *BRCA2* (located at chromosome 13: 32889345–32889428). Primer and probe sequences are as follows: for *BRCA1*, forward primer – 5'-GGAGTTTGGGGTAAGTAGTTT-GTAAG-3'; reverse primer – 5'-TTCCCTACCCCAAACAAATT-3';

methylated probe – 5'-VIC-ACTACGTCCCCGCAA-MGBNFQ-3'; unmethylated probe – 5'-6FAM-ACTACATCCCCACAAAC-MGBNFQ-3'; for *BRCA2*, forward primer – 5'-GTTGGAGTAAA-AAGAAAGGGATGG-3'; reverse primer – 5'-CCTTAAAAATCCC-AAACCACCC-3'; methylated probe – 5'-VIC-AAACCGCCCCCT-ATAC-MGBNFQ-3'; unmethylated probe – 5'-6FAM-AAAACCA-CCCCTATACC-MGBNFQ-3'. The primer binding sites lack CpG dinucleotides and, therefore, the nucleotide sequences in the methylated and unmethylated DNA are identical after the bisulfite treatment. Consequently, it is possible to amplify both alleles in the same reaction tube with one primer pair. Methylation discrimination occurs during probe hybridisation by the use of two different MGB Taqman® probes. The binding site of the *BRCA1* and *BRCA2* MGB Taqman® probes both cover two CpG dinucleotides. We used a VIC-labelled MGB Taqman® probe that specifically hybridises to the sequence derived from the methylated allele, and a 6-carboxyfluorescein (FAM)-labelled MGB

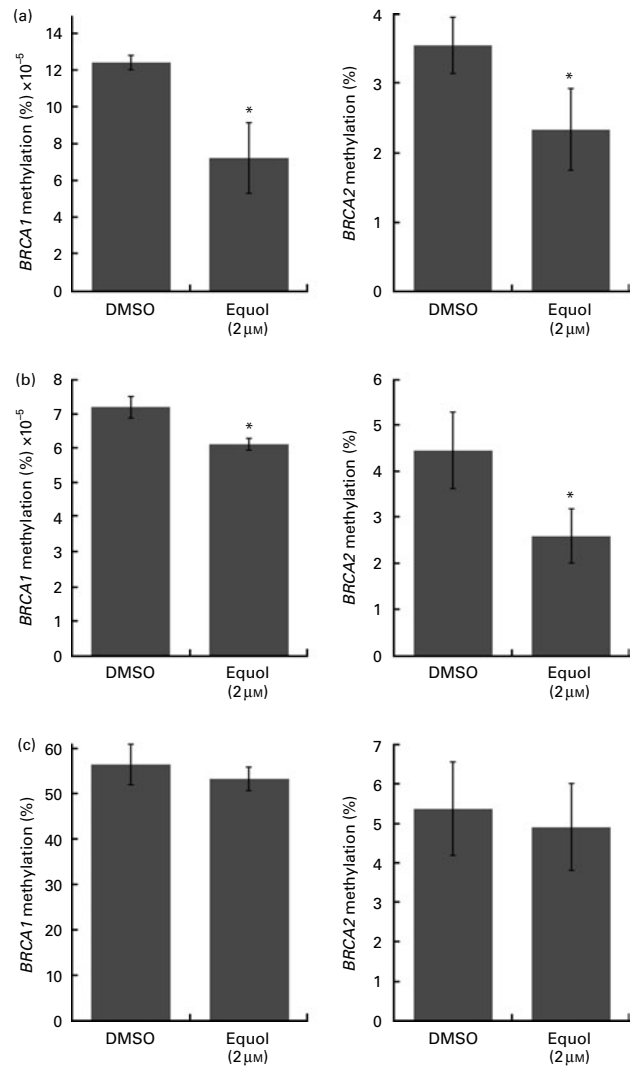


Fig. 2. Breast cancer susceptibility genes (*BRCA1* and *BRCA2*) methylation in (a) MDA-MB-231, (b) MCF-7 or (c) MCF-10a cells treated for 3 weeks with 2 μ M-*S*-equol compared with the dimethyl sulfoxide (DMSO) control. *BRCA1* and *BRCA2* methylation were decreased significantly following the *S*-equol treatment in the MDA-MB-231 and MCF-7 cells ($P < 0.05$).

Taqman[®] probe that binds to the sequence generated from the unmethylated allele. The amount of FAM and VIC fluorescence released during the PCR was measured by the real-time PCR system and is directly proportional to the amount of the PCR product generated. The cycle number at which the fluorescence signal crosses a detection threshold is referred to as C_T and the difference of both C_T values within a sample (ΔC_T) is calculated ($\Delta C_T = C_{T-FAM} - C_{T-VIC}$). All samples were measured in duplicate using the mean for further analysis. For a precise quantification of the ratio of methylated:unmethylated alleles, the ΔC_T value is determined and compared with a standard curve that exhibits a sigmoid shape with a linear part in the range of 10–90% of methylated DNA (Fig. 1). To set up the curve, we mixed bisulfite-treated and methylated control human DNA (EpiTect, ref. 59655; Qiagen) with defined ratios of bisulfite-treated and unmethylated control human DNA (EpiTect, ref. 59665; Qiagen) implemented in each run. From this, we deduced an algorithm to calculate the methylation ratio of

an unknown sample from its ΔC_T value by the Mathematica software package version 5.2 from Wolfram Research (<http://www.wolfram.com>). Student's *t* test was performed using the data obtained with QAMA, and $P < 0.05$ was considered to be statistically significant compared with the cells treated with the solvent dimethyl sulfoxide.

Western blotting

Proteins were extracted from the cells with lysis buffer containing 20 mM-Tris (pH 8), 50 mM-EDTA, 0.8% NaCl, 0.1% Triton X-100 and 1% glycerol. Protease inhibitors (1%, Protease Inhibitor Cocktail; Sigma) and phosphatase inhibitors (1%, Phosphatase Inhibitor Cocktail 2; Sigma) were added to the basic buffer extemporaneously (1% each). Then, 50 μ g proteins were electrophoresed on a SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. After 1 h blocking in Tris-Buffered Saline Tween 0.1% containing 5% milk, membranes were incubated overnight at 4°C with anti-BRCA1 (1:150 Mouse (Ab-1); Calbiochem), anti-BRCA2 (1:50 Rabbit (H-300); Santa Cruz Biotechnology[®]) or anti-actin (1:120,000 Mouse (Ab-1); Calbiochem) antibodies. The membranes were then washed three times in Tris-buffered saline Tween and incubated for 1 h with alkaline phosphatase-conjugated secondary antibody (1:2000 goat anti-mouse IgG (H&L) AP conjugate or 1:2000 goat anti-rabbit IgG (Fc) AP conjugate; Promega). Detection was then performed with the Western Blue detection system (Promega). Relative quantification of immunoblotted proteins was achieved using Quantity One software (Bio-Rad) with the local background subtraction method. A ratio between the intensity of the protein of interest and a reference protein (actin) was then calculated. The relative ratio was then calculated between each condition and the reference condition (dimethyl sulfoxide-treated cells).

Immunohistochemistry

For immunohistochemical analysis, 4 μ m alcohol-formalin-acetic acid-fixed and paraffin-embedded sections of MCF-7, MDA-MB-231 and MCF-10a cell pellets were cut using a microtome. They were mounted on silanised glass slides (Starfrost; Duiven) and dried overnight at 37°C. Slides were processed on an automated Benchmark XT immunohistochemical instrument (Ventana). In particular, sections were deparaffinised and rehydrated using EZ Prep (Ventana), and heat-induced antigen retrieval using CC1 (Ventana) was performed for 30 min. The slides were then incubated at 37°C for 44 min with anti-BRCA1 (1:20 mouse (8F7); GeneTex[®]) or anti-BRCA2 (1:20 mouse (Ab-1); Calbiochem[®]) primary antibodies. For detection, we used the UltraView universal DAB detection kit (Ventana). Signal was amplified using the Ventana amplification kit. The slides were then counterstained with haematoxylin for 3 min, rinsed in distilled water and coverslipped with an aqueous Faramount mounting media (DAKO). The primary polyclonal antibody was omitted and replaced with PBS as a negative control.

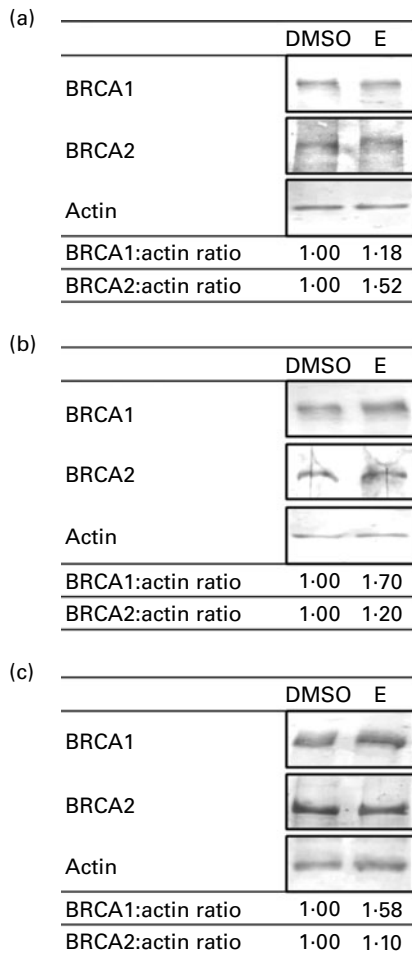


Fig. 3. Western blots with breast cancer susceptibility genes (*BRCA1* and *BRCA2*) and actin proteins extracted from (a) MCF-7, (b) MDA-MB-231 and (c) MCF-10a cells. Ratios shown correspond to relative ratios of optical densities of the bands (measured with Quantity One software; Bio-Rad) from interest proteins over actin, relatively to the control condition (dimethyl sulfoxide (DMSO)-treated cells). Cells were treated for 3 weeks with DMSO (control condition) or 2 μ M-S-equol (E).

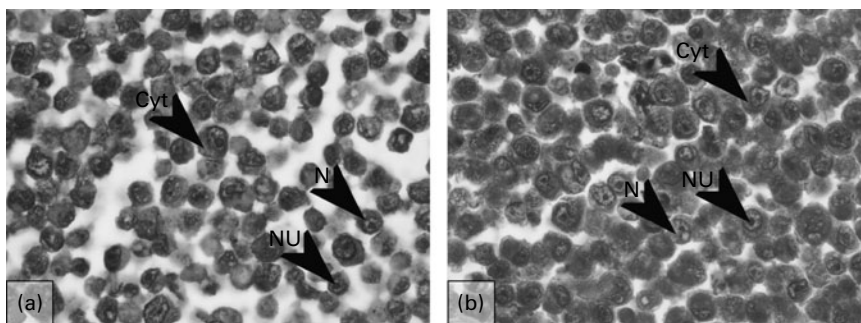


Fig. 4. Immunoperoxidase staining of MDA-MB-231 human breast cancer cell lines on paraffin-embedded sections (60×). (a) Cytoplasmic, nuclear and nucleolar staining were exhibited with 1:20 breast cancer susceptibility gene 2 (*BRCA2*) monoclonal antibody (Ab1), shown by arrowheads in untreated cells. (b) The *BRCA2* staining after 2 μM-*S*-equol treatment was considerably increased. N, nucleus; Cyt, cytoplasm; NU, nucleoli.

Results

Effect of *S*-equol on *BRCA1* and *BRCA2* CpG promoter methylation

QAMA was used to study the effects of *S*-equol on *BRCA1* and *BRCA2* CpG islands. We showed a significant decrease in the methylation of the CpG islands in the promoters of *BRCA1* and *BRCA2* following the 2 μM-*S*-equol treatment during 3 weeks in MDA-MB-231 and MCF-7 cells compared with the control (Fig. 2(a) and (b), respectively). This demethylation was not significant in MCF-10a cells (Fig. 2(c)).

Effect of *S*-equol on *BRCA1* and *BRCA2* protein expression

Western blotting was used to study the effects of *S*-equol on *BRCA1* and *BRCA2* protein expression. We showed an increase in *BRCA1* and *BRCA2* proteins following the 2 μM-*S*-equol treatment for 3 weeks in MCF-7, MDA-MB-231 and MCF-10a cell lines (Fig. 3(a)–(c), respectively). An extensive increase in *BRCA1* staining was found by immunohistochemistry in the nuclei, the cytoplasm and nucleoli in MCF-7, MDA-MB-231 and MCF-10a cell lines after 2 μM-*S*-equol exposure for 3 weeks. For *BRCA2*, the increase in staining was exhibited preferentially in the cytoplasm (Fig. 4). The results of immunohistochemistry are compiled in Table 1.

Discussion

A growing number of studies have revealed the importance of DNA methylation in cancer, with a global hypomethylation of DNA and the hypermethylation of CpG islands of oncosuppressors, leading to chromosomal instability and loss of the expression of oncosuppressors. In breast cancer, hypermethylation of the *BRCA1* and *BRCA2* genes has been found, associated with a decrease in mRNA expression for *BRCA1*. *S*-equol, an intestinal bacterial metabolite of daidzein, is a putative protective molecule for breast cancer. The present study sustains the idea that this protective effect could pass through epigenetic modulation of *BRCA1* and *BRCA2* expression. The mechanism for this effect is not yet clearly known, although studies have shown that *S*-equol can bind and activate ER.

As more and more studies have shown the effects of soya phyto-oestrogens on DNA methylation, we decided to study

the effects of *S*-equol in breast cancer cell lines on *BRCA1* and *BRCA2* methylation and consequent protein expression. We studied the effects of *S*-equol on the expression of the *BRCA1* and *BRCA2* genes that interact together in two human breast cancer cell lines (MCF-7 and MDA-MB-231) and in a fibrocystic cell line (MCF-10a). We chose an exposure of 3 weeks to *S*-equol, because this treatment has been shown to increase the number of cells blocked in the S phase⁽⁴³⁾, and *BRCA1* and *BRCA2* reach their maximal level in the late G1 and S phases in normal and tumour-derived breast epithelial cells⁽⁴⁴⁾.

An important point in the design of the present study is the use of physiological doses of *S*-equol, in the same order of magnitude as plasma concentrations found in post-menopausal women^(45,46). Long exposures were carried out to point out an eventually weak effect due to the use of such doses. The effects observed in the present study thus have better chances to be representative of real-life exposure.

We provide evidence that *S*-equol demethylates the promoters of the *BRCA1* and *BRCA2* genes in MDA-MB-231 and MCF-7 breast cancer cell lines, but not in the MCF-10a cell line. We also showed an increase in the expression of the *BRCA1* and *BRCA2* proteins in the studied cell lines following the *S*-equol treatment. The fact that demethylation occurred in

Table 1. Effects of *S*-equol on breast cancer susceptibility genes (*BRCA1* and *BRCA2*) expression in MCF-7, MDA-MB-231 and MCF-10a cell lines*

	BRCA1 (8F7)			BRCA2 (Ab-1)		
	Cyt	N	Nu	Cyt	N	Nu
MDA-MB-231						
DMSO	+	++	++	+	+	+/-
<i>S</i> -Equol (2 μM)	++	+++	++	+++	+/-	+/-
MCF-7						
DMSO	+	++	++	++	+/-	-
<i>S</i> -Equol (2 μM)	+	+++	+++	+++	+/-	-
MCF-10a						
DMSO	+	++	+	++	+/-	-
<i>S</i> -Equol (2 μM)	+	+++	+	+++	+/-	-

Cyt, cytoplasm; N, nucleus; Nu, nucleoli; DMSO, dimethyl sulfoxide.

* Cells were treated during 3 weeks with *S*-equol (2 μM). Cells were also treated with DMSO, the solvent in which *S*-equol was diluted. Then, the cells were immunostained with MoAb anti-*BRCA1* (8F7) or anti-*BRCA2* (Ab-1). Staining: negative (-); intermediate (+/-); less intensive (+); intensive (++); very intensive (+++).

MDA-MB-231 and MCF-7 cell lines but not in the MCF-10a cell line whereas protein expression increased in all the three cell lines could suggest that DNA methylation was not the only mechanism regulating *BRCA1* and *BRCA2* expression that can be modulated by *S*-equol, and thus studies on histone mark status following the *S*-equol treatment could be interesting. Indeed, many studies have shown the effects of soya phyto-oestrogens on histone modifications, and *S*-equol could have similar effects^(35,47–50). Hong *et al.*⁽⁵¹⁾ also showed that equol stimulates ER-mediated histone acetyltransferase activity. ER status and, more particularly, ER β status may play a role in the action of *S*-equol on DNA methylation, as the MCF-10a cell line lacks the ER β receptor. To our knowledge, only one study has reported an effect of equol on DNA methylation, showing a rise in the methylation of the proto-oncogene *c-H-ras* in rat pancreatic cells⁽¹⁸⁾, while more data are found for other soya phyto-oestrogens^(14,31,32,34,35,49,52–58). Such effects on oncosuppressors could help prevent cancer by restoring their expression similar to the protein expression of *BRCA1* and *BRCA2* in the present experiment. Studies on whether this demethylating effect is limited to the CpG islands in the promoter of oncosuppressors or whether it also acts on the methylation of other CG sites could be interesting, as demethylating effects on global methylation and, more particularly, repeated elements or transposable elements would be a counter effect for cancer prevention⁽⁵⁹⁾.

In summary, the present study shows that *S*-equol has a demethylating effect on the CpG islands in the promoters of *BRCA1* and *BRCA2* genes. This effect might be linked with the presence of ER but the increase in subsequent protein expression is independent of this parameter. Thus, we suppose that other mechanisms can also be implied, such as effects on histone modifications.

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