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This is the author's manuscript

Original Citation:

Availability:

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UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:

Questa è la versione dell'autore dell'opera:

[Breast Cancer Research and Treatment, volume e fascicolo: 114(3), anno: 2009,

DOI: 10.1007/s10549-008-0031-4.]

The definitive version is available at:

La versione definitiva è disponibile alla URL:

[<http://link.springer.com/article/10.1007%2Fs10549-008-0031-4>]

Molecular mechanisms of the D327N SHBG protective role on breast cancer development after estrogen exposure

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Received: 20 December 2007 Accepted: 15 April 2008 Published online: 25 April 2008

Abstract

Sex Hormone-Binding Globulin, the specific carrier for sex steroids, regulates hormone bioavailable fraction and estrogen signaling system in breast cancer cells. A common single nucleotide polymorphism in the human SHBG gene results in an amino acid substitution (Asp327Asn), which introduces an additional N-glycosylation site, and is associated with reduced breast cancer risk in postmenopausal women. The frequency of this polymorphism was evaluated in a group of patients that developed breast cancer while taking hormonal replacement therapy (HRT) for menopause, an interesting model of estrogen exposure. The polymorphism frequency was significantly higher in women taking HRT that didn't develop any breast cancer than in breast cancer patients ($P < 0.05$). To get insight into the underlying mechanisms, we compared the ability of recombinant wild type and variant (D327N) SHBG to influence estradiol effects in MCF-7 breast cancer cells. D327N SHBG was more effective than wild type protein in inhibiting estradiol-induced cell proliferation and anti-apoptosis. This depended on the fact that D327N SHBG binding to MCF-7 cells was significantly higher than that of wild type protein. As a consequence, D327N caused a larger induction of the second messenger cAMP and a deeper inhibition of the estradiol-induced Erk 1/2 phosphorylation. Our observations, demonstrating the increased efficiency of D327N SHBG in counteracting estradiol action and a significantly higher frequency of Asp327Asn polymorphism in women not developing breast cancer after estrogen exposure, first provide evidence for the mechanism of D327N SHBG protective action.

Keywords

Introduction

Sex Hormone-Binding Globulin (SHBG) is a glycoprotein of human blood that binds androgens and estradiol and regulates their bioavailable fraction [1–3]. Actually, beside its function as sex steroid carrier, it is widely accepted that SHBG plays also an active role as modulator of the steroid-signaling system in target tissues [4, 5]. Due to its unique property to regulate bioavailable estradiol, SHBG has been taken into consideration in several epidemiologic studies concerning its implication in breast cancer and it has claimed that plasma SHBG levels are inversely associated with breast cancer risk in post menopause [6, 7]. SHBG binds to membrane of breast cancer MCF-7 cells [8, 9] and, through cAMP induction and PKA activation, it inhibits the estradiol-induced cell proliferation [10]. Moreover, the protein also inhibits estradiol anti-apoptotic effect, by blocking ERK ½ activation elicited by the estradiol-membrane initiated pathway [11].

More recently, a number of studies tried to focus on the relationship between breast cancer risk and the occurrence of the variant form of SHBG characterized by an additional N-glycosylation site at residue 327 [12, 13]. The D327N SHBG is the product of a single nucleotide polymorphism (G → A) in exon 8 that causes the substitution of Asp³²⁷ with Asn, introducing thus a third N-glycosylation site in the protein [14]. In healthy white women [15, 16] the reported frequency of the Asp327Asn polymorphism of SHBG is about 10–12%. As far as breast cancer patients are concerned, Cui and coworkers [17] recently reported in a large population-based case–control study that Asp327Asn polymorphism of SHBG is significantly associated with a reduced risk of breast cancer in postmenopausal women. Moreover, a significantly higher frequency of the polymorphism was observed in postmenopausal patients with estrogen receptor-positive breast cancer than in estrogen-receptor negative [15]. Therefore, SHBG polymorphism is likely to play some role in breast cancer, although the implication of glycosylation on SHBG function in breast cancer cells is only partially known. As far as the three glycosylation sites present in the wild type protein are concerned, it was reported that the O-glycosylation site in Thr⁷ is necessary for SHBG to inhibit the effect of estradiol on both MCF-7 cell proliferation [18] and apoptosis [11], while both N-glycosylation sites (Asn³⁵¹ and Asn³⁶⁷) are not involved. The additional carbohydrate chain in Asn³²⁷ present in the variant D327N SHBG decreases the clearance rate of this protein [19] but no other modifications of SHBG function have been evaluated.

Since SHBG in breast cancer cells acts inhibiting estradiol action, it is conceivable that the reported association between the Asp327Asn polymorphism and a reduced breast cancer risk [17] as well as the higher frequency of the polymorphism in estrogen-dependent breast cancer [15] could be linked to an even more striking action of the variant protein at cell site. This might be especially valuable when the length and intensity of exposure to estrogens can increase breast cancer risk, as it occurs in postmenopausal women taking hormone replacement therapy (HRT) [20]. The present paper outlines the mechanisms of action of D327N SHBG at cell site in estrogen-dependent breast cancer cells.

Materials and methods

Subjects

Blood samples, a complete record of medical history, and appropriate informed consent were obtained for each subject enrolled in the study. All participating women had taken hormone replacement therapy (HRT) for menopause (transdermic estradiol valerate at least 50 mcg plus oral progestogens) for a period of 2–5 years. Patients and controls were referred to the Senology Unit of ASO San Giovanni Battista of Turin, to the Division of Gynaecology, Ospedale Civile of Asti, and

to the Senology Unit of the Ospedale Evangelico Valdese of Turin. Serum samples were stored at -20°C before analysis and used to measure serum SHBG concentration. Whole blood samples were used for DNA extraction and further identification of D327N SHBG polymorphism.

SHBG evaluation

Measurement of serum/medium SHBG concentration

The double-antibody Spectria SHBG IRMA (Orion Diagnostica, Espoo, Finland), that provides a specific measurement of human SHBG, was used to measure SHBG concentration in both patient serum and cell medium samples (see below), following manufacturer's instructions.

Identification of D327N SHBG polymorphism

Genomic DNA was extracted from whole blood samples of patients using a non-enzymatic method routinely used in our laboratory. As previously reported [15], the exon 8 coding sequence within the human SHBG gene was amplified by PCR using the oligonucleotide primers 5': TCC TGG ATC CGA AGC CAC CT; 3': TCC GCC TGG TAC ATT GCT AG. The PCR system contained 5 μl of 10 \times PCR buffer, 0.2 mM deoxynucleotide triphosphate (Finnzymes, Espoo, Finland), 1.25 U Taq DNA polymerase (Finnzymes, Espoo, Finland), 50 ng each of sense and antisense primers and 0.5 μg genomic DNA template in a total reaction volume of 50 μl . Amplification was carried out as follows: 1 \times (95°C , 3 min), 35 \times (95°C , 1 min; 60°C , 1 min; 72°C , 1 min), and 1 \times (72°C , 7 min). PCR products were electrophoresed on 1.5% agarose gel in the presence of ethidium bromide to monitor the specificity and amount of product. The mutation was identified by a digestion of PCR products with 20 U of the restriction enzyme *Hinf*I (Amersham Life Science, Little Chalfont, Bucks, UK) overnight at 37°C . Digestion products were visualised after electrophoresis in a 2% agarose gel.

Cell culture conditions

ER α -positive MCF-7 breast cancer cells were routinely maintained in 25 cm^2 flasks in RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA) with the addition of 10% heat inactivated FBS (Euroclone, Wetherby, West York, UK). Twenty four hours before experiments, cells were switched to RPMI 1640 without phenol red supplemented with 10% FBS, specifically treated with dextran-coated charcoal to remove steroids [21]. Chinese hamster ovary (CHO) cells, that were used to produce recombinant SHBGs, were routinely maintained in HAM F12 (Sigma-Aldrich, St Louis, MO, USA) with the addition of 10% FBS specifically treated as above described.

Recombinant SHBGs production

CHO cells were transfected with pRc/CMV expression vectors encoding wild type or D327N SHBG (additional N-glycosylation site introduced) (a kind gift of G.L. Hammond, University of British Columbia, Vancouver, British Columbia, Canada). For transfections, CHO cells were seeded in 60 mm Petri dishes ($300\text{--}500 \times 10^3$ cells/dish) in HAM-F12 with 10% FBS. Two days later, the medium was removed and RPMI 1640 without phenol red was added to each dish, and transfections were carried out with about 10 μg vector/plate in the presence of Lipofectin (Invitrogen SRL, San Giuliano Milanese, Italy), following manufacturer's recommendations. Cells were then incubated at 37°C , and FBS was added back to the medium after 6 h. Selection of transfected cells with G418

(1.0–1.25 mg/ml) was started 48 h later and continued for 20–30 days. The amount of SHBGs (either wild type or mutant) produced was evaluated in culture medium, as above described.

Western blot analysis of recombinant SHBGs

To check the type of SHBG produced by transfected cells, cell medium was subjected to western blot. Wild type SHBG or D327N SHBG containing medium was first 20× concentrated in centrifugal filter devices (Millipore, Bedford, MA, USA). Then, different samples (about 50 µg protein/lane) were subjected to SDS-PAGE (T = 8%) and electroblotted onto a PVDF membrane; the membrane was probed with, as primary antibody, a rabbit-derived polyclonal antiserum anti-human SHBG (1:500 dilution, kindly provided by G.L. Hammond, University of British Columbia, Vancouver, British Columbia, Canada). Proteins were detected with enhanced chemiluminescence detection system (Pierce, Rockford IL, USA) following manufacturer's instructions. Bands were photographed using Kodak 1D Image Analysis software.

Cell proliferation assay

MCF-7 cells were seeded at 25×10^3 cells/well in 24 multiwell plates (Corning, New York, NY, USA) in culture medium plus 10% FBS. After 24 h in RPMI 1640 without phenol red supplemented with dextran-coated charcoal treated 10% FBS, they were treated as follows: (a) cells under basal conditions; (b) cells treated with estradiol (Sigma-Aldrich, St. Louis MO, USA) at concentrations ranging from 1 to 100 nM; (c) cells treated with 50 nM human SHBGs (wild type or D327N), (before use, SHBG was charcoal-treated to remove the dihydrotestosterone (DHT) used for storage) for 40 min, followed by medium without any addition; (d) cells treated with 50 nM SHBGs (wild type or D327N) for 40 min followed by estradiol treatment (concentrations ranging from 1 to 100 nM). All the treatments were repeated every 24 h for three consecutive days. After 72 h, cell number was counted in a Burker's chamber. Each condition was repeated in triplicate in each experiment.

Apoptosis detection

MCF-7 cells were seeded in 96 multiwell plates and treated as previously described. Apoptosis was then evaluated using cell death detection ELISA^{PLUS} (Roche, Basel, Switzerland) following manufacturers' instructions. This assay is based on a quantitative sandwich-enzyme-immunoassay-principle using monoclonal antibodies directed against DNA and histones, respectively, allowing the specific determination of mono- and oligonucleosomes in the cytoplasm fraction of cell lysates. Apoptosis was expressed as an enrichment factor, calculated as a fraction of the absorbance of treated cells with respect to the untreated control.

SHBG-cell interaction assay

To evaluate the ability of both SHBGs to bind to MCF-7 cells, cells were seeded in 96 multiwell plates (about 10^4 cells/well) and incubated for 40 min at 37°C with 50 nM SHBG either wild type or D327N. At completion, SHBGs were removed, and cells incubated with 100 µl of [¹²⁵I]-labelled monoclonal antibody against human SHBG (the same used for IRMA SHBG determination) at 37°C for 1 h; then, antibody was discarded, and wells washed with ice-cold saline for three times. Plates were placed at -20°C overnight; cells were then recovered on filters with a cell harvester (Nunc, Roskilde, Denmark) and the filter radioactivity was measured. SHBG concentration on filters was calculated against a standard curve prepared from seven SHBG dilutions ranging from 0 to 200 nM.

cAMP evaluation

Cells were seeded at 45×10^4 cells/well in six multiwell plates (Corning, New York, NY, USA) and treated as follows: (a) cells under basal conditions; (b) cells treated with 10 nM estradiol; (c) cells treated with 50 nM human SHBGs (wild type or D327N) for 40 min, followed by medium without any addition; (d) cells treated with 50 nM SHBGs (wild type or D327N) for 40 min followed by 10 nM estradiol for 15 min. After treatments cAMP was evaluated using cAMP Biotrak Enzymeimmunoassay (EIA) System (Amersham Biosciences, UK).

Western blotting for Erk-1/2 and phospho Erk-1/2

10^6 MCF-7 cells were seeded in 75 cm^2 flasks and treated as above for 3 days. After treatment, cells were lysed in RIPA buffer (PBS, pH = 7.4, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 100 $\mu\text{g}/\text{ml}$ PMSF, 30 μl aprotinin, 100 mM NaVO_4), extracted at 4°C for 30 min, and centrifuged at 4°C for 20 min at $15,000\times g$. Equal amounts of protein (50 μg protein/lane) were subjected to SDS-PAGE (T = 8%) and electroblotted onto a PVDF membrane; the membrane was probed with the following primary antibodies: anti-MAP Kinase 1/2 (Erk 1/2-CT, 1:5000 dilution, Upstate, Lake Placid, NY, USA) and anti-phospho MAP Kinase 1/2 (Erk 1/2, 1:2500 dilution, Upstate, Lake Placid, NY, USA).

Chemiluminescence detection was performed following ECL manufacturer's instructions. Bands were photographed with 1D Kodak Digital Science software.

Statistical analysis

Data are expressed throughout as means \pm SD, calculated from at least three different experiments. Statistical comparisons between groups were performed with analysis of variance (one-way ANOVA) and the threshold of significance was calculated with the Bonferroni post-test for selected pairs of columns. The frequency of SHBG polymorphism in patient groups was calculated and compared with the Yates' corrected for continuity χ^2 test. Significance test used a two-tailed P-values and statistical significance was attained for $P < 0.05$.

Results

Study of D327N SHBG in menopausal patients

SHBG serum levels and the presence of D327N polymorphism were evaluated in two groups of patients: patients that developed breast cancer during HRT and patients taking HRT but without neoplastic disease occurrence (Table 1). No significant difference in SHBG serum levels was observed between the two groups (breast cancer patients vs. healthy women; n.s.). 22% of healthy women were found to be heterozygous for D327N mutation, while only 12% of breast cancer patients carried the mutation ($\chi^2 = 2.870$; $P < 0.05$).

Table 1

SHBG serum levels and percent of D327N mutation carriers in HRT women

	HRT breast cancer patients (n = 31)	HRT healthy women (n = 36)
Age (yrs)	61 ± 6	56 ± 6
SHBG serum level (nM)	64.4 ± 42.5	51.4 ± 23.9
D327N mutation carriers (%)	12	22

Evaluation of SHBGs produced by transfected CHO cells

The concentration of SHBGs (wild type or D327N) in the medium produced by transfected CHO cells was evaluated as described above. Before use, medium was concentrated 20×. Mean concentration of wild type SHBG was 301 ± 62 nM (n = 3); mean concentration of D327N SHBG was 379 ± 68 nM (n = 3).

As reported in Fig. 1, the Western blot analysis of wild type and D327N SHBGs confirmed that the two proteins were correctly glycosylated, as expected; in fact, wild type SHBG was characterized by two bands (52 and 49 kDa), whereas D327N SHBG presented the additional heavier band. We could not see any difference between D327N SHBG from serum and CHO medium, as previously reported [14], since the third heavy band migrated in both cases at about 56 kDa.

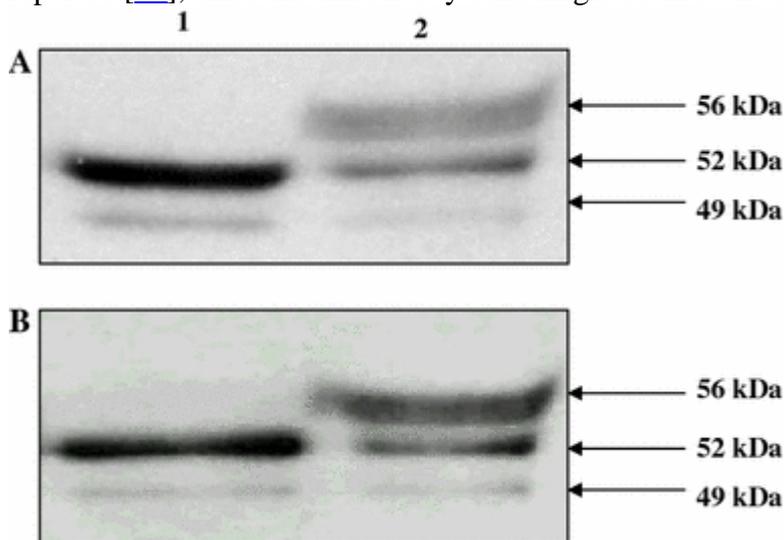


Fig. 1

Western blot of wild type and D327N SHBGs. Recombinant SHBGs produced by CHO cells transfected as described in Materials and Methods section were recovered from medium and evaluated for correct glycosylation (panel A); in comparison SHBGs from patient serum is reported in panel B; lanes 1, wild type SHBG; lanes 2, D327N SHBG

Effect of wild type and D327N SHBGs on estradiol-induced proliferation of MCF-7

As shown in Fig. 2, in MCF-7 cells, estradiol significantly increased cell number in a dose-dependent manner; wild type SHBG markedly reduced E₂-induced cell proliferation. Notably, D327N SHBG was significantly more efficient since it completely inhibited cell proliferation at each estradiol concentration.

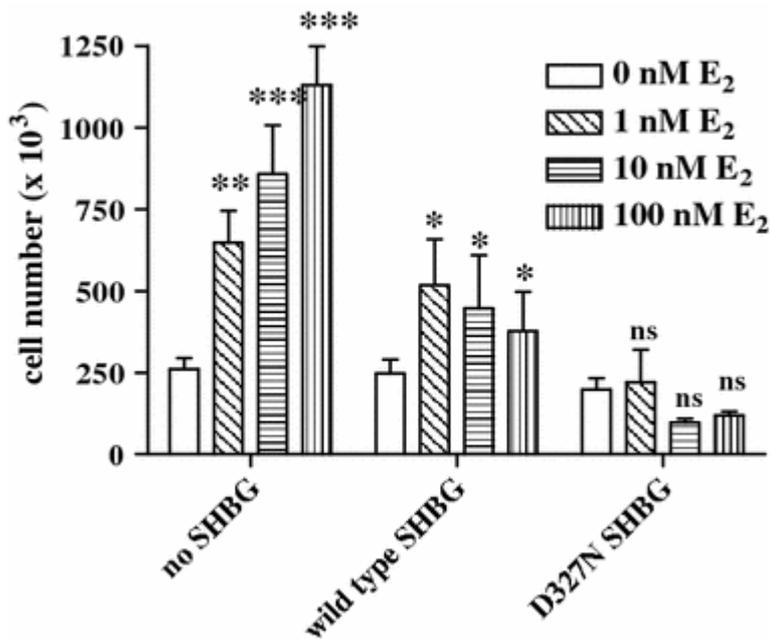


Fig. 2

Effect of wild type and D327N SHBGs on breast cancer cell proliferation. MCF-7 breast cancer cells were treated with increasing estradiol concentrations in the absence and in the presence of 50 nM recombinant SHBGs. Cell number was evaluated after 72-h treatment. The figure reports the mean \pm SD from three different experiments. Statistical significance: n.s., not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Effect of wild type and D327N SHBGs on estradiol anti-apoptotic action

As reported in Fig. 3, human wild type SHBG was able to inhibit the estradiol anti-apoptotic effect in MCF-7 cells, confirming our previous observations [11]. D327N SHBG was also able to reverse estradiol anti-apoptotic effect.

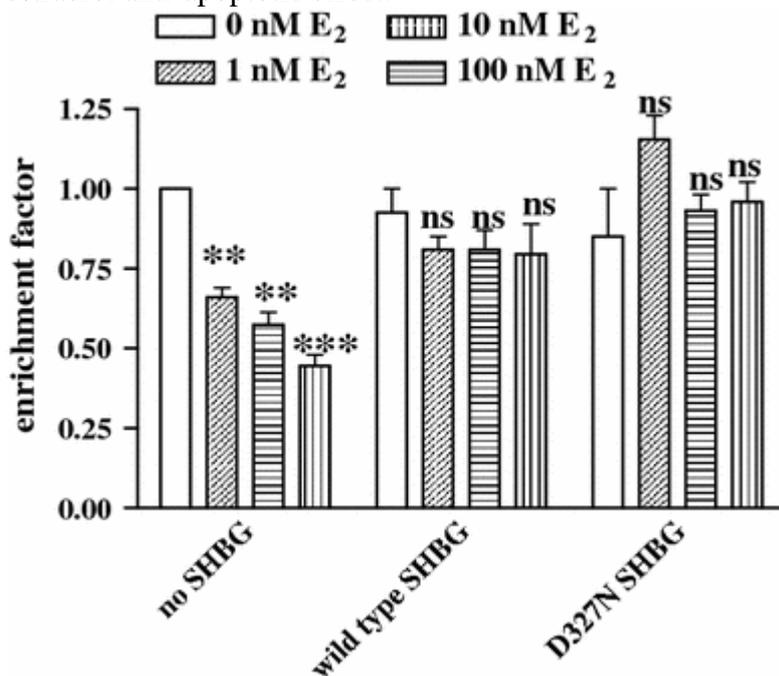


Fig. 3

Effect of wild type and D327N SHBGs on breast cancer cell apoptosis induction. MCF-7 breast cancer cells were treated with increasing estradiol concentrations in the absence or in the presence

of 50 nM recombinant SHBGs. Apoptosis is expressed as enrichment factor, calculated as a fraction of the absorbance of treated cells with respect to the untreated control (untreated cell absorbance 0.131 ± 0.006 , $n = 3$). The figure reports the mean \pm SD from three different experiments. Statistical significance: n.s., not significant; ** $P < 0.01$; *** $P < 0.001$

Wild type and D327N SHBGs cell interaction

To further understand the different effects of wild type and D327N SHBGs, we evaluated the ability of both SHBGs to bind to MCF-7 cells. The amount of D327N SHBG bound to MCF-7 cells was significantly higher than that of wild type SHBG (bound D327N SHBG 43.1 ± 6.32 nmol/ 10^5 cells vs. bound wild type SHBG 18.93 ± 0.35 nmol/ 10^5 cells; $P < 0.01$, $n = 3$).

Effect of wild type and D327N SHBGs on cAMP generation

cAMP generation was evaluated in MCF-7 cells as reported in Fig. 4. As expected, estradiol alone had no effect on cAMP production; wild type SHBG, followed by estradiol treatment caused a significant increase in intracellular cAMP. Estradiol, in cells pre-treated with D327N SHBG, determined a significant increase of cAMP reaching levels significantly higher than those obtained with wild type protein ($P < 0.01$).

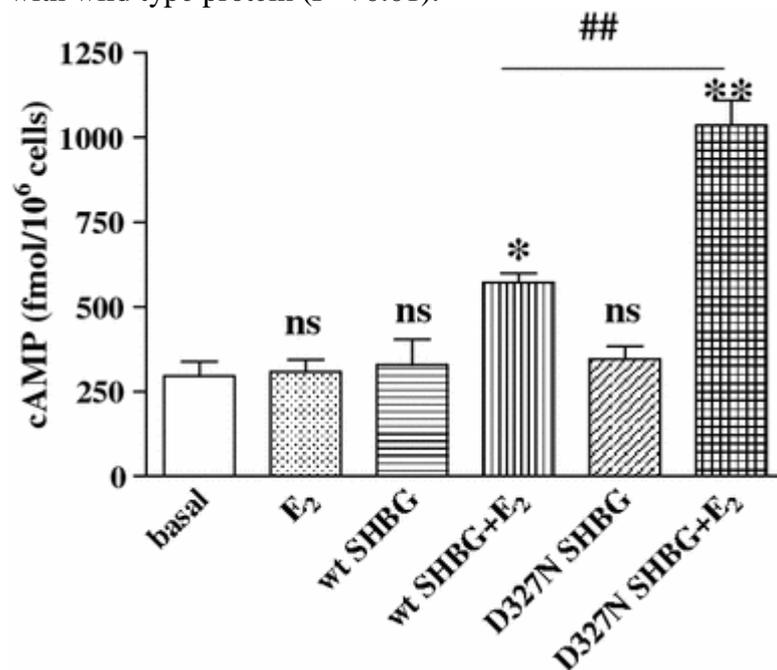


Fig. 4

Effect of wild type and D327N SHBGs on breast cancer cell cAMP production. cAMP production was evaluated in MCF-7 breast cancer cells treated with 10 nM estradiol in the absence or in the presence of 50 nM recombinant SHBGs. The figure reports the mean \pm SD from three different experiments. Statistical significance versus basal, n.s. not significant; * $P < 0.05$; ** $P < 0.01$; Wild type SHBG + E₂ versus D327N SHBG + E₂, ## $P < 0.01$

Effect of wild type and D327N SHBGs on ERK 1/2 phosphorylation

Figure 5 reports the effect of estradiol and wild type and D327N SHBGs on Erk 1/2 phosphorylation. Estradiol, as expected, induced Erk 1/2 phosphorylation; both SHBGs inhibited estradiol effect and D327N SHBG was more efficient than wild type protein.

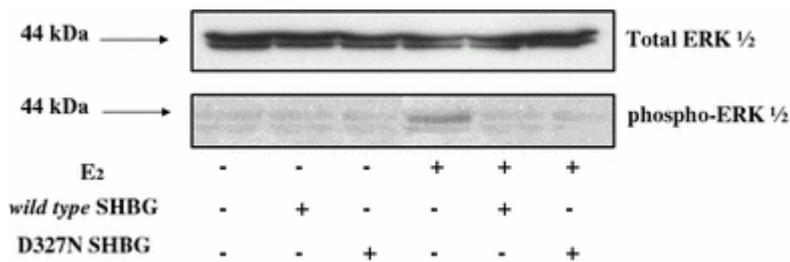


Fig. 5

Effect of wild type and D327N SHBGs on breast cancer cell ERK 1/2 phosphorylation. Western blot of total ERK 1/2 (upper panel) and phospho-ERK 1/2 (lower panel) in MCF-7 cells treated with 10 nM estradiol in the absence or in the presence of 50 nM recombinant SHBGs

Discussion

The single nucleotide polymorphism at Asp327Asn is the more common SHBG polymorphism and it is world-wide distributed even with different ethnic frequency [16]. Here, we reported the Asp327Asn polymorphism in 22% of healthy women taking HRT while only 12% of patients developing breast cancer during HRT presented it. The association of the Asp327Asn polymorphism with breast cancer risk had already been investigated but results are contradictory. In a study on both familial and sporadic breast cancer in Polish and Nordic populations [22] the 327Asn allele carriers presented an overall reduced breast cancer risk, but statistical significance was not attained. As well, in their paper Dunning and coworkers [23] reported no significant association between the Asn variant and breast cancer risk, even though they observed increased serum SHBG levels and a reduced estradiol to SHBG ratio in the same subjects. More recently, Cui et al. [17] in a large population-based case–control study (1,106 cases, 1,180 controls) observed in postmenopausal women a significant association of the Asp327Asn polymorphism with a reduced breast cancer risk that was possibly modified by the estrogen receptor status. Our present results on women taking HRT suggest, in agreement with a previous report [15], a protective role of D327N SHBG in estrogen-dependent breast cancer.

The mechanism by which D327N SHBG exerts its protective role in relation to estrogen action might involve both modification of serum protein levels and its direct effect at cell site. In fact, the single nucleotide polymorphism at Asp327Asn is associated with higher levels of circulating SHBG in hirsute women [24], in normal British postmenopausal women [23], and more recently in a multiethnic cohort of postmenopausal women [16]. These observations are all consistent with an early report indicating that the D327N SHBG has a lower clearance rate than wild type protein [19], thereby contributing to the observed increased serum levels. We could not observe any significant increase of serum SHBG concentration in Asn allele carriers. The protective role of the Asp327Asn polymorphism in breast cancer development could not, therefore, be due to modification of serum SHBG levels. Actually, in the present study we show that D327N SHBG is much more effective than wild type protein in counteracting estradiol action principally at cell site.

Besides acting as a plasma carrier protein, SHBG acts directly in MCF-7 breast cancer cells, inhibiting estradiol-induced cell proliferation [10] and anti-apoptotic effect [11]. In our study the D327N SHBG produced by CHO cells retains its capability of inhibiting estradiol-induced MCF-7 cell proliferation, as we already reported [18], and it resulted even more effective than wild type protein. Moreover, D327N SHBG is able to reverse the antiapoptotic effect of estradiol and again it is more effective than wild type protein at least in MCF-7 cells. The more pronounced effect of mutated SHBG in inhibiting estradiol effects is not likely to be caused by differences in steroid binding. It was reported that the introduction of a third N-glycosylation in the C-terminus of SHBG as it occurs in D327N SHBG did not modify the steroid binding characteristics of the protein [25]

and we also observed no differences in the binding of estradiol to SHBG, wild type or mutated protein (N.Fortunati, L.Costantino, personal data).

The inhibition of estradiol-induced proliferation and anti-apoptosis in breast cancer cells requires the interaction of SHBG with membranes. Therefore, we studied the interaction of both wild type and D327N SHBGs with MCF-7 cells. As expected, both proteins bound to cells, and a significant higher amount of D327N SHBG with respect to wild type interacted with MCF-7 cells. As a consequence, this higher cell binding determined a significant higher efficacy in inducing the second messenger cAMP and in inhibiting the estradiol-induced phosphorylation of Erk ½. The induction of cAMP and the inhibition of the effect of estradiol on MAP kinase pathway were demonstrated to be the fundamental mechanism used by SHBG to interfere with estradiol action [26]. In fact, after binding to MCF-7 cell membranes, SHBG triggers downstream signaling, starting with an increase in intracellular cAMP, followed by induction of PKA activity [10] that in turn can inhibit the MAP kinase pathway such as estradiol-induced Erk activation [11], a key MAP kinase involved in cell proliferation control. It was thus suggested that in breast cancer cells the key regulator of SHBG inhibition of estradiol effects is SHBG-cell interaction and that both estradiol and SHBG membrane-initiated pathways are subject to cross-talk at MAP kinase level with the final result of inhibiting estradiol-mediated cell growth and antiapoptosis [26]. The data presented in this study highlight how the D327N form of SHBG is a more powerful agent in controlling estradiol effects than wild type protein, being more effective in binding to breast cancer cell, in inducing cAMP and in inhibiting estradiol-induced Erk phosphorylation.

In conclusion, the present paper further supports the protective role of D327N SHBG in breast cancer, focusing in particular on the role of mutated SHBG in sheltering breast cells from estrogen exposure. Beside the reported lower clearance rate of the mutated protein, our in vitro data demonstrate that D327N SHBG is more efficient at cell site than wild type protein, being more pronounced its anti-estrogenic effects (anti-proliferative, pro-apoptotic) since the Asn327Asp mutation confers to the protein a higher capacity to bound breast cancer cells and to trigger downstream signaling leading to estradiol effect inhibition.

Acknowledgements

The present paper was partially supported by a grant from Regione Piemonte, Ricerca Sanitaria Finalizzata 2002 to N.F. and by the Special Project “Oncology”, Compagnia San Paolo, Turin to G.B. Authors express their gratitude to Geoffrey L. Hammond, University of British Columbia, Vancouver, British Columbia, Canada, for providing pRc/CMV expression vectors encoding wild type SHBG or D327N SHBG and anti-SHBG rabbit polyclonal antiserum, and to Gabriele Brondino for help in statistical analysis.

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