Relatively High Expression Ratio of Sex Hormone-binding Globulin Exon VII Splicing Variant to Wild-type mRNA in Human Uterine Cervical Cancers

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We have demonstrated the intracellular expression of sex hormone-binding globulin (SHBG) exon VII splicing variant mRNA in human uterine cervical cancer using reverse transcription-polymerase chain reaction-Southern blot and DNA sequencing analyses. Analysis of the missing base pairs proved 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 the variant protein might be different from that of the wild-type SHBG. In uterine cervical cancers, the wild-type mRNA levels were lower (P<0.01) and the ratio of the SHBG variant to wild-type mRNA levels was higher (P<0.01) than in the normal cervix. In cervical adenocarcinomas, the wild-type mRNA levels were higher (P<0.05) and the ratio of the SHBG variant to wild-type mRNA levels was lower (P<0.05) than in cervical keratinizing squamous cell carcinomas. There was no difference in expression among the clinical stages of cervical cancers. These results suggest that a relative increase of intracellular variant SHBG protein in human uterine cervical cancers might be involved in the disruption of the normal estrogen dependence.

Key words: Sex hormone-binding globulin — mRNA — Splicing variant — Uterine cervical cancer

There is clinical evidence of an important role for sex steroids in the reproductive events of the uterine cervix.1, 2) The presence of steroid hormone receptors in normal cervix3–5) and cervical cancer6–8) has been reported, suggesting that the cellular biological characteristics of these tissues might be associated with the actions of sex steroid hormones.

Sex hormone-binding globulin (SHBG) is a homodimeric glycoprotein, chiefly binding to estrogen and androgen in the circulation.9) SHBG is responsible for the transport of these hormones in human blood, and alteration of its concentration is thought to modify the bioavailability of these steroids. However, abundant evidence indicates that SHBG-steroid complex also plays a direct role in intracellular steroidal actions in the target cells.10–14) In addition, the expression of SHBG mRNA has been demonstrated in female reproductive organs and tumors, including uterine cervical cancer,15–19) indicating its intracellular presence.

The human SHBG gene is organized into eight exons separated by seven introns.20) Screening of a human testis cDNA library revealed the presence of other clones markedly different from the full-length SHBG cDNA.20) One of them, lacking a 208-base pair region, is 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.21) This variant is coexpressed in human uterine endometrium,22) but its biological implication remains to be clarified.

The aim of the present study was to investigate the presence of SHBG splicing variant and its biological implication in human uterine cervical cancers.

MATERIALS AND METHODS

Materials Specimens of normal and cancer tissues of the uterine cervix were obtained by hysterectomy from 51 patients (age 43–66 years) at the Department of Obstetrics and Gynecology, Gifu University School of Medicine from June 1996 to May 1997. The patients had not received any previous therapy. Consents for the study were obtained from the patients and the Research Committee for Human Subjects, Gifu University School of Medicine. The specimens cut for analyses was a wedge of glandular cervical tissue beginning at the squamocolumnar junction and continuing to the junction with the uterine endometrium. A part of these tissues was submitted for histological classification,23) and the remainder was immediately frozen in liquid nitrogen and later prepared for the subsequent experiments. Clinical staging was performed according to International Federation of Gynecology criteria.24)

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was isolated from each specimen by the acid guanidium thiocyanate-phenol-chloroform extraction method.25) The total RNA (3 µg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RT, 200 U, Gibco BRL, Gaithersburg, MD) in 50 µl Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 40 U of RNAsin (Toyobo, Osaka), 10 mM dithiothreitol, and 0.5 mM deoxyribonucleoside triphosphates (dNTPs), using random hexamer (50 ng, Gibco BRL) in 20 µl volume for 60 min at 37°C. The reaction mixture was incubated for 5 min at 95°C to inactivate MMLV-RT.

PCR with reverse-transcribed RNAs as templates (1 µl) and specific primers (5 pmol) either for SHBG (SHBG-5′: 655–674, exon VI, 5′-ATTCCCCAGCCCTCATGCGA-3′...
and SHBG-3’: 1119–1138, exon VIII, 5’-AAGGTCACTG- 
GCAATTGCT-3’) or for glyceraldehyde-3-phosphate de-
hydrogenase (GAPDH-5’: 71–96, exon I, 5’-TGAAGGTC-
GGAAGTCAAGGATTTGT-3’ and GAPDH-3’: 1030– 
1053, exon VIII, 5’-CATGTGGCCATGAGGTCCAC-
CAC-3’) was carried out using a DNA Thermal Cycler 
(Perkin-Elmer Cetus, Norwalk, CT) with 0.5 U of AmpliTaq 
DNA polymerase (Perkin-Elmer Cetus) in a buffer contain-
ing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2 
and 0.2 mM dNTPs in 20 µl volume. 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 organiza-
tion of human SHBG and GAPDH genes.20, 26

Southern blot analysis Amplified PCR products were 
subjected to 1.2% agarose gel electrophoresis at 100 V, and 
then capillary-transferred to a nylon membrane (Millipore, 
Burlington, MA) for 20 h, using a 10x 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/cm2 at 254 nm). The membrane was prehy-
bridized in a hybridization buffer (1 M NaCl, 50 mM Tris-
HCl pH 7.6 and 1% sodium dodecyl sulfate) at 42°C for 2 
h, and then hybridized in the same solution with biotiny-
lated specific oligonucleotide probes (SHBG probe 1: 686– 
705, exon VI, 5’-TTCTCTTTGACCTGAGACT-3’ and 
SHBG probe 2: 961–980, exon VII, 5’-AAGCTTCA-
AGGCGGTCTT-3’) (10 pmol/µl, synthesized by 
Rikaken Co., Ltd., Nagoya) at 42°C for 16 h. Finally, the 
membrane was washed with 0.5x SSC at 65°C. The 
detection reaction for hybridized biotin was performed 
using a Pex chemiluminescence 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 in terms 
of total integrated optical density (IOD) using Bio Image 
(Millipore Corporation, Bedford, MA). The IOD data are 
in arbitrary units calculated by Bio Image.

DNA sequence analysis Amplified PCR products were 
electrophoresed with 1.2% agarose gel. The DNA frag-
ments were eluted from excised agarose gel slices by a 
Gene Clean II kit (BIO 101 Inc., Vista, CA). Single-
stranded DNA used as a template was purified by an Auto-
road 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 sequencer with a 
fluorescein-tagged Cy5 primer and Autoread kit (Pharmacia).

**Statistics** Statistical analysis was performed with Student’s t test. Differences were considered significant when 
*P* was less than 0.05. Data were expressed as mean±SD.

**RESULTS**

To determine the deleted exon of SHBG mRNA, we car-
rried out Southern blot analysis using two different oligonu-
ucleotide probes corresponding to partial sequences of exons 
VI and VII. Two different sizes of PCR products for 
SHBG mRNA were observed in all samples: the slower-
migrating band corresponded to the full-length SHBG 
mRNA (548 base pairs, nucleotides 591 to 1138 of the 
SHBG), whereas the faster-migrating band probably corre-
sponded to exon VII splicing variant SHBG mRNA (approximately 350 base pairs). Both the slower- 
and faster-migrating bands were detected using SHBG probe 1 
coding a part of exon VI, while only the slower-migrating 
band was detected using SHBG probe 2 coding a part of 
exon VII (Fig. 1), suggesting that at least a part of exon VII 
is deleted in the variant. The absence of exon VII was con-
firmed by DNA sequencing analysis.

According to procedures previously described by us,15) 
semi-quantitative analysis of SHBG mRNA levels was per-
fomed. There was no significant difference between the 
levels of SHBG variant mRNA in normal cervix (1.1±0.68 
corrected IOD) and in cervical cancers (1.19±0.68 
corrected IOD) (Fig. 2).

The levels of SHBG wild-type mRNA were histologi-
cally classified as follows: keratinizing squamous cell car-
cinomas, 0.39±0.18 corrected IOD; small cell non-kerati-

![Fig. 1. RT-PCR-Southern blot analysis of SHBG mRNA expression in normal and cancer tissues of the uterine cervix. 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).](image-url)

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IOD; large cell non-keratinizing squamous cell carcinomas, 0.42±0.14 corrected IOD; adenocarcinomas, 0.65±0.24 corrected IOD (Fig. 3).

The level of SHBG variant mRNA in cervical cancers showed no significant difference with histological classification (keratinizing squamous cell carcinomas, 1.52±0.98 corrected IOD; small cell non-keratinizing squamous cell carcinomas, 0.79±0.33 corrected IOD; large cell non-keratinizing squamous cell carcinomas, 0.94±0.09 corrected IOD; adenocarcinomas, 1.19±0.38 corrected IOD). The level of SHBG variant mRNA was not correlated to the clinical stage of cervical cancers (stage I, 0.92±0.46 corrected IOD; stage II, 1.23±0.66 corrected IOD; stages III and IV, 1.45±0.84 corrected IOD).

The ratio of SHBG exon VII splicing variant to wild-type mRNA levels in each specimen was analyzed densitometrically. The ratio was significantly (P<0.01) higher in cervical cancers (2.84±1.36 corrected IOD) than in normal

Fig. 2. Level of SHBG variant and wild-type mRNAs in normal and cancer tissues of the uterine cervix. Each circle on the figure shows the average of three different parts of each individual sample. The mRNA levels are expressed as corrected IOD. **P<0.01, compared with normal cervical tissues.

Fig. 3. Levels of SHBG wild-type and variant mRNAs in cervical cancers, classified histologically. One circle on the figure shows the average of three different parts of each individual sample. The mRNA levels are expressed as corrected IOD. K, keratinizing squamous cell carcinoma; SNK, small cell nonkeratinizing squamous cell carcinoma; LNK, large cell nonkeratinizing squamous cell carcinoma; A, adenocarcinoma. *P<0.05, compared with keratinizing squamous cell carcinoma.
The ratios of variant to SHBG wild-type mRNA levels in normal and cancer tissues of the uterine cervix (A) and in cervical cancers classified histologically (B). One circle on the figure shows the average of three different parts of each individual sample. The mRNA levels are expressed as corrected IOD. K, keratinizing squamous cell carcinoma; SNK, small cell non-keratinizing squamous cell carcinoma; LNK, large cell non-keratinizing squamous cell carcinoma; A, adenocarcinoma. **P<0.01, compared with normal cervix. *P<0.05, compared with keratinizing squamous cell carcinoma.

**DISCUSSION**

The following evidence favors an association of estrogen with cervical malignancy. Estrogen receptor has been identified in normal and cancerous tissues of the cervix. The growth of a human uterine cervical cancer cell line was stimulated by estradiol, and the estradiol-stimulated growth could be inhibited by tamoxifen, progesterone and medroxyprogesterone acetate, which exert anti-estrogenic action. The conversion of estradiol to 16α-hydroxyestrone, a plausible risk factor for estrogen-responsive tumors, is elevated in cervical epithelial cells infected with human papillomavirus. Further, estrogen itself has been shown to transactivate the viral genome in human papillomavirus containing malignant cell lines and to induce cervical carcinogenesis in the female reproductive tract of transgenic mice. Additionally, much evidence indicates that intracellular SHBG-steroid complex plays a direct role in the steroidial interaction in steroid target cells. A recent study suggested that SHBG is capable of being synthesized in uterine cervical cancer.

When a human adult testis library was screened for SHBG cDNA, three distinct cDNAs were obtained, one of which corresponds to the human SHBG cDNA sequence. One of the other clones lacks exon VII of the SHBG gene, and the third contains a sequence inversion at the 3′ end. In addition, since the different cDNAs in the human testis library have never been observed in cDNA libraries isolated from human adult and fetal livers, it would appear that they are tissue-specific products of alternative splicing within a single gene.

In the present study, we have demonstrated the presence of SHBG variant mRNA in human uterine cervical cancers, like that previously seen in human testis. The variant lacks the entire exon VII, thereby being potentially defective in steroid binding ability, since this exon codes for a part of the steroid binding domain. SHBG forms a dimer in the cell and the entire coding sequence may be needed to generate an active protein. There are examples of dominant-negative activity by mutant nuclear receptors in human malignancies. Namely, the mutant receptor forms dimers with wild-type receptors, resulting in inactivation. Thus, SHBG variant might inactivate intracellular SHBG function. Therefore, the SHBG variant expression in uter-
mRNA in uterine cervical cancers showed no significant difference with histological classification. Relatively high expression of SHBG wild-type to variant mRNA in cervical adenocarcinoma might conserve the activity of SHBG-related steroidal mechanisms as compared with other histological types and might be correlated with glandular differentiation of uterine cervical cancers.

In conclusion, the present study demonstrates coexpression of SHBG exon VII splicing variant mRNA with the wild-type mRNA and an increased expression ratio of the SHBG variant to wild-type mRNA in cervical cancers. This change might be involved in the disruption of normal estrogen dependence.

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