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

Ryou Misao[1] Yoshihito Nakanishi, Jiro Fujimoto and Teruhiko Tamaya

Department of Obstetrics and Gynecology, Gifu University School of Medicine, 40 Tsukasamachi, Gifu 500

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 cervix[3–5] and cervical cancer[6–8] 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 specimen 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 M Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 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′-ATTCCCCAGCCTCATGCAGA-3′

and SHBG-3′: 1119–1138, exon VIII, 5′-AAGCGTCAGT-GCCATTGCCT-3′) or for glyceraldehyde-3-phosphate de-hydrogenase (GAPDH-5′: 71–96, exon I, 5′-TGAAGGTC-GGAGTCAACGGATTTGGT-3′ and GAPDH-3′: 1030–1053, exon VIII, 5′-CATGTGGGCCATGAGGTCCAC-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 MgCl$_2$ 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 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 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′-TTCTCTTTGGACCTGGGACT-3′ and SHBG probe 2: 961–980, exon VII, 5′-AAGCCTCA-AGGGCGTCTCTT-3′) (10 pmol/μl, synthesized by Rikaken Co., Ltd., Nagoya) 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 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 Stu-dent'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-ried out Southern blot analysis using two different oligonu-cleotide 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-formed. There was no significant difference between the levels of SHBG variant mRNA in normal cervix (1.11±0.68 corrected IOD) and in cervical cancers (1.19±0.68 cor-rected 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-nizing squamous cell carcinomas, 0.37±0.30 corrected
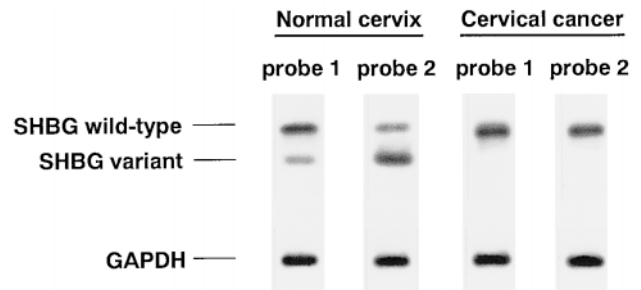

Fig. 1.　RT-PCR-Southern blot analysis of SHBG mRNA expres-sion in normal and cancer tissues of the uterine cervix. Total RNA isolated from each tissue was reverse-transcribed and amplified with primers specific to SHBG or GAPDH gene. Southern blot analysis was performed as described in "Materials and Methods." Lane A: Southern blot hybridization of RT-PCR products using SHBG probe 1 (a part of exon VI). Lane B: Southern blot hybrid-ization of RT-PCR products using SHBG probe 2 (a part of exon VII).
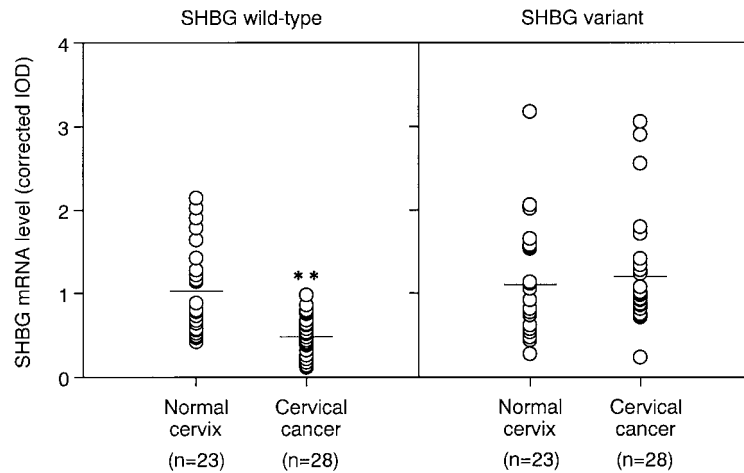
Fig. 2. Level of SHBG variant and wild-type mRNAs in normal and cancer tissues of the uterine cervix. Each circle on the figure shows the average of three different parts of each individual sample. The mRNA levels are expressed as corrected IOD. **$P<0.01$, compared with normal cervical tissues.
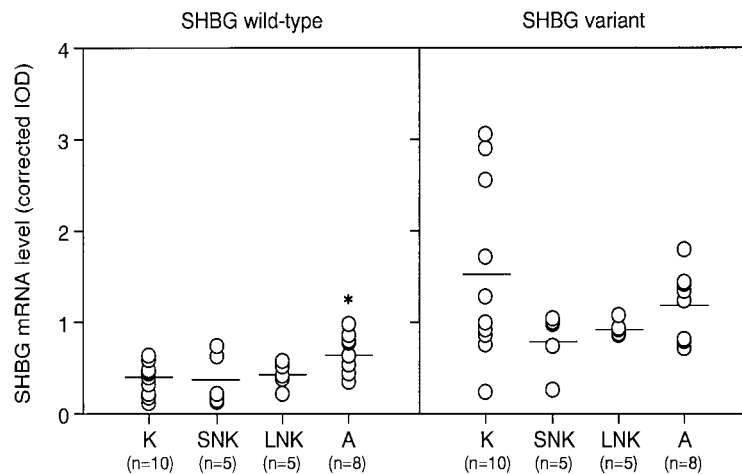

Fig. 3. Levels of SHBG wild-type and variant mRNAs in cervical cancers, classified histologically. One circle on the figure shows the average of three different parts of each individual sample. The mRNA levels are expressed as corrected IOD. K, keratinizing squamous cell carcinoma; SNK, small cell nonkeratinizing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

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
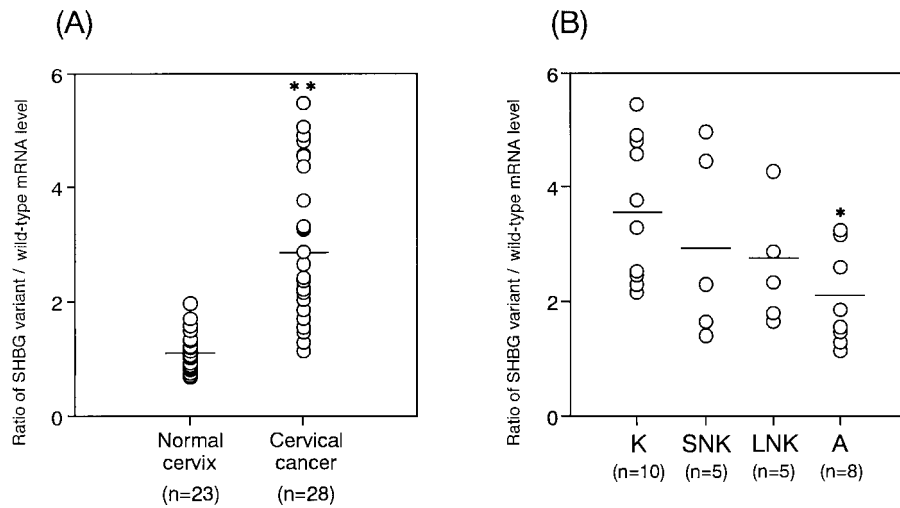
Fig. 4. The ratio of variant to SHBG wild-type mRNA levels in normal and cancer tissues of the uterine cervix (A) and in cervical cancers classified histologically (B). One circle on the figure shows the average of three different parts of each individual sample. The mRNA levels are expressed as corrected IOD. K, keratinizing squamous cell carcinoma; SNK, small cell nonkeratinizing squamous cell carcinoma; LNK, large cell nonkeratinizing squamous cell carcinoma; A, adenocarcinoma. **$P<0.01$, compared with normal cervix. *$P<0.05$, compared with keratinizing squamous cell carcinoma.

cervix (1.10±0.34 corrected IOD) (Fig. 4A). The ratios expressed according to histological classification were as follows: keratinizing squamous cell carcinomas, 3.58±1.31 corrected IOD; small cell non-keratinizing squamous cell carcinomas, 2.94±1.75 corrected IOD; large cell non-keratinizing squamous cell carcinomas, 2.54±1.14 corrected IOD; adenocarcinomas, 2.07±0.90 corrected IOD. The difference of the ratio between keratinizing squamous cell carcinoma and adenocarcinoma was significant ($P<0.05$) (Fig. 4B). The ratio of SHBG variant to wild-type mRNA levels was not related to the clinical stage of cervical cancers (stage I, 2.29±1.25 corrected IOD; stage II, 2.72±1.09 corrected IOD; stages III and IV, 3.59±1.48 corrected IOD).

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.[3–8] 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.[27] 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.[28] Further, estrogen itself has been shown to transactivate the viral genome in human papillomavirus containing malignant cell lines[29] and to induce cervical carcinogenesis

in the female reproductive tract of transgenic mice.[30] Additionally, much evidence indicates that intracellular SHBG-steroid complex plays a direct role in the steroidal interaction in steroid target cells.[10–14] A recent study suggested that SHBG is capable of being synthesized in uterine cervical cancer.[19]

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.[20] 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[31, 32] and fetal[33] livers, it would appear that they are tissue-specific products of alternative splicing within a single gene.[20]

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.[20] 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.[21] SHBG forms a dimer in the cell and the entire coding sequence may be needed to generate an active protein.[21] There are examples of dominant-negative activity by mutant nuclear receptors in human malignancies.[34] 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-

ine cervical cancers might influence the development and growth of the cancers.

SHBG wild-type mRNA expression in uterine cervical cancers was lower than that in normal cervix.[19] In the present study, there was no significant difference between the levels of SHBG variant mRNA in normal cervix and cervical cancers. Therefore, it naturally follows that the relative ratio of SHBG variant to wild-type mRNA was higher in cervical cancers than in normal cervix. The increased ratio of SHBG variant to wild-type mRNA might lead to disruption of the action of intracellular SHBG and thus to an alteration in the hormone-responsive character, compared with normal cervix. The levels of SHBG variant 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.

REFERENCES

1) Pommerenke, W. T. Cyclic changes in the physical and chemical properties of cervical mucus. *Am. J. Obstet. Gynecol.*, **52**, 1023–1031 (1946).

2) MacDonald, R. R. Cyclic changes in cervical mucus. *J. Obstet. Gynaecol. Br. Commonw.*, **76**, 1090–1099 (1969).

3) Terenius, L., Lindell, A. and Persson, H. Binding of estradiol-17β to human cancer tissue of the female genital tract. *Cancer Res.*, **31**, 1895–1898 (1971).

4) Sanborn, B. M., Held, B. and Kuo, H. S. Hormonal action in human cervix-II: specific progestogen binding proteins in human cervix. *J. Steroid Biochem.*, **7**, 665–672 (1976).

5) Soutter, W. P., Pergoraro, E. J., Green-Thompson, R. W., Naidoo, D. V., Joubert, S. M. and Philpott, R. H. Nuclear and cytoplasmic oestrogen receptors in squamous carcinoma of the cervix. *Br. J. Cancer*, **44**, 154–159 (1981).

6) Gao, Y. L., Twiggs, L. B., Leung, B. S., Yu, W. C. Y., Potish, R. A., Okagaki, T., Adcock, L. L. and Prem, K. A. Cytoplasmic estrogen and progesterone receptors in primary cervical carcinoma: clinical and histopathologic correlates. *Am. J. Obstet. Gynecol.*, **146**, 299–306 (1983).

7) Hunter, R. E., Longcope, C. and Keough, P. Steroid hormone receptors in carcinoma of the cervix. *Cancer*, **60**, 392–396 (1987).

8) Fujiwara, H., Tortolero-Luna, G., Mitchell, M. F., Koulos, J. P. and Wright, T. C. Adenocarcinoma of the cervix: expression and clinical significance of estrogen and progesterone receptors. *Cancer*, **79**, 505–512 (1997).

9) Mercier-Bodard, C., Alfsen, A. and Baulieu, E. E. Sex steroid binding plasma protein. *Acta Endocrinol.*, **147**, 204–224 (1970).

10) Strel'chyonok, O. A., Avvakumov, G. V. and Survilo, L. I. A recognition system for sex hormone binding protein-estradiol complex in human decidual endometrium plasma membranes. *Biochim. Biophys. Acta*, **802**, 459–466 (1984).

11) Hryb, D. J., Khan, M. S. and Roser, W. Testosterone-estradiol-binding globulin binds to human prostatic cell membranes. *Biochem. Biophys. Res. Commun.*, **128**, 432–440 (1985).

12) Nakhla, A. M., Khan, M. S. and Rosner, W. Biologically active steroids activate receptor-bound human sex hormone-binding globulin to cause LNCaP cells to accumulate adenocine 3′5′-monophosphate. *J. Clin. Endocrinol. Metab.*, **71**, 398–404 (1990).

13) Mercier-Bodard, C., Radanyi, C., Roux, C., Groyer, M. T., Robel, P., Dadoune, J. P., Petra, P. H., Jolly, D. J. and Baulieu, E. E. Cellular distribution and hormonal regulation of h-SBP. *J. Steroid Biochem.*, **27**, 297–307 (1987).

14) Sinnecker, G., Hiort, O., Kwan, P. W. L. and de Lellis, R. A. Immunohistochemical localization of sex hormone binding globulin in normal and neoplastic breast tissue. *Horm. Metab. Res.*, **22**, 47–50 (1990).

15) Misao, R., Itoh, N., Mori, H., Fujimoto, J. and Tamaya, T. Sex hormone-binding globulin mRNA levels in human uterine endometrium. *Eur. J. Endocrinol.*, **131**, 623–629 (1994).

16) Misao, R., Hori, M., Ichigo, S., Fujimoto, J. and Tamaya, T. Levels of sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) messenger ribonucleic acid (mRNA) in ovarian endometriosis. *Reprod. Nutr. Dev.*, **35**, 155–165 (1995).

17) Misao, R., Nakanishi, Y., Ichigo, S., Hori, M., Fujimoto, J. and Tamaya, T. Expression of sex hormone-binding globulin mRNA in human endometrial cancers. *J. Steroid Biochem. Mol. Biol.*, **52**, 517–522 (1995).

18) Misao, R., Nakanishi, Y., Fujimoto, J., Hori, M., Ichigo, S. and Tamaya, T. Expression of sex hormone-binding globulin mRNA in human ovarian cancers. *Eur. J. Endocrinol.*, **133**, 327–334 (1995).

19) Misao, R., Nakanishi, Y., Fujimoto, J., Hori, M., Ichigo, S. and Tamaya, T. Expression of sex hormone-binding globulin mRNA in uterine cervical cancers. *Tumor Biol.*, **18**, 6–12 (1997).

20) Hammond, G. L., Underhill, D. A., Rykse, H. M. and Smith, C. L. The human sex hormone-binding globulin gene contains exons for androgen-binding protein and two other testicular messenger RNAs. *Mol. Endocrinol.*, **3**, 1869–1876 (1989).

21) Petra, P. H. The plasma sex steroid binding protein (SBP or SHBG): a critical review of recent developments on the structure, molecular biology and function. *J. Steroid Biochem. Mol. Biol.*, **40**, 735–753 (1991).

22) Misao, R., Nakanishi, Y., Fujimoto, J. and Tamaya, T. Expression of sex hormone-binding globulin exon VII splicing variant mRNA in human uterine endometrium. *J. Steroid Biochem. Mol. Biol.*, **62**, 385–390 (1997).

23) Poulsen, H. E. and Taylor, C. W. Histological typing of female genital tract tumours. *In* "International Histological Classification of Tumours 13" (1975). World Health Organization, Geneva.

24) Announcement. Changes in definitions of clinical staging for carcinoma of the cervix and ovary: FIGO. *Am. J. Obstet. Gynecol.*, **156**, 263–264 (1987).

25) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).

26) Arcali, P., Martinelli, R. and Salvatore, F. The complete sequence of a full length cDNA for human liver glyceraldehyde-3-phosphate dehydrogenase: evidence for multiple mRNA species. *Nucleic Acids Res.*, **12**, 9179–9189 (1984).

27) White, J. O., Jones, R. N., Croxtall, J. D., Gleeson, R. P., Krausz, T., Pervez, S., Jamil, A., Guida, L., Beesley, J. E. and Soutter, W. P. The human squamous cervical carcinoma cell line, HOG-1, is responsive to steroid hormones. *Int. J. Cancer*, **52**, 247–251 (1992).

28) Auborn, K. J., Woodworth, C., DiPaolo, J. A. and Bradlow, H. L. The interaction between HPV infection and estrogen metabolism in cervical carcinogenesis. *Int. J. Cancer*, **49**, 867–869 (1991).

29) Mitrani-Rosenbaum, S., Tsvieli, R. and Tu-Kuspa, R. Oestrogen stimulates differential transcription of human papillomavirus type 16 in SiHa cervical carcinoma cells. *J. Gen. Virol.*, **70**, 2227–2232 (1989).

30) Arbeit, J. M., Howley, P. M. and Hanahan, D. Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice. *Proc. Natl. Acad. Sci. USA*, **93**, 2930–2935 (1996).

31) Hammond, G. L., Underhill, D. A., Smith, C. L., Goping, I. S., Harley, M. J., Musto, N. A., Cheng, C. Y. and Bardin, C. W. The cDNA-deduced primary structure of human sex hormone-binding globulin and location of its steroid-binding domain. *FEBS Lett.*, **215**, 100–104 (1987).

32) Gershagen, S., Fernlund, P. and Lundwall, A. A cDNA coding for human sex steroid hormone-binding globuin: homology to vitamin K-dependent protein S. *FEBS Lett.*, **219**, 129–135 (1987).

33) Que, B. G. and Petra, P. H. Characterization of a cDNA coding for sex hormone-binding protein of human plasma. *FEBS Lett.*, **219**, 405–409 (1987).

34) Yen, P. M. and Chin, W. W. Minireview; molecular mechanism of dominant negative activity by nuclear hormone receptors. *Mol. Endocrinol.*, **8**, 1450–1454 (1994).