

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

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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 dedifferentiation. These results suggest that dedifferentiation of 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).

Evidence of an important role 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.

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

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 leiomyoma (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 specimen by the acid guanidinium thiocyanate-phenol-chloroform extraction method (21). The total RNA (3 μ g) were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (200 units; Life Technologies, Inc., Gaithersburg, MD) in 50 M 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 μ l) and 5 pmol of specific primers either for SHBG (SHBG-5': 655-674, exon VI, 5'-ATTCCCCAGCTCATGCAGA-3' and SHBG-3': 1119-1138, exon VIII, 5'-AAGCGTCAGTGCCATTGCCT-3') or for GAPDH (GAPDH-5': 71-96, exon I, 5'-TGAAGGTCGGAGTCAACGGATTGGT-3' and GAPDH-3': 1030-1053, exon VIII, 5'-CATGTGGCCATGAGGTCCACCAC-3') was carried out using a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) with 0.5 unit of Ampliqaq 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-transferred to a nylon membrane (Millipore, Burlington, MA) for 20 h, using a 10 \times SSC solution (1 \times 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 UV irradiation (33,000 μ J/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'-TTCTCTTTGGACTGGGACT-3' and SHBG probe 2: 961-980, exon VII, 5'-AAGCTCAAGGGCGTCTCTT-3', as shown in Fig. 1; 10 pmol/ μ l; synthesized by Rikaken Co. Ltd., Nagoya, Japan) at 42°C for 16 h. 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 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

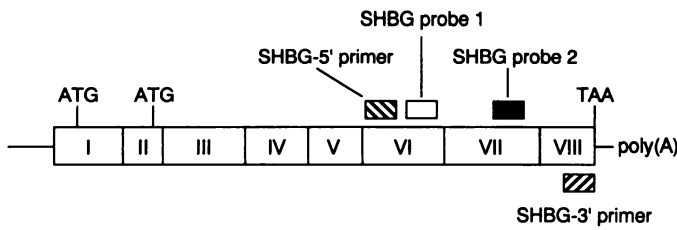


Fig. 1. Schematic exon (I to VIII) presentation of human SHBG cDNA. Location of oligonucleotide primers for PCR amplification and probes for Southern blot analysis are shown in this schema.

(Pharmacia, Uppsala, Sweden). Sequencing reactions were performed using the dideoxy chain-termination method by automated methods, using a Pharmacia A.L.F. express DNA sequencer with a fluorescein-tagged Cy5 primer and Autoread kit (Pharmacia).

Western Blotting. The specimens were homogenized in an extraction buffer of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, and 0.2 mM phenylmethylsulfonyl fluoride with a Polytron (Brinkmann Instruments, Westbury, NY) and incubated on ice for 45 min to extract the SHBG proteins. The supernatants were used for Western blot experiments. Protein was determined according to the method of Lowly *et al.* (23) using BSA as a standard. All samples (2 mg/ml) were reduced in the presence of mercaptoethanol and subsequently separated on a 16% SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose membranes for 2.5 h at 60 V in a buffer of 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol. The membrane was incubated successively with rabbit anti-human SHBG (Dakopatts, Glostrup, Denmark), followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit IgG at room temperature for 1 h. The primary and secondary antibodies were used at 1:400 and 1:1000 dilution, respectively. The detection reaction was performed using enhanced chemiluminescence detection reagent (Amersham International, Buckinghamshire, UK). Kodak XAR-5 film (Eastman Kodak) was exposed to the membrane for 5 min. The strength of the recorded signal on film was analyzed densitometrically using Bio Image (Millipore).

Statistics. Statistical analysis was performed with the Kruskal-Wallis test. Differences were considered significant when *P* was less than 0.05. Data were expressed as mean \pm SD.

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 of 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

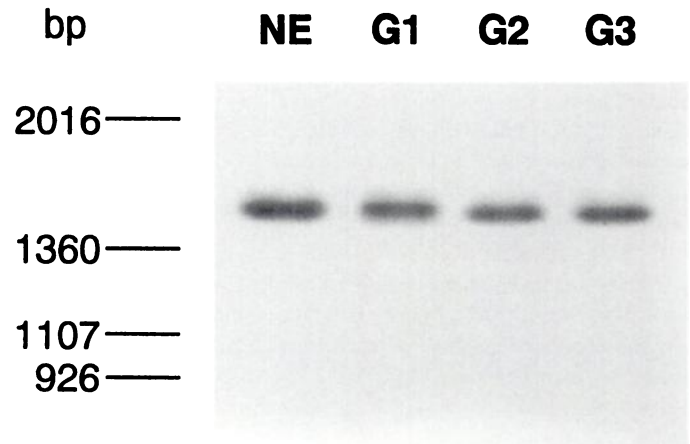
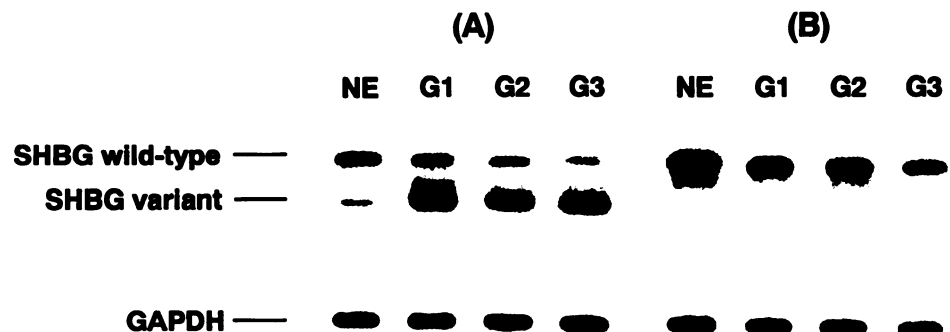


Fig. 3. PCR-Southern blot analysis of genomic DNA in uterine endometria and endometrial cancers. Genomic DNA (1 ng/ μ l) isolated from each tissue was amplified in PCR with primers specific to SHBG or GAPDH gene. Southern blot analysis was performed as described in "Materials and Methods." NE, normal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

Fig. 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.



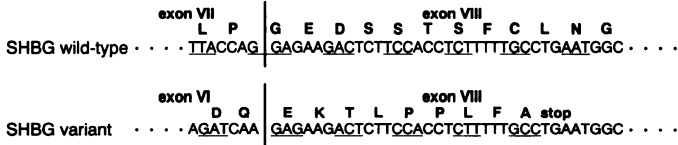


Fig. 4. A comparison of nucleotide sequence of SHBG wild-type and its variant type lacking the entire exon 7 in human uterine endometrial cancer by DNA sequence analysis using an autosequencer. Corresponding amino acid sequence (single letter code) is shown in boldface. Splicing of exon VI to VIII generates a frame shift and a translation termination codon.

(Fig. 5). 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 dedifferentiation 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 dedifferentiation 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 M_r 45,000 and M_r 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 dedifferentiation (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.

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

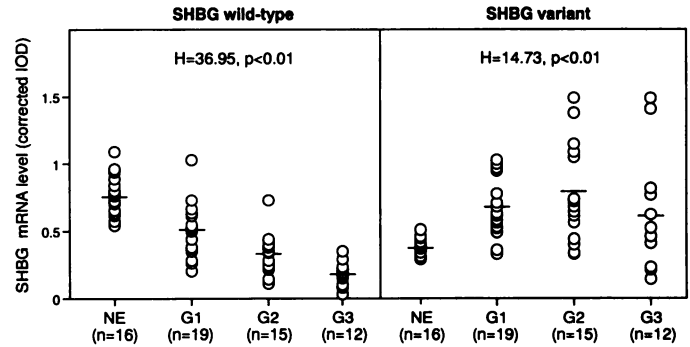


Fig. 6. Level of SHBG wild-type and variant mRNAs in uterine endometria and endometrial cancers. Each circle on the figure shows the average of three different parts of each individual sample. NE, normal endometrium; G1, well-differentiated adenocarcinoma of the endometrium; G2, moderately differentiated adenocarcinoma of the endometrium; G3, poorly differentiated adenocarcinoma of the endometrium.

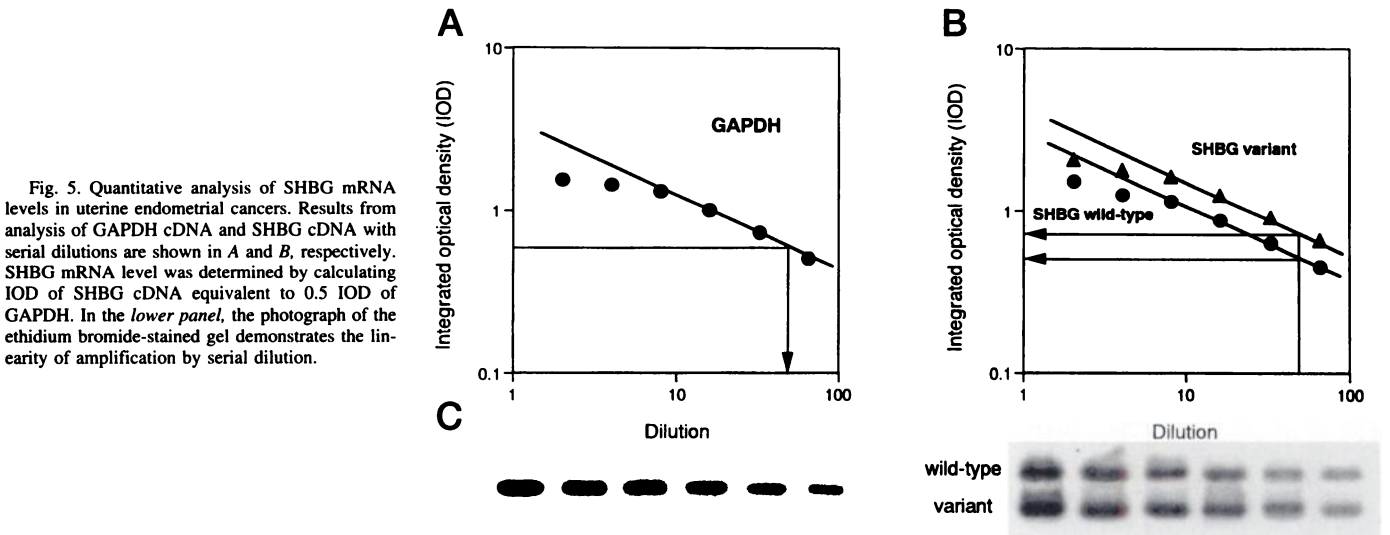


Fig. 5. Quantitative analysis of SHBG mRNA levels in uterine endometrial cancers. Results from analysis of GAPDH cDNA and SHBG cDNA with serial dilutions are shown in A and B, respectively. SHBG mRNA level was determined by calculating IOD of SHBG cDNA equivalent to 0.5 IOD of GAPDH. In the lower panel, the photograph of the ethidium bromide-stained gel demonstrates the linearity of amplification by serial dilution.

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

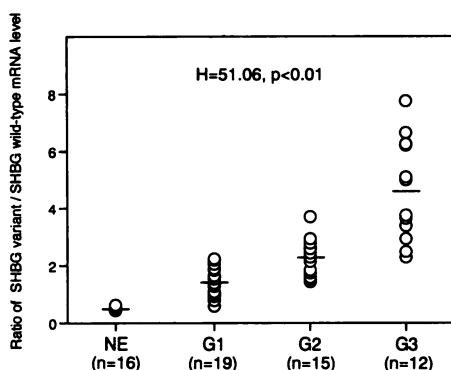


Fig. 7. The ratio of SHBG variant to SHBG wild-type mRNA levels in uterine endometria and endometrial cancers. One circle on the figure shows the average of three different parts of each individual sample. NE, normal endometrium; G1, well-differentiated adenocarcinoma of the endometrium; G2, moderately differentiated adenocarcinoma of the endometrium; G3, poorly differentiated adenocarcinoma of the endometrium.

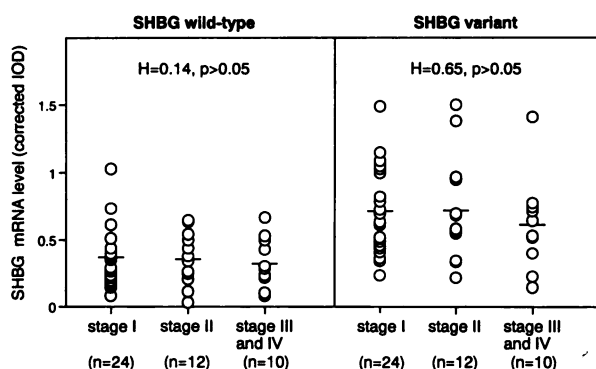


Fig. 8. Level of SHBG wild-type and variant mRNAs in uterine endometrial cancers classified by clinical staging. Each circle on the figure shows the average of three different parts of each individual sample.

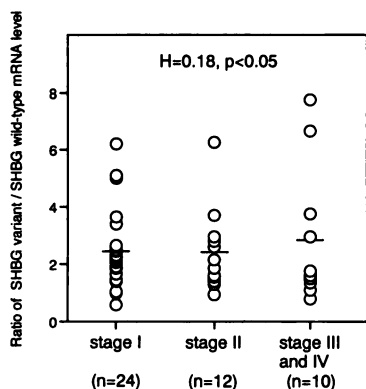


Fig. 9. The ratio of SHBG variant to SHBG wild-type mRNA levels in uterine endometrial cancers classified by clinical staging. One circle on the figure shows the average of three different parts of each individual sample.

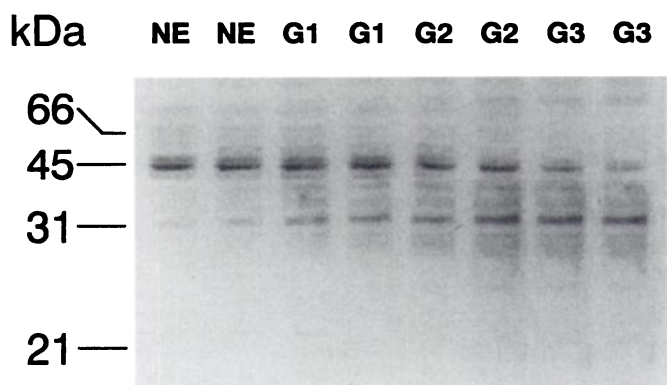


Fig. 10. Western blot analysis of SHBG in uterine endometrial cancers. Proteins were separated in the SDS-PAGE and electrotransferred to nitrocellulose membranes as described in "Materials and Methods." NE, normal endometrium; G1, well-differentiated adenocarcinoma of the endometrium; G2, moderately differentiated adenocarcinoma of the endometrium; G3, poorly differentiated adenocarcinoma of the endometrium.

demonstrated yet, its molecular size appears to be approximately M_r 30,000 (19, 31). Two predicted bands of SHBG wild-type and variant (approximately M_r 45,000 and M_r 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 dedifferentiation. 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.

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