Soy Phytoestrogens Modify DNA Methylation of GSTP1, RASSF1A, EPH2 and BRCA1 Promoter in Prostate Cancer Cells

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Abstract. Background: The aim of this study was to determine the effects of soy phytoestrogens on the methylation of promoter genes in prostate tumors. The incidence of prostate cancer in Asia is thirty percent lower than in Western countries. Since soy phytoestrogens represent a large portion of the Asian diet, evidence suggests their protective effect against prostate cancer. Materials and Methods: In three human prostate cancer cell lines, methylation-specific-PCR was used to determine the effect of soy isoflavones (genistein and daidzein), compared to known demethylating agent 5-azacytidine as control in the promoter regions of glutathione S-transferase P1 (GSTP1), Ras association domain family 1 (RASSF1A), ephrin B2 (EPHB2) and breast cancer 1 (BRCA1) genes. In parallel, immunohistochemistry was used to assess the effects of genistein, daidzein and 5-azacytidine treatment on the corresponding protein expression. Results: All studied promoters, with the exception of that for BRCA1, were strongly methylated without treatment. After treatment by phytoestrogens, demethylation of GSTP1 and EPHB2 promoter regions was observed and an increase in their protein expression was demonstrated by immunohistochemistry. Conclusion: Epigenetic modifications of DNA, such as the promoter CpG island demethylation of tumor suppressor genes, might be related to the protective effect of soy on prostate cancer.

Prostate cancer is the fourth cause of mortality in France, with incidence increasing by approximately 5% per year (1). In spite of widespread screening, the advanced stages of the disease depend on only one palliative treatment (2). On a worldwide scale, great disparities exist concerning the incidence and the mortality from prostate neoplasm (3, 4). The epidemiological data show the incidence of prostate cancer in Asian countries to be up to 30 times lower than in Western Europe and North America. These differences suggest the potential protective effect of environmental factors and, in particular nutrition.

Just like certain genetic mutations are responsible for the decreases in the oncosuppressor expression in familial cancer, so are epigenetic mechanisms that seem to be involved in prostatic carcinogenesis (5). These mechanisms are reversible modifications of chromatin without modification of the sequence of the DNA that controls gene expression. These epigenetic mechanisms include histone modifications, modulating changes of chromatin structure, accessibility of the genome to the transcriptional machinery (6), and promoter methylation.

In the human genome, DNA methylation occurs almost exclusively at the level of a cytosine in a CpG dinucleotide. The majority of such dinucleotides are methylated, with the exception of those located within specific areas, called CpG islands, associated with gene promoters (7). The methylation of the latter areas would result in the inactivation of the corresponding genes by preventing the binding of transcription factors, thus precluding transcription (8). This
methylation is dependent on specific enzymes: the DNA methyltransferases (DNMT) (9). Methylation is reversible using inhibitors of DNMT such as 5-aza-2’-deoxycytidine (10) or procainamide (11).

Demethylating agents that have the ability to modulate the expression of genes of interest by epigenetic mechanisms are promising in prostate cancer prevention research. They are also interesting in therapeutic treatment. Soy phytoestrogens, genistein and daidzein, seem to be implicated in the change of incidence of prostate cancer based upon foods consumed. They potentially have a protective effect against developing this neoplasm. Their consumption is very important in Asian countries (approximately 30 mg/day of soy isoflavones) while this neoplasm. Their consumption is very important in Asian countries (approximately 30 mg/day of soy isoflavones) while it is practically zero in the rest of the world (12). These two molecules have an antiproliferative effect on prostate tumor cells in vitro, demonstrated by an arrest of the cell cycle in G2/M phase after genistein treatment and in G0/G1 phase with daidzein (13) on prostate carcinogenesis in animal models (14). A potentializing effect on chemotherapeutic treatment of prostate, pulmonary, mammary and pancreatic neoplasms was also reported (15). Recent work studying the effect of a phytoestrogenic treatment on cells of esophageal carcinoma and prostate cancer has shown a re-expression of p16, retinoic acid-related receptors β (RAR β) and O-6-methylguanine-DNA methyltransferase (MGMT) genes by demethylation of their promoters under the effect of genistein (16). The molecular mechanisms explaining this effect are not well known.

In this work, we studied four genes implicated in prostate cancer whose loss of expression can be related to DNA methylation: (i) glutathione S-transferase π-1 (GSTP1) is a phase II detoxification enzyme, coded by a gene located at 11q3. Different authors have shown a loss of expression of GSTP1 in 90% of prostate tumors by methylation of the CpG islands of its promoter (17, 18); (ii) RAS-association domain family 1, isoform A (RASSF1A), is a tumor suppressor gene whose loss of expression is found in lung cancer, clear cell renal carcinoma, and 74% of prostate tumors. This loss is related to CpG island methylation and deacetylation of histones in the promoter of the gene, located at 3p21(19); (iii) ephrin B2 (EPHB2) is a tumor suppressor gene located at 1q36,1 whose nonsense mutation exists in 10% of sporadic prostate tumors (20); (iv) breast cancer 1 (BRCA1) is an oncosuppressor implicated, just like BRCA2, in DNA repair, cell cycle regulation and transcription control of certain genes. A BRCA1 mutation was initially described in familial and sporadic forms of breast cancer (21). A change in this gene can multiply the relative risk of prostate cancer two-fold (22).

Here, we report the effects of soy phytoestrogen treatment on the methylation of the promoters of genes of human prostate cancer cell lines compared with the expression of the corresponding proteins.

Materials and Methods

Cell lines. Prostate cell lines (PC-3, DU-145, LNCaP) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). PC-3 cells were derived from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old man. They do not express the androgen receptor. They were grown in Ham’s F12K medium (ATCC) supplemented with 10% of fetal bovine serum, 1% of glutamine and 0.1% of gentamicin. DU-145 cells were derived from a cerebral metastasis of a grade II prostatic adenocarcinoma from a 69-year-old man. They also do not express the androgen receptor. They were grown in Eagle’s minimum essential medium (ATCC) supplemented with 10% of fetal bovine serum, 1% of glutamine and 0.1% of gentamicin. LNCaP cells were derived from a lymph node metastasis of a prostatic adenocarcinoma from a 50-year-old man. They do express the androgen receptor. They were grown in RPMI-1640 medium (ATCC) supplemented with 10% of fetal bovine serum, 1% of glutamine and 0.1% of gentamicin.

Cell treatments. Each cell line was plated at 1x10^6 cells per T-75 flask. The DU-145, PC-3 and LNCaP cells were maintained in medium supplemented with 40 μM genistein (Sigma-Aldrich, St. Louis, MO, USA) or with 110 μM daidzein (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) for the treated cells. These concentrations were determined previously by flow cytometry analysis and corresponded to cell cycle arrest in G2/M (23). 5-Azacytidine (Sigma-Aldrich) was used as a DNMT inhibitor and was solubilized in DMSO at a concentration of 2 μM. A control was performed with DMSO alone and one without any treatment. The cells were collected after 48 h by trypsinisation.

DNA extraction. Genomic DNA extraction was carried out with the Non-organic DNA Extraction Kit S4520 (Millipore Corporate, Billerica, MA). After recovering the cells, 9 ml of wash buffer 1x are added to resuspend the pellet. After a 15-minute incubation at room temperature, the cells were centrifuged at 1000xg for 20 minutes. The supernatant was discarded; the cells were resuspended in 3 ml of suspension buffer I. Lysis buffer I (80 μl) and 50 μl of protein-digesting enzyme were added to the suspension. The samples were incubated for two hours at 50°C. After adding 1 ml of protein-precipitating agent, a 15-minute centrifugation at 1000xg was carried out. The supernatant thus obtained was mixed with two volumes of absolute ethanol. The precipitated DNA is recovered using a Pasteur loop, dried for five minutes at room temperature, and dipped in 5 ml 70% ethanol. The DNA was resuspended in 300 μl of suspension buffer II. The quantity of DNA was determined using the NanoDrop™ (ND-8000, NanoDrop Technologies, Wilmington, DE, USA).

Sodium bisulfite DNA modification. The modification with sodium bisulfite was carried with MethylDetector™ kit (Active Motif, Carlsbad, CA, USA). The extracted DNA is deposited in a 96-well plate at a rate of 2 ng of DNA in 13 μl of water; 120 μl of conversion buffer and 7 μl hydroquinone are also added to each well. The DNA undergoes a modification with the thermocycler (GeneAmp™, PCR System 2700; Applied Biosystems, Foster, CA, USA) under the following conditions: 94°C for 3 minutes, 50°C for 9 hours and stored at 4°C. The 140 μl of converted DNA was then mixed with 500 μl DNA binding buffer, deposited on a purification column and centrifuged at 10000 rpm for 30 seconds. After
emptying the collection tube, 200 μl DNA wash buffer were deposited on the column and a new centrifugation was carried out under the same conditions. The collection tube was emptied and 200 μl of desulfonation buffer were deposited on the columns which were then left to incubate for 20 minutes at room temperature. After centrifugation of the samples, the column was deposited on a new collection tube and 50 μl of elution buffer were added directly to this one. After a 3-minute incubation at room temperature, the columns were again centrifuged at 10000 rpm during 30 seconds. The solution in the collection tube contained the converted DNA. A check of the conversion was carried out by nested PCR using specific primers for the p16 gene, according to the recommendations of the manufacturer.

**Methylation-specific PCR (MSP).** The primers (Table I) were designed using MethPrimer (http://www.urogene.org/methprimer/). The CpG islands were determined within the DNA sequence of each studied gene according to preset criteria: a percentage in GC higher than 50% and a size higher than 100 bp. The selected primers needed to correspond to a CpG island within the promoter of the gene, or close to this one, and had the highest possible Tm. For each studied gene, we obtained two pairs of primer, each having a forward and reverse primer. One corresponds to the converted sequence with methylated DNA and thus none converted CpGs, and the other corresponds to the converted sequence with unmethylated DNA and thus converted CpGs.

The pairs of primers corresponding to each studied gene (Eurogentec S.A, 4102 Seraing, Belgium) were used to carry out a methylation-specific PCR, for each cell line and each condition. On a 96-well plate; 19.3 μl of water, 3 μl of 10 μM primers (1.5 μl of forward primer and 1.5 μl of reverse primer) corresponding to the methylated or demethylated CpG islands, 0.5 μl of 10 mM dNTP (Applied Biosystems, Foster, CA, USA), 3 μl of 10X PCR master mix (geneamp PCR buffer; Applied Biosystems) and 0.5 μl of 5 U/μl Taq polymerase (AmpliTaq™, DNA polymerase; Applied Biosystems) were deposited per well. Four μl of DNA having undergone a sodium bisulfite conversion were added to this mix.

The plate was then placed in a thermocycler, with the following program: a phase of initial denaturation at 94˚C for 3 minutes followed by 45 cycles including a phase of denaturation at 94˚C for 30 seconds, a phase of hybridization at 50 or 55˚C for 30 seconds and a phase of elongation at 72˚C for 30 seconds, and finally a phase of final elongation at 72˚C for 4 minutes. The plate was then maintained at 4˚C. Ten μl of each methylation-specific PCR were deposited on a 2.5% agarose gel (Agarose, type II; Sigma-Aldrich).

Electrophoresis was performed in Tris-borate-EDTA (TBE, Ultrapure™; Invitrogen Carlsbad, CA, USA). At the end of the migration, the amplicons were revealed by ultraviolet (Fisher Bobblock Scientific, Illkirch, France).

**Immunohistochemistry.** Treated and untreated cells were washed with PBS and pelleted. Paraffin sections of 4 μm were cut using a microtome, assembled on glass slides and dried overnight at 37˚C. After dewaxing and rehydration with alcohol and distilled water, a method of heat induction of the antigen was used. Several stages were programmed on an automat (Ventana Medical Systems, Tucson, AZ, USA). The slides were then incubated at 37˚C for 30 minutes with primary antibody, anti-BRCA1 antibody (Mouse, GSTX7011; Genetex, Irvine, CA, USA), anti-EPHB2 (Rabbit, Sc-28980, Santa Cruz Biotechnologies, Santa Cruz, CA, USA); anti-GSTP1 (Mouse, Sc-66000; Santa Cruz Biotechnologies) or anti-RASSF1A (Rabbit, Sc-28563; Santa Cruz Biotechnologies). All the
Figure 2. Increased staining of nuclear GSTP1 expression, after 5-azacytidine (b), genistein (c), and genistein combined with 5-azacytidine treatment (d), compared to the control cells (a), by immunohistochemistry on PC-3 cell line (×40).

Figure 3. Increased staining of nuclear GSTP1 expression, after 5-azacytidine (b), genistein (c) and daidzein treatment (d), compared to the control cells (a), by immunohistochemistry on DU-145 cell line (×40).
antibodies were used with a dilution of 1:20. A secondary antibody coupled to an avidin-conjugated peroxidase complex was then added according to the protocol of the Ventana automat. The slides were then counterstained with hematoxylin for 3 minutes, rinsed with distilled water and mounted with Faramount aqueous medium (DAKO, Glostrup, Denmark). The negative control was produced by replacing the first antibody by PBS (4).

Results

Methylation status of the promoters of the genes studied with MSP. Amplification by MSP was obtained with the primers targeting the methylated promoters of GSTP1, RASSF1A, EPHB2 and BRCA1, for all the cell lines. An amplification starting from sodium bisulfite-converted DNA, corresponding to the nonmethylated promoter, was observed only with the BRCA1 primers.

Partial demethylation of the CpG island in the promoter of GSTP1 was observed in all cell lines after genistein treatment. This effect was apparent in PC-3 cells treated with 5-azacytidine+daidzein (Figure 1A) and a complete demethylation of this promoter was observed after treatment with 5-azacytidine, 5-azacytidine+genistein and 5-azacytidine+daidzein in DU-145 line (Figure 1B). In the LN CaP line, a demethylation of the GSTP1 promoter, equivalent to that observed after genistein treatment, was obtained with daidzein, 5-azacytidine alone, and 5-azacytidine+daidzein (Figure 1C). A demethylation of the CpG island of the promoter of EPHB2 was observed in the PC-3 line after treatment by 5-azacytidine alone, and 5-azacytidine and soy phytoestrogens (Figure 1A), as after genistein treatment in the DU-145 line (Figure 1B). In the same way, the treatment by 5-azacytidine alone, and 5-azacytidine and soy phytoestrogens induced a demethylation of the CpG islands of the promoter of RASSF1A in the LNCaP line (Figure 1C). The various treatments did not have an effect on the methylation of the promoter of BRCA1, whatever the studied cell line. In all the cases, these partial demethylations resulted in a reduction of the amplification of bisulfite converted DNA with methylated DNA targeting probes, visible after migration on agarose gel and revelation with ultraviolet.

Protein expression corresponding to the genes studied with immunohistochemistry. An increase in the nuclear expression of GSTP1, compared to the control condition, in PC-3 cells treated with 5-azacytidine (Table II and Figure 2) and in line DU-145 treated by genistein, daidzein, or 5-azacytidine (Table III and Figure 3) was observed. The expression of EPHB2 was also increased in the nucleus of line PC-3 treated by 5-azacytidine alone, and 5-azacytidine and genistein (Table II), as in the cytoplasm of DU-145 cells after treatment by genistein, daidzein, 5-azacytidine combined with phytoestrogens, but not 5-azacytidine alone (Table III and Figure 4). The various treatments did not
in vivo 24: 393-400 (2010)

involve notable modification of proteins studied in the line LNCaP (Table IV). No modification of the expression of BRCA1 and RASSF1A, whatever the cell line or the studied condition (Table II, III and IV), was observed.

**Discussion**

The methylation of the CpG islands in promoters of oncosuppressors leading to their loss of expression is a phenomenon described in certain neoplastic pathologies, particularly prostate cancer (5). There has been an increase in research related to restoration of their function under the influence of demethylating agents and in relation to anti-tumor therapy and preventative medications for these pathologies.

Many studies have been concerned with soy phytoestrogens. These agents seem to take part in the existing epidemiological imbalance between Western and Asian countries and may have a preventative effect on the occurrence of neoplasms such as prostate or breast cancer.

Based on current published data, we studied the potential demethylating effect of genistein and daidzein on human cell lines of prostate cancer. We compared these data with results from cells treated with a known demethylating agent, 5-azacytidine.

In our study and in control assays, the promoters of all genes studied were strongly methylated in all the cell lines with the exception of BRCA1, whose promoter was found to be methylated and unmethylated in equivalent proportions. The methylation of GSTP1 (17), RASSF1A (24) and BRCA1 (25) in prostate cancer has already been reported.

In contrast, EPHB2 is generally unmethylated in neoplastic pathologies (26) and its methylation in cell lines of prostate cancer has not yet been studied.

Demethylation of the promoter of RASSF1A in LNCaP cells and EPHB2 in PC-3 cells was obtained after a treatment with 5-azacytidine alone, and 5-azacytidine when combined with a phytoestrogen. Although reactivation of RASSF1A after 5-azacytidine treatment of gastric cancer cells has been reported (27), it has never been described in relation to prostate cancer cells. We did not observe a re-expression of RASSF1A after demethylation of its promoter. This suggested that the loss of expression in prostate cancer utilizes mechanisms other than the methylation of CpG islands on its promoter.

On the other hand, by immunohistochemistry a re-expression of EPHB2 was obtained in the nuclei of PC-3 cells after 5-azacytidine alone and when combined with genistein. This was also the case by immunohistochemistry, in the cytoplasm of DU-145 cells after treatment by soy phytoestrogens regardless of their treatment with 5-azacytidine. These results were in agreement with the possible demethylation of the promoter of EPHB2 with these treatments, which induced an overexpression of the protein.

### Table II. Protein expression in the PC3 cell line as analyzed by immunohistochemistry.

<table>
<thead>
<tr>
<th></th>
<th>GSTP1</th>
<th>RASSF1A</th>
<th>EPHB2</th>
<th>BRCA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt</td>
<td>N</td>
<td>Cyt</td>
<td>N</td>
<td>Cyt</td>
</tr>
<tr>
<td>Control</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>Genistein</td>
<td>+</td>
<td>++/−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Daidzein</td>
<td>+</td>
<td>−</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>Azacytidine</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Az. + Ge.</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>++</td>
</tr>
<tr>
<td>Az. + Da.</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>++</td>
</tr>
</tbody>
</table>

GSTP1, EPHB2 and BRCA2 are upregulated in the nuclei after 5-azacytidine treatment, compared to the control. The same result is observed for EPHB2 after 5-azacytidine combined with genistein treatment. Cyt: Cytoplasmic; N: nuclear; −: negative; +/−: weakly intense; +: intense; ++: very intense; +++: strongly intense.

### Table III. Protein expression in the DU-145 cell line as analyzed by immunohistochemistry.

<table>
<thead>
<tr>
<th></th>
<th>GSTP1</th>
<th>RASSF1A</th>
<th>EPHB2</th>
<th>BRCA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt</td>
<td>N</td>
<td>Cyt</td>
<td>N</td>
<td>Cyt</td>
</tr>
<tr>
<td>Control</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>Genistein</td>
<td>+</td>
<td>++/−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Daidzein</td>
<td>+</td>
<td>−</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>Azacytidine</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Az. + G</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>+++</td>
</tr>
<tr>
<td>Az. + D</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+++</td>
</tr>
</tbody>
</table>

Nuclear up-regulation of GSTP1 expression as observed after genistein, daidzein or azacytidin treatment, compared to the control. Cytoplasmic expression of EPHB2 was up-regulated by phytoestrogens alone or when combined with azacytidine. The cytoplasmic expression of BRCA2 was up-regulated by azacytidine alone. Cyt: Cytoplasmic; N: nuclear; −: negative; +/−: weakly intense; +: intense; ++: very intense; +++: strongly intense.

### Table IV. Protein expression in the LN-CaP cell line as analyzed by immunohistochemistry.

<table>
<thead>
<tr>
<th></th>
<th>GSTP1</th>
<th>RASSF1A</th>
<th>EPHB2</th>
<th>BRCA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt</td>
<td>N</td>
<td>Cyt</td>
<td>N</td>
<td>Cyt</td>
</tr>
<tr>
<td>Control</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−/−</td>
</tr>
<tr>
<td>Genistein</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
</tr>
<tr>
<td>Daidzein</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Azacytidine</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Az. + G</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>−/−</td>
</tr>
<tr>
<td>Az. + D</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
</tbody>
</table>

The different treatments did not have notable effects on the analyzed protein levels. Cyt: Cytoplasmic; N: nuclear; −: negative; +/−: weakly intense; +: intense.
We observed a demethylation of the promoter of GSTP1 after treatments of the PC-3 by 5-azacytidine combined with daidzein; in DU-145 by 5-azacytidine, and 5-azacytidine combined with phytoestrogens, and LNCaP, regardless of the treatment, except with 5-azacytidine combined with genistein. These results agreed with the observation made in immunohistochemistry of a re-expression of GSTP1 in the nuclei of PC-3 cells after 5-azacytidine treatment and of DU-145 cells treated with genistein, daidzein and 5-azacytidine. Nevertheless, this effect was not observed in the LNCaP line. The demethylation of the promoter of GSTP1 in the LNCaP line after genistein treatment associated with a demethylating agent had already been reported (28). However, to our knowledge, this effect had never been described after genistein treatment alone or after daidzein treatment. Moreover for this particular cell line in our study, the addition of an agent such as 5-azacytidine did not seem to increase the demethylating potential of the phytoestrogens. We demonstrated that genistein and daidzein at the concentrations used had a maximum effect on this promoter. This is, however, not the case for the DU-145 cells treated with 5-azacytidine combined with phytoestrogens, genistein or daidzein alone. In our study, according to the cell type and the promoter considered, the effect of soy phytoestrogens represents all or part of the effect observed with a treatment combined with the 5-azacytidine. MSP is not a quantitative amplification technique, thus it is not possible for us to determine the proportion of the demethylating effect for which phytoestrogens and 5-azacytidine are responsible.

A study using PCR in real time after immunoprecipitation of methylated DNA should be undertaken in order to quantify the demethylating potential of genistein and daidzein. In addition, it would be interesting to determine through which mechanisms phytoestrogens have an inhibiting effect on DNA methylation, through the utilization of antibodies directed against methyl cytosine with the technique of methylated DNA immunoprecipitation and subsequent hybridization on CpG islands chips.

Lastly, the study of the effects of genistein and daidzein on prostatic cancer cells from in vivo studies, biopsies or parts of radical prostatectomies would be interesting to consider. It is probable that although these tissues would be difficult to analyze due to their heterogeneity, the effect of phytoestrogens would be more pronounced, and their anaplasia would be less comparable with the immortalized lines.

Conclusion

The promoters of GSTP1, RASSF1A, EPHB2 are strongly methylated in different human tumor prostate cell lines. Treatments with genistein or daidzein appear to demethylate the promoters of GSTP1 and EPHB2, resulting in an increase of their expression.

Thus, soy phytoestrogens could play a role in the protective effect observed in epidemiological studies.

Acknowledgements

A. Vardi was a recipient of a grant from l’Association Française d’Urologie. R. Bosviel is a recipient of a grant CPER, Crédits du Conseil Régional D’Auvergne/Axe Nutrition et Cancer. N. Rabiau is a recipient of a grant CIFRE from Soluscience S.A., Clermont-Ferrand, France. We thank Timothy H. Gunnels for assisting with the English translation of this study.

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Received October 23, 2009
Revised May 28, 2010
Accepted June 4, 2010