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Expression of sex hormone-binding globulin exon 7 splicing variant mRNA in secondary spreading lesions of gynecological cancers

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Abstract

This study was designed to determine the clinical implications of intracellular expression of sex hormone-binding globulin (SHBG) wild-type and exon 7 splicing variant mRNAs in secondary spreading lesions of gynecologic cancers using the reverse transcription-polymerase chain reaction-Southern blot and DNA sequencing analyses. Compared with primary cancers, a relative increase in SHBG variant mRNA to wild-type mRNA expression was observed (4/10 cases of uterine endometrial cancers, 5/10 cases of uterine cervical cancers, 6/10 cases of ovarian cancers) or the expression of SHBG wild-type and variant mRNAs could not be detected (5/10 cases of uterine endometrial cancers, 3/10 cases of uterine cervical cancers, 4/10 cases of ovarian cancers). On the other hand, alteration to a relative increase in SHBG wild-type mRNA expression in the metastatic lesions occurred in only 3 cases (1/10 cases of uterine endometrial cancers and 2/10 cases of uterine cervical cancers) analyzed.

These results suggest that the transcription of SHBG mRNA and the regulation of its splicing might be altered with metastatic potential, and this status might be involved in a change in steroidal dependency of metastatic lesions.

Keywords: Sex hormone-binding globulin; mRNA; Splicing variant; Metastatic tumor; Gynecologic cancer

1. Introduction

In human plasma, sex hormone-binding globulin (SHBG) is a glycoprotein binding to steroid hormones such as estrogen and androgen [1]. Much evidence indicates that the SHBG–steroid complex directly plays a role in intracellular steroidal actions in the target cells [2–4]. The expression of SHBG mRNA has been demonstrated in female reproductive organs and cancers of the uterine endometrium, cervix and ovary [5–8]. The human SHBG gene consists of eight exons separated by seven small introns [9]. Screening of a human testis cDNA library revealed the presence of a clone lacking a 208-base pair region as an exon 7 splicing variant of the SHBG gene [9]. This clone encodes for a truncated form of SHBG, which lacks a part of the steroid-binding domain [10]. Recently, SHBG exon 7 splicing variant mRNA has been detected in normal and primary cancer tissues of uterine endometrium, cervix and ovary [11–13], plausibly being involved in the development and growth of these cancers. In these cancers, the ratio of SHBG variant to wild-type mRNA levels is higher than in normal tissues [11–13]. This prompted us to investigate the status of SHBG wild-type and variant mRNA expression in primary cancers and their corresponding metastatic lesions.

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Table 1
Status of SHBG wild-type and variant mRNA expressions in uterine endometrial cancers. G1, well-differentiated adenocarcinoma of the endometrium; G2, moderately differentiated adenocarcinoma of the endometrium; G3, poorly differentiated adenocarcinoma of the endometrium; ADS, adenosquamous carcinoma of the endometrium. Clinical stagings were according to International Federation of Obstetrics and Gynecology (FIGO) classification. ND, not detected. All data show the mean ratio in 4 different parts of each individual tumor

Case No.	Serum estradiol level (pg/ml)	Serum progesterone level (ng/ml)	Serum SHBG level (nmol/l)	SHBG variant/wild-type mRNA ratio		Histological type	Stage
				primary	metastatic		
1	20	0.6	76	3.8	ND	G2	IIIc
2	10	0.8	35	2.6	3.2	G1	Ib
3	45	0.7	68	1.4	ND	ADS	IIIc
4	35	0.7	66	4.1	2.6	G2	Ic
5	28	0.4	54	3.1	ND	G2	Ib
6	34	0.8	89	1.9	ND	G1	IVb
7	68	1.8	58	2.4	4.1	G3	IVa
8	20	0.5	43	3.6	ND	G3	IIb
9	10	0.8	70	1.6	2.8	G1	IIIb
10	15	0.7	58	3.9	6.9	G3	IIb

2. Materials and methods

2.1. Materials

30 patients (age 31–68 years) underwent operations for cancers of the uterine endometrium, cervix or ovary at the Department of Obstetrics and Gynecology, Gifu University School of Medicine from March 1997 to August 1998, as indicated in Tables 1–3. None of the patients had received any pre-operative therapy. Agreements for the study were obtained from the patients and the Research Committee for Human Subjects, Gifu University School of Medicine. The cancer tissues obtained after resection were immediately frozen in liquid nitrogen and later prepared for the subsequent experiments. Clinical staging was performed according to the International Federation of Obstetrics and Gynecology (FIGO) criteria [14]. Blood samples were drawn just before the surgery and the plasma was stored at −20°C until assayed.

2.2. Determination of serum estradiol-17β, progesterone and SHBG

Serum estradiol-17β and progesterone levels were determined by radioimmunoassay using an E_2 kit, Daiichi II (Daiichi Radioisotope Laboratories, Tokyo) and a progesterone kit (Nihon DPC, Tokyo), respectively [5]. The serum SHBG level was determined by time-resolved fluoroimmunoassay using a DELFIA SHBG kit (Wallacoy, Turku, Finland) [5].

Table 2
Status of SHBG wild-type and variant mRNA expressions in uterine cervical cancers. LNK, large cell non-keratinizing squamous cell carcinoma of the cervix; K, keratinizing squamous cell carcinoma of the cervix; SNK, small cell non-keratinizing squamous cell carcinoma of the cervix. Clinical stagings were according to International Federation of Obstetrics and Gynecology (FIGO) classification. ND, not detected. All data show the mean ratio in 4 different parts of each individual tumor

Case No.	Serum estradiol level (pg/ml)	Serum progesterone level (ng/ml)	Serum SHBG level (nmol/l)	SHBG variant/wild-type mRNA ratio		Histological type	Stage
				primary	metastatic		
11	10	0.8	69	1.0	2.0	K	IIb
12	87	7.2	72	2.9	3.5	LNK	Ib
13	114	12	78	2.2	1.5	K	IIIb
14	43	1.6	56	3.7	2.0	K	IVa
15	104	16	89	0.8	1.3	SNK	IIIc
16	58	15	56	3.9	ND	LNK	IIIb
17	46	6.4	43	1.1	ND	SNK	IIIb
18	71	7.4	73	1.3	3.3	LNK	IIIb
19	89	4.9	98	4.1	ND	K	IIb
20	15	0.9	49	3.5	4.8	SNK	IIb

Table 3
Status of SHBG wild-type and variant mRNA expressions in ovarian cancers. SCAD, serous cystadenocarcinoma of the ovary; MCAD, mucinous cystadenocarcinoma of the ovary. Clinical stagings were according to International Federation of Obstetrics and Gynecology (FIGO) classification. ND, not detected. All data show the mean ratio in 4 different parts of each individual tumor

Case No.	Serum estradiol level (pg/ml)	Serum progesterone level (ng/ml)	Serum SHBG level (nmol/l)	SHBG variant/wild-type mRNA ratio		Histological type	Stage
				primary	metastatic		
21	43	7.8	39	2.1	3.5	SCAD	IIIc
22	12	3.3	70	2.5	3.1	SCAD	IIIc
23	105	10	65	0.4	1.5	MCAD	IIIc
24	78	6.2	114	2.2	ND	SCAD	IV
25	98	11	89	0.4	0.9	SCAD	IIIc
26	47	7.6	69	0.4	ND	SCAD	IV
27	129	14	97	1.9	2.8	SCAD	IV
28	36	8.8	44	1.4	ND	MCAD	IIIc
29	20	0.8	73	1.8	2.4	SCAD	IIIc
30	14	0.4	77	1.2	ND	MCAD	IV

2.3. Reverse transcription (RT)

Total RNA was isolated from each specimen by the acid guanidium thiocyanate–phenol–chloroform extraction method [15]. The total RNA (3 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RT, 200 units, Gibco BRL, Gaithersburg, MD) in 50 M Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 40 units of RNAsin (Toyobo, Osaka, Japan), 10 mM dithiothreitol and 0.5 mM deoxyribonucleoside triphosphates (dNTPs), using random hexamer (50 ng, Gibco BRL) for 60 min at 37°C. The reaction was incubated for 5 min at 95°C to inactivate MMLV-RT.

2.4. Polymerase chain reaction (PCR)

PCR with reverse-transcribed RNAs and genomic DNAs isolated from each tissue as templates (1 μl) and 5 pmol of specific primers either for SHBG (SHBG-5′: 655–674, exon 6, 5′-ATTCCCCAGCCTCATGCAGA-3′ and SHBG-3′: 1119-1138, exon 8, 5′-AAGCGTCAGTGCCATTGCCT-3′) or for glyceraldehyde-3-phosphate dehydrogenase (GAPDH-5′: 71–96, exon 1, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and GAPDH-3′: 1030–1053, exon 8, 5′-CATGTGGGCCATGAGGTCCACCAC-3′) was carried out using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) with 0.5 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus) in a buffer containing 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. GAPDH mRNA was used as an internal standard for SHBG mRNA expression. Amplification was performed for 38 cycles for SHBG PCR products and 23 cycles for GAPDH PCR products at 94°C for 45 s for denaturing, 55°C for 45 s for annealing and 72°C for 90 s for extension. Primers and oligonucleotide probes were designed according to the genomic organization of human SHBG and GAPDH genes [9,16] (Fig. 1).

2.5. Southern blot analysis

Amplified PCR products were applied to 1.2% agarose gel for electrophoresis performed at 100 V and capillary-transferred to a nylon membrane (Millipore, Burlington, MA) for 20 h, using a 10 × standard sodium citrate solution (SSC; 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). After blotting, the membrane was dried at 80°C for 15 min and then cross-linked by ultraviolet irradiation (33,000 μJ/cm^2 at 254 nm). The membrane was prehybridized in a hybridization buffer (1 M NaCl, 50 mM Tris–HCl pH 7.6, 1% sodium dodecyl sulfate) at 42°C for 2 h and then in the same solution with biotinylated specific oligonucleotide probes (probe 1: 686–705, exon 6, 5′-TTCTCTTTGGACCTGGGACT-3′ and probe 2: 961–980, exon 7, 5′-AAGCCTCAAGGGCGTCTCTT-3′, as shown in Fig. 1; 10 pmol/μl, synthesized by Rikaken Co., Nagoya, Japan) at 42°C for 16 h.

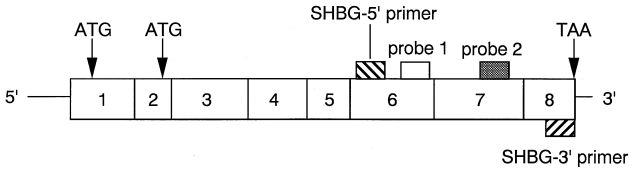

Fig. 1. Schematic exon (1 to 8) presentation of human SHBG cDNA. Location of oligonucleotide primers for PCR amplification and probes for Southern blot analysis are shown in this schema.

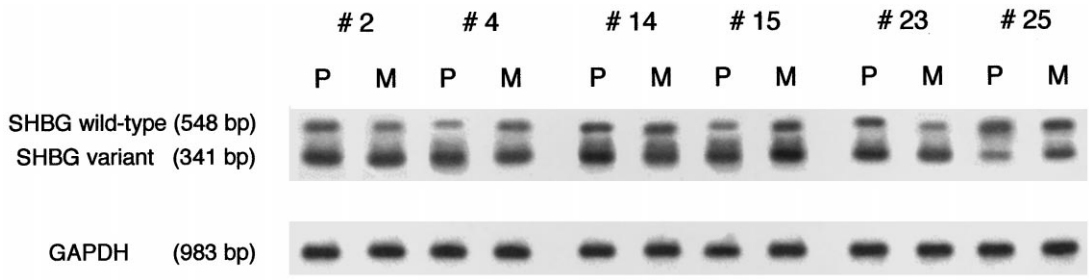

Fig. 2. RT-PCR-Southern blot analysis of SHBG wild-type and variant mRNA expressions in representative cases of cancers of uterine endometrium, cervix and ovary. Total RNA isolated from each tissue was reverse transcribed and amplified with primers specific to SHBG or GAPDH gene. Southern blot analysis was performed as described in Section 2. Results and clinical backgrounds of each case are described in Tables 1–3. #, case number; P, primary tumor; M, metastatic tumor.

Finally, the membrane was washed with $0.5 \times$ SSC at 65°C. The detection reaction for hybridized biotin was performed using a Plex chemiluminescent kit (New England BioLabs, Beverly, MA). Kodak XAR-5 film (Eastman Kodak, Rochester, NY) was exposed on the membrane for 15 min. The strength of the recorded signal on film was analyzed densitometrically by calculating the area with total integrated optical density (IOD) using Bio Image (Millipore Corporation, Bedford, MA). The IODs show arbitrary units calculated by Bio Image.

2.6. DNA sequence analysis

Amplified PCR products were electrophoresed with 1.2% agarose gel. The DNA fragments were eluted from excised agarose gel slices by a Gene Clean II kit (BIO 101, Vista, CA). Single strand DNA used as template was purified by an Autoroad Solid Phase Sequencing Kit (Pharmacia, Uppsala, Sweden). Sequencing reactions were performed using the dideoxy chain-termination method by automated methods, employing a Pharmacia A.L.F. express DNA

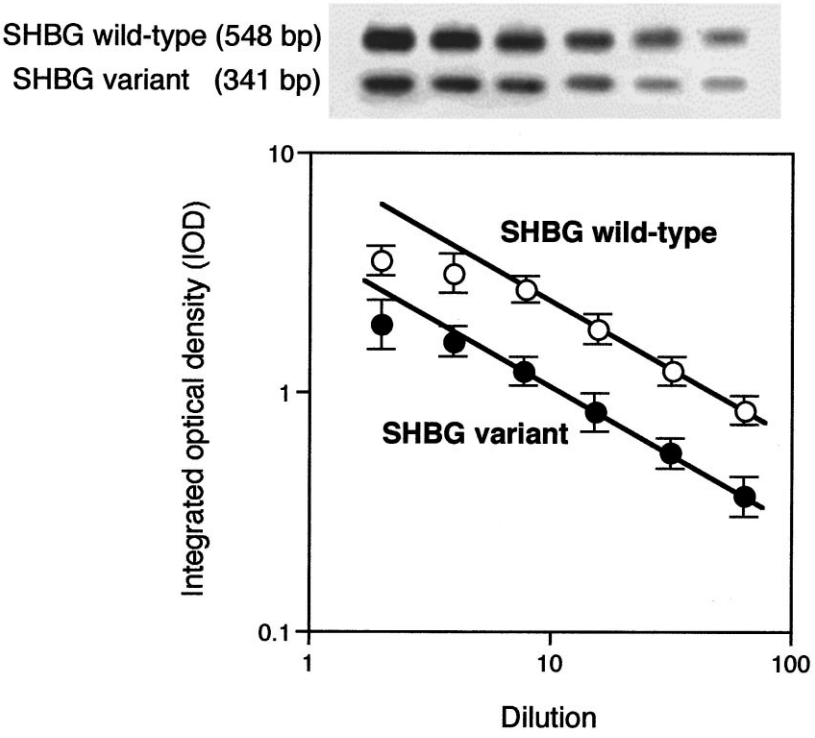

Fig. 3. Signal intensity curve for SHBG wild-type and variant mRNA levels in a series of reverse-transcribed total RNA of normal uterine endometria by reverse transcription-polymerase chain reaction (RT-PCR)-Southern blot analysis. PCR was performed with serial dilutions of reverse-transcribed total RNA (3 μg), ranging from 1/64 to 1/2 dilution. The upper panel shows the results of Southern blot analysis after RT-PCR. Data are the mean ± S.D. of eight determinations.

sequencer with a fluorescein-tagged Cy5 primer and Autoread kit (Pharmacia).

3. Results

Two different sizes of PCR products for SHBG mRNA were observed in all samples of primary cancers and in 18 out of 30 samples of metastatic cancers: the slowly migrating band corresponded to the full length of SHBG mRNA (548 base pairs, nucleotides 591 to 1138 of the SHBG), whereas the faster migrating band corresponded to exon 7 splicing variant SHBG mRNA (approximately 350 base pairs). To determine the detected exon of SHBG mRNA, we carried out Southern blot analysis using two different oligonucleotide probes corresponding to a part of a sequence in exons 6 and 7 (Fig. 1). Both the slowly and faster migrating bands were detected using probe 1 coding a part of exon 6, while only the slowly migrating band was detected using probe 2 coding a part of exon 7 (data not shown), suggesting the variant is deleted at exon 7. In the PCR-Southern blot analysis for genomic DNAs isolated from ovaries and ovarian tumors, only one band (1654 base pairs) corresponding to the full length of SHBG gene was detected without non-specific products (data not shown). To confirm the expression of SHBG exon 7 splicing variant mRNA, DNA sequencing analysis for the PCR products was carried out in all samples detected. The slowly migrating product was identical to the corresponding full sequences of SHBG cDNA, whereas the faster migrating product lacked base pairs corresponding to the entire exon 7. SHBG variant and wild-type mRNA expressions in primary cancers and their corresponding metastatic lesions of the uterine endometrium, cervix and ovary in individual cases are shown in Fig. 2 as representative Southern blot analyses.

The signal intensity curve for SHBG wild-type and variant mRNAs levels, ranging from 1/64 to 1/2 dilution of reverse-transcribed total RNA of normal uterine endometrium by RT-PCR-Southern blot analysis, was linear (Fig. 3). Therefore, the ratio of SHBG variant to wild-type mRNA levels was proved to be reliable. Moreover, we performed total RNA isolation and competitive RT-PCR-Southern blot analysis in four different part of each individual sample.

As shown in Tables 1–3, alteration to a relative increase in SHBG variant to wild-type mRNA expression in the metastatic lesions occurred in 4 out of 10 cases of uterine endometrial cancers, 5 out of 10 cases of uterine cervical cancers and 6 out of 10 cases of ovarian cancers. Conversely, alteration to a relative increase in SHBG wild-type mRNA expression in the metastatic lesions occurred in 1 out of 10 cases of uterine endometrial cancers and 2 out of 10 cases of uterine cervical cancers. Additionally, neither SHBG wild-type nor variant mRNAs were detected in 5 out of 10 cases of uterine endometrial cancers, 3 out of 10 cases of uterine cervical cancers, and 4 out of 10 cases of ovarian cancers. There was no association of these patterns with serum steroid hormone levels, SHBG levels, differentiation grades, histological types or clinical staging in the cases analyzed.

4. Discussion

The growth and function of female reproductive organs, especially the uterus and ovary, are partly regulated by sex steroids and their receptors [17,18], which is relevant to the invasion and metastasis of their corresponding cancers [19,20]. Concentrations of receptors for estrogen (ER) and progesterone (PR) in primary gynecologic cancers are higher than in their metastatic lesions [21–24]. It is well-known that ER exon 5 splicing variant (ER E5SV) is transcriptionally active without ligand binding [25]. The ratio of ER E5SV to wild-type mRNA expressions increases in some metastatic lesions of female reproductive tumors, suggesting a contribution to the metastatic potential of gynecological cancers [26]. On the other hand, two isoforms of PR, PR form A (PR-A) and form B (PR-B), have been demonstrated in various target cells [27,28]. PR-A acts as a progestin-dependent, transdominant repressor of PR-B function and other steroid receptor functions [29]. PR-B overexpresses in metastatic lesions of gynecologic cancers, probably due to damage to the transcription of PR-A mRNA [30]. These findings suggest that cellular growth regulation by sex steroids in metastatic lesions is altered in comparison with their primary tumors.

Analysis of the missing base pairs proved they corresponded to the entire exon 7 of SHBG, which is considered to encode a portion of the steroid-binding site, suggesting that this variant protein lacks the entire exon 7 and thereby steroid binding ability [10]. Moreover, absence of exon 7 replaces 118 amino acids from the carboxy-terminus of SHBG with nine different amino acid residues due to the formation of a new stop codon at residue 334 [9,11]. Much evidence indicates that intracellular SHBG–steroid complex plays a direct role in the steroidal interaction in steroid target cells [2–4] and recent studies suggest that SHBG is capable of being synthesized in gynecological tumors [6–8]. Therefore, the change in SHBG wild-type and variant mRNA coexpression in gynecological cancers might influence the development and growth of these cancers [11–13].

We demonstrated the ratio of SHBG variant to wild-type mRNA levels in primary tumors in our previous studies [11–13]. Especially the relative expression

of variant SHBG mRNA was nicely correlated with the histological grade of endometrial cancers [11]. Though the number of tissue samples was too small to show a clear correlation in the present study, these data are within the limits of our previous data. In the present study, we demonstrated the coexpression of SHBG wild-type and variant mRNAs in some metastatic lesions of cancers, as well as in their primary tumors. A relative increase in SHBG variant mRNA expression was observed or the expression of SHBG wild-type and variant mRNAs could not be detected in almost all metastatic lesions (27 out of 30 cases), suggesting deviation from normal SHBG-related intracellular steroidal action. These findings indicate that the regulation of SHBG mRNA transcription and splicing in gynecologic cancers might be altered with the process of secondary spreading of malignant lesions.

In conclusion, the present study demonstrated the deviated change of expression of SHBG wild-type and exon 7 splicing variant mRNAs in the secondary spreading of gynecologic cancers, which might lead to a change in steroidal dependency of metastatic lesions.

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