Expression of Sex Hormone-binding Globulin Exon VII Splicing Variant Messenger RNA in Human Uterine Endometrial Cancers

Ryou Misao,1 Yoshihito Nakanishi, Jiro Fujimoto, and Teruhiko Tamaya
Department of Obstetrics and Gynecology, Gifu University School of Medicine, Gifu 500, Japan

ABSTRACT
We have demonstrated the intracellular expression of sex hormone-binding globulin (SHBG) exon VII splicing variant mRNA in human uterine endometrial cancer using the reverse transcription-PCR-Southern blot and DNA sequencing analyses. Analysis of the missing base pairs proved that they corresponded to the entire exon VII, which is considered to encode a portion of the steroid-binding site, suggesting that the steroid-binding affinity of this variant might be different from that of the SHBG wild type. In uterine endometrial cancers, the wild-type mRNA levels significantly (P < 0.01) decreased, and the ratio of the SHBG variant to wild-type mRNA levels (P < 0.01) increased with the advance of histological differentiation. These results suggest that differentially expressed endometrial cancers might induce a reduction in their estrogen-dependent properties via intracellular SHBG.

INTRODUCTION
In human plasma, SHBG2 is a homodimeric glycoprotein binding to steroid hormones such as estrogen and androgen (1). SHBG is responsible for the transport of testosterone and estradiol in human blood, and alteration in its concentration is thought to modify the bioavailability of these sex steroid hormones. However, much evidence indicates that SHBG-steroid complex directly plays a role in intracellular steroidal actions in the target cells (2-6). The expression of SHBG mRNA has been demonstrated in female reproductive organs and tumors, including uterine endometrial cancer (7-12).

Evidences of important roles for estrogens in the development and growth of endometrial cancers has accumulated clinically (13-15). In particular, exposure to estrogens is implicated in the development of well-differentiated endometrial cancers, which are associated with a favorable prognosis (16). Therefore, it is suggested that intracellular SHBG influences cellular development and growth in uterine endometrial cancers on the basis of estrogen storage (10).

The human SHBG gene consists of eight exons separated by seven small introns (17, 18). cDNA library screening for the human testis reveals the presence of other clones markedly different from the full-length SHBG cDNA (17, 18). One of them lacks a 208-bp region as an exon VII splicing variant of the SHBG gene. This clone encodes for a truncated form of SHBG, which lacks a part of the steroid-binding domain (19).

The aim of the present study was to test for the presence of the splicing variant of SHBG in human endometrial cancers and to discuss the manner of intracellular steroidal action in estrogen-dependent growth of endometrial cancers from the perspective of its mRNA expression.

MATERIALS AND METHODS
Materials. The specimens of uterine endometria and cancers were obtained by hysterectomy from 16 patients with a regular menstrual cycle who had uterine leiomyomas (ages 38–47 years) and from 46 postmenopausal patients (ages 51–63 years), respectively, at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, from September 1996 to March 1997. The patients had not received any previous hormone therapy or chemotherapy. Agreements for the study were obtained from the patients and the Research Committee for Human Subjects, Gifu University School of Medicine. A part of each specimen was submitted for histological diagnosis and grading (20), and the remainder was immediately frozen in liquid nitrogen and later prepared for the subsequent experiments.

RT-PCR. Total RNA was isolated from each sample by the acid guanidinium thiocyanate-phenol-chloroform extraction method (21). The total RNA (3 µg) were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (200 units; Life Technologies, Inc., Gaithersburg, MD) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 40 units of RNasin (Toyobo, Osaka, Japan), 10 mM DTT, and 0.5 mM deoxyribonucleoside triphosphates, using random hexamer (50 ng, Life Technologies, Inc.) for 60 min at 37°C. The reaction was incubated for 5 min at 95°C to inactivate Moloney murine leukemia virus reverse transcriptase.

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 VI, 5'-ATTCCCAAGCTCTATGCGA-3' and SHBG-3': 1119-1138, exon VIII, 5'-AAGGGTGCTCTGCGCT-3') or for GAPDH (GAPDH-5': 71-96, exon I, 5'-TGAAGTTCGGACTCAAGACCTTTGTGGT-3' and GAPDH-3': 1030-1053, exon VIII, 5'-CATGGGCAGGTCACCAC-3') was carried out using a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) with 0.5 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, and 0.2 mM deoxyribonucleoside triphosphates (Fig. 1). 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 (Refs. 18, 19, and 22; Fig. 1).

Southern Blot Analysis. Amplified PCR products were applied to 1.2% agarose gel for electrophoresis performed at 100 V and capillary-transfered to a nylon membrane (Millipore, Burlington, MA) for 20 h, using a 10× SSC solution (1× SSC = 1.5 M NaCl, 0.15 mM sodium citrate, pH 7.0). After blotting, the membrane was dried at 80°C for 15 min and then cross-linked by UV irradiation (33,000 μJoules/cm2 at 254 nm). The membrane was prehybridized in a hybridization buffer [1 M NaCl, 50 mM Tris-HCl (pH 7.6), 1% SDS] at 42°C for 2 h and then in the same solution with biotinylated specific oligonucleotide probes (SHBG probe 1: 686-705, exon VI, 5'-TTTCCCTCTGGACCTGGGACT-3' and SHBG probe 2: 961-980, exon VII, 5'-AAAGCTTCAAGGGCGGCTCTTTG-3'), as shown in Fig. 1; 10 pmol/µl; synthesized by Rikaken Co. Ltd., Nagoya, Japan) at 42°C for 16 h. Finally, the membrane was washed with 0.5× 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 to the membrane for 15 min. The strength of the recorded signal on film was analyzed densitometrically by calculating the area with total IOD using Bio Image (Millipore Corporation, Bedford, MA). The IODs show arbitrary units calculated by Bio Image.

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, Inc., Vista, CA). Single-strand DNA used as template was purified by an Autoread Solid Phase sequencing kit:

Received 5/13/97; accepted 10/15/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Department of Obstetrics and Gynecology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500, Japan. Phone: 81 58-267-2631; Fax: 81 58-265-9006.

2 The abbreviations used are: SHBG, sex hormone-binding globulin; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IOD, integrated absorbance.
SHBG VARIANT IN UTERINE ENDOMETRIAL CANCERS

RESULTS

To determine the deleted exon of SHBG mRNA, we carried out Southern blot analysis using two different oligonucleotide probes corresponding to a part of a sequence in exons VI and VII (Fig. 1). Two different sizes of PCR products for SHBG mRNA were observed in all samples given as follows. The slowly migrating band corresponded to the full-length SHBG mRNA (548 bp, nucleotides 591 to 1138 of the SHBG), whereas the faster migrating band probably corresponded to exon VII splicing variant SHBG mRNA (~350 bp). Both the slowly and faster migrating bands were detected using SHBG probe 1 coding a part of exon VI, whereas only the slowly migrating band was detected using SHBG probe 2 coding a part of exon VII (Fig. 2), suggesting that the variant is deleted at exon VII. In the PCR-Southern blot analysis for genomic DNAs isolated from uterine endometria and endometrial cancers, only one band (1654 bp) corresponding to the full-length SHBG gene was detected without nonspecific products (Fig. 3). This demonstrates that there is not any genomic rearrangement in the SHBG gene in human subjects, whereas with PCR-Southern blot analysis without reverse transcriptase reaction for total RNAs isolated from uterine endometria and endometrial cancers, any band was not detected as a negative control for RT-PCR-Southern blot analysis. This demonstrates that the RT-PCR products were not derived from contaminant genomic DNA.

Furthermore, to demonstrate the expression of SHBG exon VII deleted splicing variant mRNA, DNA sequencing analysis for the PCR products was carried out in all samples. The slowly migrating product was identical to the corresponding full sequences of the SHBG cDNA, whereas the faster migrating product lacked base pairs corresponding to the entire exon VII (Fig. 4).

According to procedures described by us previously (7), semiquantitative analysis of SHBG mRNA levels was performed. After analysis by scanning densitometry, the results of RT-PCR-Southern blot analysis were plotted on a double-logarithmic scale for serial dilutions of SHBG and GAPDH cDNAs, which were reverse-transcribed from the samples. The plots appeared to be linear and parallel to each other.

Fig. 1. Schematic exon (I to VIII) 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 mRNA expression in uterine endometria and endometrial cancers. 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 "Materials and Methods." Lane A. Southern blot hybridization of RT-PCR products using SHBG probe 1 (a part of exon VI). Lane B. Southern blot hybridization of RT-PCR products using SHBG probe 2 (a part of exon VII). NE, normal endometrium; G1, well-differentiated adenocarcinoma of the endometrium; G2, moderately differentiated adenocarcinoma of the endometrium; G3, poorly differentiated adenocarcinoma of the endometrium.

Fig. 3. PCR-Southern blot analysis of genomic DNA in uterine endometria and endometrial cancers. Genomic DNA (1 ng/μl) isolated from each tissue was amplified in PCR with primers specific to SHBG or GAPDH gene. Southern blot analysis was performed as described in "Materials and Methods." NE, normal endometrium; G1, well-differentiated adenocarcinoma of the endometrium; G2, moderately differentiated adenocarcinoma of the endometrium; G3, poorly differentiated adenocarcinoma of the endometrium.

Fig. 4. Semi-logarithmic plots of RT-PCR-Southern blot analysis. Genomic DNA (1 ng/μl) isolated from each tissue was amplified in PCR with primers specific to SHBG or GAPDH gene. Southern blot analysis was performed as described in "Materials and Methods." NE, normal endometrium; G1, well-differentiated adenocarcinoma of the endometrium; G2, moderately differentiated adenocarcinoma of the endometrium; G3, poorly differentiated adenocarcinoma of the endometrium.
SHBG variant to wild-type levels increased with the advance of histological dedifferentiation. In the present study, we have demonstrated SHBG variant mRNA in human uterine endometrial cancers, in addition to that seen previously in human testis (17, 18). The variant protein lacks the entire exon VII and thereby steroid binding ability, because this exon contains a part of the steroid binding domain (19). Moreover, absence of exon VII replaces 118 amino acids from the COOH-terminus of SHBG with nine different amino acid residues due to the formation of a new stop codon at residue 334. Much evidence indicates that intracellular SHBG-steroid complex plays a direct role in the steroidal interaction in steroid target cells (2–6). Recent studies suggest that SHBG is capable of being synthesized in uterine endometrial cancer (10). Therefore, the change of SHBG mRNA levels in uterine endometrial cancer tissues (Fig. 10). Therefore, the change of

The relative quantity of SHBG mRNA was evaluated by IOD on the graph of SHBG (Fig. 5B), which was based on each value of cDNA dilutions, equivalent to the value of IOD (IOD. 0.5) on the graph of GAPDH (Fig. 5A). The mRNA level after standardization was expressed as a value of corrected IOD.

The levels of SHBG wild-type mRNA expressed, in descending order, were: normal endometrium (0.76 ± 0.16 corrected IOD), well-differentiated adenocarcinoma (G1; 0.51 ± 0.19 corrected IOD), moderately differentiated adenocarcinoma (G2; 0.34 ± 0.15 corrected IOD), and poorly differentiated adenocarcinoma (G3; 0.17 ± 0.09 corrected IOD). These expression differences during histological de-differentiation were significant (P < 0.01; Fig. 6). The level of SHBG variant mRNA in endometrial cancers showed no significant difference among the histological gradings (G1, 0.68 ± 0.22; G2, 0.80 ± 0.37; and G3, 0.61 ± 0.45 corrected IOD; Fig. 6). The level of SHBG variant mRNA in endometrial cancers was significantly higher (P < 0.01) than that in normal endometrium (0.37 ± 0.06 corrected IOD). The ratio of SHBG exon VII splicing variant mRNA to SHBG wild-type mRNA in each specimen was analyzed densitometrically. The ratios expressed, in ascending order, were: normal endometrium (0.50 ± 0.07), G1 (1.45 ± 0.46), G2 (2.29 ± 0.62), and G3 (4.63 ± 1.78). These expression differences during histological de-differentiation were significant (P < 0.01; Fig. 7). SHBG mRNA levels and the ratio of SHBG variant to wild-type mRNA had no relation to the clinical stages of endometrial cancers (Figs. 8 and 9).

In Western blot analysis, polyclonal anti-SHBG antibodies recognized a protein of approximately Mr 45,000 and Mr 30,000 extracted from uterine endometrial cancer tissues (Fig. 10). The ratio of the SHBG variant to wild-type levels increased with the advance of histological de-differentiation (normal endometrium, 0.38 ± 0.19; G1, 1.38 ± 0.41; G2, 2.18 ± 0.58; and G3, 5.02 ± 1.98).

DISCUSSION

Human SHBG gene is a simple Mendelian inheritance of an autosomal gene (24) and is consistent with recent evidence that SHBG is encoded by a single gene on the short arm of human chromosome 17 (25). When the SHBG cDNA was screened from an adult human testis library, three distinct cDNAs were obtained, one of which corresponds to the human SHBG cDNA sequence (17). One of the other clones lacks exon VII of the SHBG gene, and the other contains a sequence inversion at the 3' end. In addition, because the different cDNAs in the human testis library have never been observed in cDNA libraries isolated from either adult (26, 27) or fetal (28) human livers, it would appear that they are tissue-specific products of alternative splicing within a single gene (17). In the present study, we have demonstrated SHBG variant mRNA in human uterine endometrial cancers, in addition to that seen previously in human testis (17, 18). The variant protein lacks the entire exon VII and thereby steroid binding ability, because this exon contains a part of the steroid binding domain (19). Moreover, absence of exon VII replaces 118 amino acids from the COOH-terminus of SHBG with nine different amino acid residues due to the formation of a new stop codon at residue 334.

Fig. 6. Level of SHBG wild-type and variant mRNAs in uterine endometria and endometrial cancers. Each circle on the figure shows the average of three different parts of each individual sample. NE, normal endometrium; G1, well-differentiated adenocarcinoma of the endometrium; G2, moderately differentiated adenocarcinoma of the endometrium; G3, poorly differentiated adenocarcinoma of the endometrium.
SHBG wild-type and variant coexpression in uterine endometrial cancers might influence the development and growth of the cancers. The concentration and detection rate of steroid receptors decreases with the advance of histological dedifferentiation from G1 to G3 in endometrial cancers (29, 30). In the present study, the levels of SHBG wild-type mRNA decreased and the ratio of the SHBG variant to wild-type mRNA levels increased with the advance of histological dedifferentiation. To investigate the correlation between SHBG mRNA and protein levels in uterine endometrial cancers, Western blot analysis was carried out. Although the SHBG variant has not been demonstrated yet, its molecular size appears to be approximately Mr 30,000 (19, 31). Two predicted bands of SHBG wild-type and variant (approximately Mr 45,000 and Mr 30,000, respectively) were detected. The ratio of the SHBG variant to wild-type levels increased with the advance of histological dedifferentiation as well as the ratio of mRNA levels. This result demonstrated that the changes in SHBG protein levels were parallel to those in its mRNA levels. The decrease of SHBG wild-type and the relative increase of SHBG variant might reduce the level of intracellular SHBG-estrogen complex and be involved in altering the hormone-responsive character. Therefore, the intracellular interaction of estrogen with SHBG might gradually decrease during histological differentiation. Alternatively, malignant potential might induce the alteration of splicing in SHBG mRNA.

In conclusion, the present study demonstrates coexpression of SHBG exon VII splicing variant mRNA with its wild-type and the increased expression of the SHBG variant to wild-type in endometrial cancers, plausibly involving the loss of the estrogen-dependent properties.

REFERENCES

11. Misao, R., Nakashiri, Y., Fujimoto, J., Hori, M., Ichigo, S., and Tamaya, T. Expres-
Expression of Sex Hormone-binding Globulin Exon VII Splicing Variant Messenger RNA in Human Uterine Endometrial Cancers

Ryou Misao, Yoshihito Nakanishi, Jiro Fujimoto, et al.

Cancer Res 1997;57:5579-5583.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/24/5579

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.