Expression of sex hormone-binding globulin mRNA in human ovarian cancers

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To know the role of sex hormone-binding globulin (SHBG) in the intracellular steroid actions in human ovarian cancers, the expression of SHBG mRNA as a substitute for intracellular SHBG expression was investigated in normal ovarian tissues and ovarian tumors. In the present study, we used competitive reverse transcription–polymerase chain reaction–Southern blot analysis to evaluate SHBG mRNA levels. The expression of SHBG mRNA was detected in all normal ovaries and benign and malignant ovarian tumors analyzed. There were no significant differences in the mean SHBG mRNA levels among the three types of tissue. The expression in normal ovaries was significantly higher (p < 0.01) in premenopause, suggesting the predominance of a sex steroid hormone effect on ovarian SHBG synthesis. Relative overexpression of SHBG mRNA was observed in six out of 22 cases (27%) of ovarian cancer (three cases of endometrioid adenocarcinoma, two cases of serous cystadenocarcinoma and one case of mucinous cystadenocarcinoma) in comparison with normal ovaries and benign ovarian tumors. There was no difference in expression among the clinical stages of ovarian cancers. These data suggest that normal human ovaries and ovarian tumors might synthesize SHBG intracellularly, ovarian cancers might conserve an estrogen-associated property via SHBG and the regulation of intracellular SHBG expression might be changed in some cancers.

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In previous studies it has been suggested that 17β-estradiol (1–3), progesterone (1, 4), testosterone and dihydrotestosterone (5) are related to ovarian function, and estrogen, progesterin and androgen binding sites have been detected and described in human normal ovary and benign and malignant ovarian tumors (6–13). Some ovarian cancers respond favorably to tamoxifen (14–18), megestrol acetate (15) and medroxyprogesterone acetate (19).

Previously it was assumed that the free fraction of serum steroid hormones was biologically active, and the sex hormone-binding globulin (SHBG)-bound fraction was not available within the target cells. In the last decade, SHBG binding sites were detected in the cell membrane of human decidual endometrium (20) and prostate (21). Immunochemical detection of SHBG has been reported in human endometrium, prostate (22) and mammary glands (23). In addition to hepatic cells (24, 25). Moreover, the expression of SHBG mRNA has been demonstrated in human uterine endometrial and prostatic cancer cell lines (26, 27) and human normal uterine endometrium (28). These studies indicate that SHBG might be synthesized in the target cells and directly play a role in intracellular steroidal actions.

The aim of the present study was to determine the SHBG mRNA expression in human ovarian cancers by competitive reverse transcription–polymerase chain reaction–Southern blot analysis (RT-PCR-SBA) in order to analyze the possibility of an alternative regulation of intracellular steroidal mechanisms.

Material and methods

Materials

Normal ovaries were obtained by oophorectomy from 15 patients with uterine leiomyoma or uterine cervical cancer (age 25–68 years) at the Department of Obstetrics and Gynecology, Gifu University School of Medicine from March 1994 to November 1994. The specimens of ovarian tumors were obtained from 34 patients (age 24–70 years) who had not received any therapy. In histopathological examination of the samples, 22 malignant and 12 benign neoplastic lesions were identified. Agreements for the study were obtained from the patients and the Research Committee for Human Subjects, Gifu University School of Medicine. Part of these tissues (tissues of whole normal ovaries, capsules on benign ovarian cysts and cancer lesions) were immediately frozen in liquid nitrogen and later prepared for the sequencing. RNA isolation and RT-PCR-SBA.
Preparation of internal standard recombinant RNA

A scheme for synthesis of internal standard recombinant RNA (rcRNA) is shown in Fig. 1. Deoxyribonucleic acid construction of the internal standard was originated and synthesized by PCR from a BamH/EcoRI fragment of v-erbB (Clontech Laboratories, Palo Alto, CA) with two sets of oligonucleotide primers containing T7 promoter and SHBG specific primer sequences. The sequences of the first set of primers for the first PCR were as follows: 5′-TGTAGAATCAAATCCGGGGACGCAAGTGAAATCTCCTCG-3′ and 3′-GATGTTTGA- CGTAACGTCTTTCCACCACAAGAGAAGACC-5′. The sequences of the second set of primers for secondary PCR were as follows: 5′-TAATACGACTCACTATAGGTGTAGAATCAAATCCGGGA-3′ and 3′-TTCCACCA-CAGAGAAGACC-5′ (29, 30). The described two sets of primers were synthesized by Rikaken Co. Ltd. (Nagoya, Japan).

The first PCR was conducted in a final vol of 50 µl containing PCR buffer (50 mmol/l KCl, 10 mmol/l TRIS·HCl (pH 8.3), 1.5 mmol/l MgCl2), 0.2 mmol/l deoxyribonucleoside triphosphates (dNTPs), 2 ng of Bam H/Eco RI DNA fragment of v-erbB, 10 pmol each of

**Fig. 1.** Scheme for synthesis of internal standard recombinant RNA.
the first set of PCR primers and 2.5 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The second PCR was conducted in a final volume of 100 µl containing PCR buffer, 0.2 mmol/l dNTPs, 20 pmol each of the second set of PCR primers and 5 units of Amplitaq DNA polymerase. The mixtures were amplified for 28 cycles of PCR at 95°C for 45 s for denaturing, 60°C for 45 s for annealing and 72°C for 90 s for extension in a DNA Thermal Cycler (Perkin-Elmer Cetus).

The second PCR product was purified with a Gene Clean II Kit (BIO 101 Inc, La Jolla, CA) and transcribed using 100 units of T7 RNA polymerase (Gibco BRL, Gaithersburg, MD) in a final vol of 100 µl containing T3/T7 buffer (40 mmol/l TRIS-HCl (pH 8.0), 8 mmol/l MgCl2, 2 mmol/l spermidine-(HCl), 25 mmol/l NaCl), 0.1 mol/l dithiothreitol (DTT), 10 mmol/l ribonucleoside triphosphates, 40 units of RNase inhibitor (Promega, Madison, WI), 20 mmol/l template DNA and 10 µCi of [α-32P]UTP (New England Nuclear Co., Boston, MA) as a tracer. The reaction was incubated at 37°C for 1 h and then treated with 70 units of RNase-free DNase (Takara Shuzo Co. Ltd, Kyoto, Japan) at 37°C for 5 min to remove the DNA template. Subsequently, products were extracted with water-saturated phenol/chloroform and passed through a Sephadex G50 column (Boehringer Mannheim, Mannheim, Germany). The amount of transcribed internal marker RNA was calculated from the total radioactivity of the transcribed RNA.

Competitive RT-PCR

Total RNA was isolated from ovary, ovarian tumor and liver as a positive control and monocytes as a negative control by the acid guanidium thiocyanate–phenol–chloroform extraction method as described by Chomczynski and Sacch (31). Peripheral monocytes were isolated from heparinized peripheral blood (32).

To obtain a standard curve each time, the total RNA (3 µg) and a series of diluted recombinant RNA

![Fig. 2. Strategy for Southern blot to detect two specific genes and analyze the intensity of two bands.](image)

![Fig. 3. Expression of sex hormone-binding globulin (SHBG) mRNA in human liver, ovary and monocyte. Lane 1 (L), human liver; lane 2 (O), premenopausal ovary; lane 3 (M), peripheral monocyte. The reverse transcription–polymerase chain reaction containing SHBG specific primers was carried out in the presence of 3 µg of the respective total RNA and 10−2 pg internal standard recombinant RNA. (A) The results of Southern blot analysis after competitive RT-PCR. (B) Analysis of total RNA from each sample by gel electrophoresis.](image)
(10⁻¹–10⁻³ pg) were reverse-transcribed in 20 μl vol for 1 h at 37°C with a mixture of 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco BRL) and the following reagents: 50 mmol/l TRIS·HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl₂, 40 units of RNAsin (Toyobo, Osaka, Japan), 10 mmol/l DTT, 0.5 mmol/l dNTPs and 30 pmol 3’-end specific primer (SHBG-3’, as detailed below). The reaction was incubated for 5 min at 95°C to inactivate M-MLV reverse transcriptase.

The sequences of primers to amplify the SHBG gene were as follows: 5’-TGTAGAATCAAATCCCGGGA-3’ (SHBG-5’; 591–610, Exon VII) and 3’-TTCCACCCACAGAGAAGACC-5’ (SHBG-3’; 790–809, Exon V) (29) (Rikaken Co, Ltd.). The sizes of PCR products for SHBG mRNA and internal standard rcRNA are 219 and 440 bp, respectively. Polymerase chain reaction with reverse-transcribed RNAs as templates (1 μl) and 5 pmol of each specific primer was carried out using a DNA Thermal Cycler (Perkin-Elmer Cetus) with 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) in a buffer containing 50 mmol/l KCl, 10 mmol/l TRIS·HCl (pH 8.3), 1.5 mmol/l MgCl₂ and 0.2 mmol/l dNTPs in 20 μl vol. Amplification was performed for 38 cycles of PCR at 94°C for 45 s for denaturing, 55°C for 45 s for annealing and 72°C for 90 s for extension.

**Southern blot analysis (Fig. 2)**

Amplified PCR products (8 μl) with 2 μl of loading dye mix were electrophoresed with 1.2% GTG agarose gels (FMC BioProducts, Rockland, ME) in a 100-V constant voltage field for 50 min and capillary-transferred to a nylon membrane (Immobilon-S; Millipore, Burlington, MA) for 20 h using 10 X standard sodium citrate solution (SSC; 1.5 mol/l NaCl, 0.15 mol/l sodium citrate, pH 7.0). After blotting, the membrane was dried at 75°C and then cross-linked by UV irradiation (33000 μJ/cm² at 254 nm). The membrane was prehybridized in hybridization buffer (1 mol/l NaCl, 50 mmol/l TRIS·HCl (pH 7.6), 1% sodium dodecyl sulfate) at 42°C for 2 h, then in the same solution with the biotinylated SHBG gene-specific oligonucleotide probe (5’-TTCTCTTGGAGACCTGGGACT-3’) and biotinylated internal standard gene-

![Fig. 4. Quantitative analysis of sex hormone-binding globulin (SHBG) mRNA in uterine endometrium by competitive reverse transcription-polymerase chain reaction–Southern blot analysis (RT-PCR-SBA).](image)

**Fig. 4.** Quantitative analysis of sex hormone-binding globulin (SHBG) mRNA in uterine endometrium by competitive reverse transcription–polymerase chain reaction–Southern blot analysis (RT-PCR-SBA). The RT-PCR reactions containing SHBG specific primers, in the presence of total RNA and serial diluted internal standard recombinant RNA (rcRNA) at the range 10⁻²–10⁻¹ pg, were carried out. Panel (A) shows the result of Southern blot analysis after competitive RT-PCR. The signal intensity in Southern blot was determined. In panel (B), data are plotted to calculate the SHBG mRNA level as the log ratio of rcRNA/SHBG mRNA in total RNA isolated from samples vs log rcRNA.
normal ovaries, benign ovarian tumors and ovarian cancers. One circle on the figure shows the average of three different parts of each individual sample. The SHBG mRNA levels were expressed as pg internal standard recombinant RNA (rcRNA)/mg total RNA.

**Statistics**

Statistical analysis was performed with Student’s t-test. Differences were considered significant when p was less than 0.05. All data were expressed as means ± sd.

**Results**

In the competitive RT-PCR-SBA for SHBG mRNA, only two predicted sizes of DNA fragments were detected.
Fig. 7. The levels of sex hormone-binding globulin (SHBG) mRNA in the histological subgroups of ovarian cancers. One circle on the figure shows the average of three different parts of each individual sample. The SHBG mRNA levels were expressed as pg internal standard recombinant RNA (rcRNA)/mg total RNA. Two cases of Krukenberg tumor metastasized from gastric cancer.

without non-specific products. The SHBG mRNA was detected in higher expression in human liver as a positive control than in normal ovary (Fig. 3), while SHBG mRNA was not detected in human monocytes as a negative control in 38 cycles of PCR. The amount of SHBG mRNA was determined using a standard curve and a serial dilution of rcRNA in competitive RT-PCR-SBA, as shown in Fig. 4. We collected solid parts of ovarian tumors and confirmed a neighboring part of each sample histologically. Moreover, we performed total RNA isolation and competitive RT-PCR-SBA in three different parts of each individual sample.

The SHBG mRNA was detected in all normal ovaries and benign and malignant ovarian tumors analyzed (Fig. 5). There were no significant differences in the mean SHBG mRNA levels among the three types of tissue (normal ovaries, 1.21 ± 0.79 pg rcRNA/mg total RNA; benign ovarian tumors, 1.43 ± 0.49 pg rcRNA/mg total RNA; ovarian cancers, 2.23 ± 1.93 pg rcRNA/mg total RNA), while six cases of ovarian cancer showed relative overexpression of SHBG mRNA (Fig. 5).

The SHBG mRNA level in normal ovaries of premenopausal women (1.80 ± 0.55 pg rcRNA/mg total RNA) was significantly (p < 0.01) higher than that of postmenopausal women (0.55 ± 0.34 pg rcRNA/mg total RNA).

Fig. 8. The levels of sex hormone-binding globulin (SHBG) mRNA in clinical stages for ovarian cancers. One circle on the figure shows the average of three different parts of each individual tumor. The SHBG mRNA levels were expressed as pg internal standard recombinant RNA (rcRNA)/mg total RNA.
total RNA) (Fig. 6), suggesting the predominance of a sex steroid hormone effect on normal ovarian SHBG synthesis.

There was no significant correlation between SHBG mRNA levels and histological classifications in benign (Fig. 6) or malignant (Fig. 7) ovarian tumors, while six ovarian cancers (three cases of endometrioid adenocarcinoma, two cases of serous cystadenocarcinoma and one case of mucinous cystadenocarcinoma) relatively overexpressed SHBG mRNA (Fig. 7). Especially noteworthy is that all three endometrioid adenocarcinomas were characterized by a relatively high expression of mRNA. No correlation was found between SHBG mRNA levels and clinical stages for ovarian cancers (Fig. 8).

Discussion

The expressions of estrogen and progesterone receptors in human normal ovaries and either benign or malignant ovarian tumors have been described in many studies (6–12). In rat or canine ovary, chronic administration of sex steroid hormones (androgen or estrogen) has been shown to induce ovarian neoplasms (33, 34). It has been reported that tamoxifen (14–18), megestrol acetate (15) and medroxyprogesterone acetate (19) are partially effective in inhibiting the growth of some advanced ovarian cancers. These studies suggest that the growth of ovarian cancers might be associated with steroid actions.

Generally, SHBG binds estrogen with relatively high affinity as a transporter for steroid hormones in the circulation. However, in the last decade, much evidence has suggested that the SHBG–steroid complex plays a direct role in the intracellular steroid interaction in steroid target cells (20–25). Uterine endometrial SHBG might be synthesized under the influence of estrogen and progesterone and be involved as a storehouse of estrogen in the regulatory system of intracellular steroidal actions (28). As shown in the present study, the predominance of SHBG expression in premenopausal ovaries indicates the presence of a sex steroid hormone effect on ovarian SHBG synthesis. Therefore, it is supposed that SHBG likely influences the cellular development and growth associated with estrogen.

In our previous study, SHBG mRNA in normal uterine endometrium was present in a copy concentration too low to be evaluated by Northern blot analysis (28). Therefore, we necessarily performed RT-PCR. In the present study, we used RT-PCR-SBA to detect the SHBG mRNA in normal ovaries and ovarian tumors, which had a copy number of SHBG mRNA lower than normal uterine endometria (28). We could not analyze the intracellularly synthesized SHBG level in such tissues by conventional immunohistochemical and Western blot studies, because SHBG in the samples from such tissues could not exclude the possibility of endocytosis (35) and/or contamination with serum SHBG. We were able to achieve the accurate quantitation of mRNA expression by competitive RT-PCR-SBA, in which the target RNA in the sample and the synthesized cRNA as an internal control were reverse-transcribed and amplified simultaneously with the same efficiency. In the competitive RT-PCR-SBA, only two predicted DNA fragments were detected without non-specific products. We confirmed their specificity using inter-primer probes (data not shown), and their signal intensity using SHBG-5' as shown in Fig. 2.

Many investigators have reported that estrogen induces follicular development and granulosa cell differentiation. The expression of SHBG mRNA in such tissue attracts interest, and the possibility that intracellular SHBG might be involved in the regulation of normal ovarian folliculogenesis is considered. In benign ovarian tumors, as in normal tissues, relative overexpression of SHBG mRNA was not observed in the present study. Relative overexpression of SHBG mRNA was observed in some ovarian cancers, suggesting deviation in the regulatory unit of SHBG mRNA transcription in the process of neoplastic change, and change in the regulation of intracellular estrogenic action involving SHBG synthesis, as observed in some uterine endometrial cancers (36).

In conclusion, although the present study is preliminary, ovarian malignant tumors may conserve the activity of SHBG-related steroidal mechanisms.

References


