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Report

The additionally glycosylated variant of human sex hormone-binding globulin (SHBG) is linked to estrogen-dependence of breast cancer *

Marzia Becchis[1], Roberto Frairia[1], Patrizia Ferrera[1], Annamaria Fazzari[1], Sabrina Ondei[1], Alda Alfarano[2], Claudio Coluccia[3], Nicoletta Biglia[4], Piero Sismondi[4], and Nicoletta Fortunati[1]
[1]*Laboratorio di Endocrinologia, Dip. Fisiopatologia Clinica & II UOADU Medicina Generale, Azienda Ospedaliera S. Giovanni Battista;* [2]*Laboratorio di Biologia Molecolare, Dip. Scienze Biomediche & Oncologia Umana;* [3]*IV UOADU Chirurgia Generale, Azienda Ospedaliera S.Giovanni Battista;* [4]*Cattedra di Ginecologia Oncologica, University of Turin, Turin, Italy*

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Summary

Sex Hormone-Binding Globulin (SHBG), the plasma carrier for androgens and estradiol, inhibits the estradiol-induced proliferation of breast cancer cells through its membrane receptor, cAMP, and PKA. In addition, the SHBG membrane receptor is preferentially expressed in estrogen-dependent (ER+/PR+) breast cancers which are also characterized by a lower proliferative rate than tumors negative for the SHBG receptor. A variant SHBG with a point mutation in exon 8, causing an aminoacid substitution (Asp 327 → Asn) and thus, the introduction of an additional N-glycosylation site, has been reported. In this work, the distribution of the SHBG variant was studied in 255 breast cancer patients, 32 benign mammary disease patients, and 120 healthy women. The presence of the SHBG mutation was evaluated with PCR amplification of SHBG exon 8 and Hin**f I** restriction fragment length polymorphism (RFLP) procedure. This technique allowed us to identify 54 SHBG variants (53 W/v and 1 v/v) in breast cancer patients (21.2%), 5 variants (4 W/v and 1 v/v) in benign mammary disease patients (15.6%), and 14 variants (W/v) in the control group (11.6%). The results of PCR and RFLP were confirmed both by nucleotide sequence of SHBG exon 8 and western blot of the plasma SHBG. No differences in the mean plasma level of the protein were observed in the three populations. The frequency of the SHBG variant was significantly higher in ER+/PR+ tumors and in tumors diagnosed in patients over 50 years of age than in the control group. This observation suggests the existence of a close link between the estrogen-dependence of breast cancer and the additionally glycosylated SHBG, further supporting a critical role of the protein in the neoplasm.

Introduction

Estrogen-dependent breast cancer induction and development is controlled by estradiol, which after binding to its nuclear receptor (ER) induces cell proliferation [1, 2]. Estrogen-dependent breast cancer is characterized by the expression of ER and by the estradiol-induction of the progesterone receptor (PR). It is also well-known that tumors expressing both ER and PR have a better prognosis, and, in addition, may be easily manipulated using anti-estrogenic compounds, which represent one of the most powerful therapeutic strategies of the disease [3, 4]. It has been suggested that the function of ER can also be modulated by the second messenger cAMP, induced by the activation of a number of membrane receptors [5, 6].

Among these, for the last years we have been mainly concentrating on the membrane receptor for Sex Hormone-Binding Globulin (SHBG or SBP), [7], which has been shown to be a potential tool in the control of estradiol-induced proliferation of breast cancer cells. In fact, the receptor (SHBG-R) is expressed on membranes of MCF-7 cells (estrogen-dependent breast cancer cultured cells), [8, 9] and also on membranes of estrogen-dependent breast cancer tissue samples [10].

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We have also observed that SHBG, through SHBG-R, cAMP, and PKA, inhibits the estradiol-induced proliferation of MCF-7 cells [11] and that breast cancer tissue samples positive for SHBG-R are characterized by a lower proliferative rate than negative samples [10].

A variant form of plasma SHBG, characterized by an additional N-glycosylation site [12, 13] due to a point mutation in exon 8 (Asp 327 → Asn), has been described [14]. Bocchinfuso et al. [15] reported that the additional glycosylation of the variant SHBG does not influence the steroid binding properties and the immunological characteristics of the protein, while Avvakumov et al. [16] demonstrated that the carbohydrate moiety of the wild type SHBG is critical for a correct binding of the glycoprotein to its receptor.

The aim of this study was to analyze the presence of the variant SHBG in a breast cancer patient population in order to evaluate any possible association between the mutated protein and the neoplasm.

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. Women affected with breast cancer ($n = 255$) were referred to both the IV UOADU Chirurgia Generale, Azienda Ospedaliera S.Giovanni Battista, Torino, and to the Cattedra di Ginecologia Oncologica, Università di Torino, while women affected with benign mammary disease ($n = 32$) were referred to the Cattedra di Ginecologia Oncologica. Control subjects were healthy women ($n = 120$) with no sign or history of actual/previous endocrine and neoplastic diseases (in particular, obese subjects were excluded). Serum samples were stored at -20°C before analysis. White blood cells were isolated from EDTA-treated blood for DNA extraction and analysis.

SHBG immunoassay

Serum SHBG concentrations were evaluated using the double-antibody SHBG immunoradiometric assay (Farmos Diagnostica, Oulunsalo, Finland).

Analysis of SHBG exon 8

PCR amplification and restriction fragment length polymorphism (RFLP)

Genomic DNA was extracted from whole blood samples using a non enzymatic method routinely used in our laboratory [17]. 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. Amplification reactions contained 0.1M Tris–HCl pH 8.8, 0.5M KCl, 25 mM $MgCl_2$, BSA 2 mg/ml, 2 mM each deoxyribonucleotide (Boehringer Mannheim Gmbh, Mannheim, Germany), 50 pmol of each primer, 0.5 μg genomic DNA template, and 1 U Taq DNA polymerase (Amersham Life Science, Little Chalfont, Bucks., UK) in a total reaction volume of 100 μl. The reaction mixtures were incubated at 95°C for 1 min and then subjected to 35 cycles of amplification (denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min). Aliquots (10 μl) of each PCR product were analyzed on 1.4% agarose (Sigma Chemical, St. Louis MO, USA) gels to monitor the specificity (based on the predicted size) and amount of product.

The mutation was identified by a digestion of PCR products with 20 U of the restriction enzyme Hin**f I** (Amersham Life Science, Little Chalfont, Bucks., UK) overnight at 37°C. Digestion products were visualized after electrophoresis in a 2% agarose gel.

Nucleotide sequence of SHBG exon 8

The DNA templates were treated with two hydrolytic enzymes before sequencing, to remove the excess dNTPs and primers from DNA produced by amplification. 10 U of exonuclease I and 2 U of shrimp alkaline phosphatase (Amersham Life Science, Little Chalfont, Bucks., UK) were added to 5 μl of PCR amplification mixture and incubated at 37°C for 15 min and then at 80°C for 15 min in the thermal cycler. Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Little Chalfont, Bucks., UK) was used to sequence three templates identified as homozygous, heterozygous, and wild type SHBG in presence of [α^{33}P] ddNTP (450 μCi/ml) (Amersham Life Science, Little Chalfont, Bucks., UK). The primers for sequence analysis were identical to the primers used for PCR amplification. Sequencing mixtures were subjected to 50 cycles of amplification (the same conditions used for PCR amplification). Sequence reaction products were separated on 6% polyacrylamide gels containing 8.3M urea.

Analysis of SHBG variants by western blotting

Serum containing normal or mutated SHBG (previously identified with PCR and RFLP) was applied to 2 ml columns of Blue Sepharose CL-6B (Sigma Chemical, St. Louis MO, USA) pre-equilibrated in

0.05 M Tris–HCl pH 7.0, 0.1 M KCl, in order to selectively remove albumin. Eluates were 50× concentrated (Minicon B devices, Amicon Division, Beverly MA, USA) and the protein concentration of each sample was determined using the DC Protein Assay Kit (Bio Rad Labs., Richmond CA, USA).

Samples were then diluted with electrophoresis loading buffer (125 mM Tris–HCl pH 6.8, 4.6% SDS, 0.05% DTT, 10% glycerol, 0.01% bromophenol blue) and boiled at 90°C for 10 min. The samples (40 μg protein/well) were then subjected to SDS-PAGE (5% stacking gel and 12% resolving gel). Proteins were electrophoretically transferred from the gel onto PVDF membranes (Amersham Life Science, Little Chalfont, Bucks., UK). The membrane was first incubated overnight with 3% gelatin in TTBS (20 mM Tris, 500 mM NaCl, pH 7.5, plus 0.05% Tween-20) to block the nonspecific sites; subsequently, it was incubated for 4 h with a rabbit-derived polyclonal antiserum against human SHBG (kindly provided by G.L. Hammond, London Regional Cancer Centre, London, Ontario, Canada) diluted 1:500 in 1% gelatin in TTBS, followed by 1 h incubation with 1:10,000 secondary horseradish peroxidase-labelled antibody. Immunoreactive proteins were detected with the chemiluminescence ECL detection system (Amersham Life Science, Little Chalfont, Bucks., UK) according to the manufacturer's instructions.

Data analysis and statistics

All analyses used the Graphpad Instat PC program (Graphpad Software Inc., San Diego CA, USA). Breast cancer patients were *a priori* grouped on the basis of age at diagnosis ($<$ or $>$ 50 years), on tumor histology, and tumor estradiol and progesterone receptor (ER and PR) pattern; benign breast disease patients were grouped on the basis of the histology of the lesion. The frequency of the SHBG variant was calculated in the different populations/groups and compared with the Yates' corrected for continuity χ^2 test. All significance tests used a two-tailed p value and statistical significance was attained for $p < 0.05$.

Results

Analysis of SHBG variants

The three populations under study were screened for the presence of the SHBG variant using the PCR amplification followed by Hin**f I** RFLP of the exon 8. PCR amplification of SHBG exon 8 was obtained from genomic DNA of 255 breast cancer patients, 32 benign mammary disease patients, and 120 women in the control group. The electrophoretic analysis of each PCR product on 1.4% agarose gels revealed a single band with a molecular weight of about 300 bp, according to the predicted size (294 bp). To identify the point mutation in exon 8, each amplification product was submitted to digestion with the restriction endonuclease Hin**f I**, which recognizes the sequence G ↑ ANTC. The GAC → AAC mutation of SHBG gene exon 8 destroys one of the two Hin**f I** sites of the exon. Exon 8 Hin**f I** digestion will, therefore, result in three fragments (138, 92, and 64 bp) for subjects carrying the SHBG wild type (W/W); in two fragments (156 and 138 bp) for homozygous subjects (v/v); and in four fragments (156, 138, 92, and 64 bp) for heterozygous subjects (W/v), as shown in Figure 1.

In order to confirm that the previously described point mutation of codon 327 [14] was responsible for the different RFLP patterns we observed, the nucleotide sequence of SHBG exon 8 was obtained for one wild type, one heterozygous, and one homozygous patient. Direct sequencing, both upstream and downstream, showed the reported G → A substitution at codon 327 in the v/v patient, while the W/v presented both G and A in this position (Figure 2).

As reported in Figure 3, the western blot analysis of the plasma SHBG of one W/W, one v/v, and two W/v patients confirmed that the mutation of exon 8 generated the predicted electrophoretic phenotypes [12]. In fact, W/W SHBG was characterized by two bands (52 and 49 kDa), whereas W/v and v/v protein presented an additional 56 kDa band, which was more evident and abundant in the homozygous phenotype.

Analysis of SHBG variant distribution

Table 1 reports the characteristics and the frequency of SHBG variant in the three populations under study. The frequency of SHBG variant in breast cancer patients was higher than in the the control group even though statistical significance was not attained. The v allele (mutated) frequencies were 0.108 in breast cancer patients, 0.094 in benign mammary disease patients, and 0.058 in the control group, respectively. Mean plasma concentrations of SHBG were similar in all the three populations. In particular, plasma SHBG of the two homozygous patients found (one breast cancer patient and one benign mammary disease patient) was 29 and 35 nM, respectively.

The distribution of SHBG variant in the benign mammary disease patients, grouped on the basis of the lesion histology, revealed that in all 5 variant

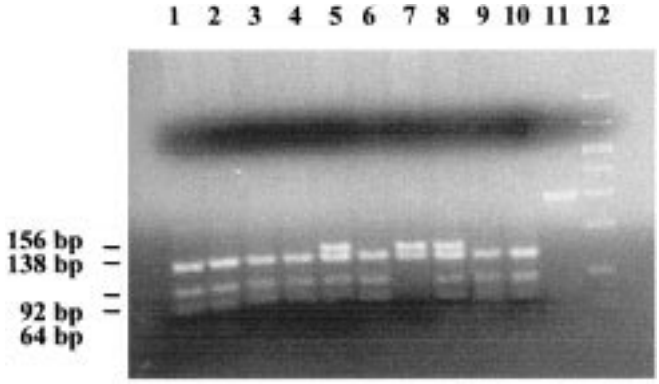

Figure 1. Hin**f I** RFLP analysis of SHBG exon 8 from genomic DNA of breast cancer patients: detection of D327N point mutation. Exon 8 of SHBG gene was amplified with PCR and digested with Hin**f I**, as described in Materials and methods. Hin**f I** digestion of SHBG exon 8 results in three fragments for subjects carrying the wild type SHBG (W/W), in two fragments for homozygous subjects (v/v), and in four fragments for heterozygous subjects (W/v). Lanes 1–4, 6, 9, 10: W/W subjects; lane 7: v/v subject; lanes 5, 8: W/v subjects; lane 11: undigested DNA; lane 12: DNA markers.

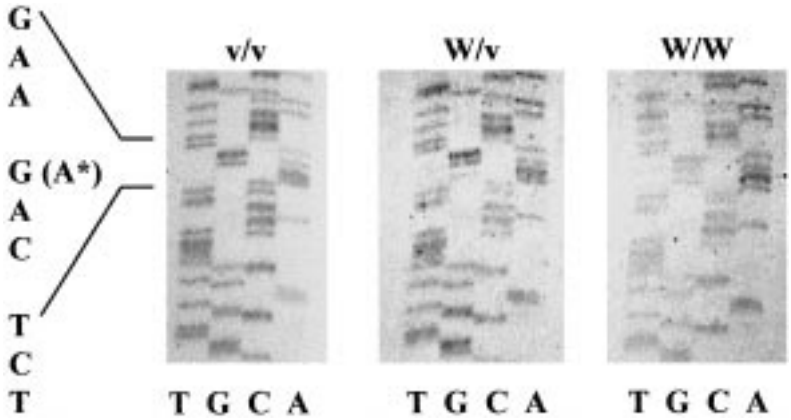

Figure 2. Partial nucleotide sequence of SHBG exon 8. PCR amplified exon 8 of DNAs from one v/v, one W/v, and one W/W breast cancer patient were sequenced as described in Materials and methods. The position of the point mutation (∗) is indicated.

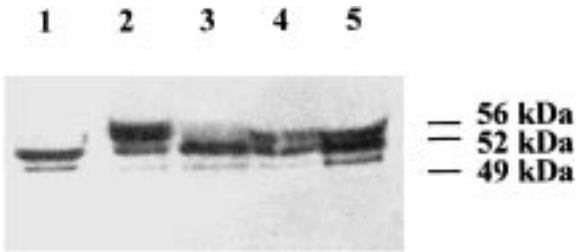

Figure 3. Western blot of plasma SHBG from breast cancer patients. Lane 1: purified human SHBG used as control; lane 2: v/v SHBG; lane 3: W/W SHBG; lanes 4, 5: W/v SHBG. Size markers are shown on the right.

carriers (4 W/v and 1 v/v) fibroadenosis was diagnosed (Table 2). The number of cases is, unfortunately, too small to give a particular meaning to this observation.

The distribution of SHBG variant in breast cancer patient subgroups, identified as previously described, is reported in Tables 3, 4, and 5, respectively. The SHBG variant frequency was compared within each subgroup, be it with the total breast cancer patients, be it with the control group. No significant difference was observed when grouping patients on the basis of tumor histology. The SHBG variant was significantly more frequent ($p < 0.05$) in patients in which breast cancer was diagnosed after 50 years, than in the control group. As far as the tumor ER/PR pattern was concerned, the frequency of SHBG variant was significantly higher in ER+/PR+ patients ($p < 0.05$) than in the control group.

Table 1. Characteristics and percent of variant SHBG of the three populations under study

	n	Age (years) m ± SD	Plasma SHBG (nM) m ± SD	Variant SHBG* (*n*)	Variant SHBG (%)
Breast cancer patients	255	58 ± 21	46.3 ± 24.6	53 W/v 1 v/v 54 total	21.2
Benign mammary disease patients	32	46 ± 15	44.1 ± 23.5	4 W/v 1 v/v 5 total	15.6
Control group	120	45 ± 18	45.2 ± 15.4	14 W/v 14 total	11.6

*Variant SHBG: breast cancer patients vs. benign mammary disease patients, $\chi^2 = 0.14$, $p = 0.708$, NS; breast cancer patients vs. control group, $\chi^2 = 3.032$, $p = 0.081$, NS; benign mammary disease patients vs. control group, $\chi^2 = 0.05$, $p = 0.818$, NS.

Table 2. Benign mammary disease patients ($n = 32$). The patients were grouped on the basis of histology and the percent of variant SHBG calculated for each subgroup

Histology	*n*	% out of total	Variant SHBG (*n*)	Variant SHBG (% in the group)
Fibroadenosis	17	53.2	5	29.4
Cyst	11	34.4	0	0
Microcalcifications	2	6.2	0	0
Not determined	2	6.2	0	0

Table 3. Breast cancer patients ($n = 255$). The patients were grouped on the basis of the age at diagnosis and the percent of variant SHBG was calculated for each group

Age at diagnosis	*n*	% out of total	Variant SHBG* (*n*)	Variant SHBG (% in the group)
< 50 yr	73	28.6	11	15
> 50 yr	182	71.4	43	23.6

*Variant SHBG: breast cancer patients < 50 yr vs. control group, $\chi^2 = 0.143$, $p = 0.705$, NS; breast cancer patients > 50 yr vs. control group, $\chi^2 = 4.091$, $p = 0.043$; (control group, total $n = 120$, mutated SHBG $n = 14$).

Table 4. Breast cancer patients ($n = 255$). The patients were grouped on the basis of tumor histology and the percent of variant SHBG was calculated for each group

Histology	*n*	% out of total	Variant SHBG (*n*)	Variant SHBG (% in the group)
Infiltrating ductal Ca	192	75.3	40	20.8
Infiltrating lobular Ca	42	16.5	10	23.8
Ductal-lobular Ca	7	2.7	2	28.6
In situ ductal Ca	5	2	2	40
In situ lobular Ca	2	0.8	0	0
Infiltrating tubular Ca	3	1.2	0	0
Papillary Ca	2	0.8	0	0
Medullary Ca	1	0.35	0	0
Comedo Ca	1	0.35	0	0

Table 5. Breast cancer patients ($n = 255$). The patients were grouped on the basis of tumor estrogen and progesterone receptor pattern and the percent of variant SHBG was calculated for each group

ER/PR	*n*	% out of total	Variant SHBG* (*n*)	Variant SHBG (% in the group)
+/+	147	58	36	24.5
−/−	54	21.2	11	20.4
+/−	44	17.2	7	15.9
−/+	10	3.6	0	0

*Variant SHBG: breast cancer patients +/+ vs. control group, $\chi^2 = 4.284$, $p = 0.038$; breast cancer patients −/− vs. control group, $\chi^2 = 1.133$, $p = 0.287$, NS; breast cancer patients +/− vs. control group, $\chi^2 = 0.135$, $p = 0.712$, NS; breast cancer patients −/+ vs. control group, $\chi^2 = 0.273$, $p = 0.601$, NS; (control group, total $n = 120$, mutated SHBG $n = 14$).

Discussion

The data reported in the present paper suggest for the first time that the additionally glycosylated SHBG variant is more frequent in estrogen-dependent breast cancer patients than in the healthy controls.

A large number of subjects were screened for the present study using the PCR and Hin**f I** RFLP of SHBG exon 8. As confirmed by nucleotide sequence and western blot, this technique is sensitive enough to identify both heterozygous and homozygous subjects. In addition, the method we used is quite simple and not excessively time-consuming, and, for this reason, is advisable in large-scale screening.

The function of the SHBG variant has not been specifically investigated. Nevertheless, our observations provide some interesting guidelines for future studies regarding the functional relationship between breast cancer and SHBG glycosylation.

In particular, our data pointed out that the SHBG variant is related to some characteristics of the tumor, such as estrogen-dependence. In fact, only ER+/PR+ tumors presented an SHBG variant frequency significantly higher than the control group. The close link between estrogen-dependent breast cancer and SHBG function at the cell site was proposed by previous studies on cultured MCF-7 cells [11]. The wild type SHBG was described to inhibit the estradiol-induced proliferation of MCF-7 cells through its membrane receptor. Moreover, the SHBG receptor is expressed preferentially on ER+/PR+ neoplasms, which are additionally characterized by a lower proliferative rate than tumors negative for the SHBG receptor [10]. The distribution of the SHBG variant in breast cancer patients seems to follow the SHBG receptor distribution, indirectly suggesting an increased activity of the additionally glycosylated protein at the cell site. The present data do not offer any direct evidence regarding the receptor binding and activation of the SHBG variant, but several authors [15, 18] have already speculated as to this possibility. It has been recently described that the SHBG variant has a reduced clearance rate [18], and this could partially account for the hypothesized increased cell function. Unfortunately, only the SHBG obtained from homozygous subjects presented such a prolonged half-life, while in the breast cancer patients we evaluated only one homozygous patient, out of 54 SHBG variant carriers, was found. However, studies are in progress to understand the mechanisms of action of the additionally glycosylated SHBG at the receptor level in breast cancer cells.

The data presented in this paper also suggest that the SHBG variant is more frequent in patients in which breast cancer was diagnosed after 50 years of age. It has been reported that breast cancers arising in postmenopause are less aggressive [19]. The SHBG variant seems again to be related to tumors with a better prognosis.

In conclusion, the present study contains evidence that the additionally glycosylated variant of plasma SHBG is more frequent in estrogen-dependent breast cancer and suggests that it is linked to a better prognosis of the neoplasm.

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Address for offprints and correspondence: Nicoletta Fortunati, Lab. Endocrinologia, Via Genova 3, 10126 Torino, Italy; *Tel:* 39-11-633-6627; *Fax:* 39-11-667-0436; *E-mail:* fortunat@iol.it