ORIGINAL ARTICLE

Functional characterization of seven single-nucleotide polymorphisms of the steroid sulfatase gene found in a Japanese population

Jun Matsumoto¹, Noritaka Ariyoshi², Itsuko Ishii¹ and Mitsukazu Kitada²

Steroid sulfatase (STS) is an enzyme that hydrolyzes steroid sulfates such as dehydroepiandrosterone sulfate (DHEA-S) and estrone sulfate. STS has a key role in the synthesis of steroid hormones in placenta and breast cancer cells. Recently, we have identified six novel single-nucleotide polymorphisms (SNPs) and one nonsynonymous SNP (V476M) in the *STS* gene in a Japanese population. To clarify the effects of SNPs in the 5'-flanking region or 5' untranslated region on transcriptional activity, a reporter gene assay was conducted. In addition, DHEA-S desulfatase activity of a variant (Met at codon 476)-type enzyme was compared with that of the wild (Wd)-type enzyme in COS-1 cells. The transcriptional activities were significantly decreased (155A) and increased (–2837A and –1588C) in MCF-7 cells. On the other hand, no significant difference was found in expression levels of STS protein or specific activities of DHEA-S desulfation between Wd and the variant enzymes. This is the first report on the effects of various SNPs in the *STS* gene detected in Japanese healthy subjects.

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INTRODUCTION

Steroid sulfatase (STS, EC 3.1.6.2, also known as aryl sulfatase C) is a membrane-bound microsomal enzyme that is responsible for hydrolysis of steroid sulfates such as dehydroepiandrosterone sulfate (DHEA-S) and estrone sulfate (E1-S), leading to the production of their unconjugated active forms. STS is a member of a superfamily comprising 16 different mammalian sulfatases.¹ In humans, STS is predominantly expressed in the placenta, where it is required for estrogen formation from DHEA-S during pregnancy,^{2,3} although STS is believed to be ubiquitously distributed in other tissues at low levels.⁴ Recent studies have revealed that STS is also highly expressed in estrogen receptor-positive breast cancer tissue and that it may have a key role in growth of tumor cells by production of active forms of estrogens.^{4–7}

The human *STS* gene (GenBank accession No. M23945) consists of 10 exons spread over 146 kb in the distal short arm of the X chromosome (Xp22.3-Xpter). The human *STS* gene has been cloned, sequenced and characterized, and its functional structure has been studied.^{8–10} The *STS* gene encodes a membrane-bound precursor protein (or polypeptides) with an apparent molecular size of 63 kDa that is processed to a mature 61-kDa protein.¹¹ The deduced amino-acid sequence of this enzyme comprises 583 amino-acid residues, and STS contains four possible N-linked glycosylation sites, two of which, Asn47 and Asn259, are utilized.^{8,12}

Several point mutations in the *STS* gene have been reported in patients with X-linked ichthyosis (XLI), which is an X-linked disorder of cutaneous hyperkeratinization due to lack of STS activity.^{8,13–20} Approximately 80% of the patients have complete deletion of the *STS* gene, and the remaining patients have point mutations. In addition, recent reports demonstrated that some single-nucleotide polymorphisms (SNPs) within the *STS* gene were significantly associated with susceptibility of attention deficit hyperactivity disorder (ADHD).^{21,22} However, because these SNPs are located in introns, it is unlikely that loss of STS expression or function is one of the determinants for ADHD, suggesting that a candidate gene responsible for ADHD may exist near the *STS* gene.

Furthermore, recent studies have revealed that the expression level of STS mRNA in breast tissues was related to the risk of breast cancer,^{23–26} and inhibition of STS activities by an STS inhibitor (STX64 and BN83495) is an attractive therapeutic strategy for estrogen-dependent breast and endometrial cancers.^{27,28} Overexpression of STS has been thought to accelerate proliferation of estrogen receptor-positive cancer cells by supplying active forms of estrogens via desulfation of estrogen sulfates.

To date, there is no report about SNPs affecting STS activities in healthy subjects, although there are some reports about causal mutations of XLI. Recently, we discovered six novel SNPs including one nonsynonymous one (1647G>A, V476M), which is in coding

¹Department of Clinical Pharmacology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan and ²Division of Pharmacy, University Hospital, Chiba University School of Medicine, Chiba, Japan
Correspondence: Dr N Ariyoshi, Division of Pharmacy, University Hospital, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan.
E-mail: ariyoshi@faculty.chiba-u.jp

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region of the *STS* gene (Figure 1).²⁹ In the present study, we investigated the effects of seven SNPs found in the *STS* gene in a healthy Japanese population on expression and catalytic activities of *STS*.

MATERIALS AND METHODS

Human genomic DNA samples

Genomic DNA isolated from unrelated Japanese healthy volunteers and the DNAs, which was confirmed possessing SNPs (−2837G>A, −2427G>A, −1588T>C, −1117T>C, −21G>A and 155G>A) in the *STS* gene by direct sequencing analysis in our previous study,²⁹ was used. This study was approved by the Life-Ethics Committee of Chiba University School of Medicine (Approval No. 5–24), and written informed consent was obtained from all participants.

Cell culture

Human placental choriocarcinoma cell line BeWo, human breast cancer cell line MCF-7 and monkey kidney cell line COS-1 were obtained from RIKEN BioResource Center (Tsukuba, Japan). BeWo cells were cultured in Ham's F-12 medium supplemented with 100 U ml^{−1} penicillin, 100 µg ml^{−1} streptomycin (Wako, Tokyo, Japan) and 10% fetal bovine serum (Thermo Fisher Scientific, Yokohama, Japan). MCF-7 and COS-1 cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 100 U ml^{−1} penicillin, 100 µg ml^{−1} streptomycin, non-essential amino acid (Wako) and 10% fetal bovine serum. All cell lines were cultured in a humidified 5% CO₂ atmosphere at 37 °C.

PCR and plasmid construction

The 5'-flanking region and 5' untranslated region (about 3 kb from a translational initiation site) of the *STS* gene and 1.8 kb of the full-length of cDNA encoding the *STS* protein (Dnaform, Yokohama, Japan) were amplified by PCR using KOD-Plus-Ver.2 (Toyobo, Osaka, Japan) with a specific primer set possessing recognition sites for restriction enzymes. A coding variant (1647G>A, V476M) was generated by site-directed mutagenesis. These PCRs were conducted in a reaction mixture of 50 µl containing 1.5 mM MgSO₄ and 0.2 mM dNTPs with 0.3 µM of each primer. PCR conditions consisted of initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C

for 20 s, annealing at 58 °C for 15 s and extension at 68 °C for 2 or 3 min. The primers used in this study are summarized in Table 1. Both of the DNA fragments from wild (Wd) and mutant types of the 5'-flanking region and 5' untranslated region of the *STS* gene were cloned into the pGL4.10 [*luc2*] vector (Promega, Madison, WI, USA) after restriction digestion by *KpnI* and *XhoI* (pGL-*STS* Wd, −2837A, −2427A, −1588C, −1117C, −21A and 155A). Full-length of cDNAs encoding Wd and the variant *STS* proteins were cloned into the pALTER-MAX vector (Promega) at *EcoRI* and *Sall* sites, respectively (pALTER-*STS* Wd and V476M). The results of site-directed mutagenesis and plasmid constructions were confirmed by sequencing analysis.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from each cell line by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the quality of the isolated RNA was checked by ribosomal RNA ratio and absence of degraded bands with a Formaldehyde-Free RNA Gel Kit (AMRESCO, Cochran Solon, OH, USA). Reverse transcription of 2.5 µg of total RNA was performed with a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions. The amount of *STS* and *RPL13A* mRNAs were measured using THUNDERBIRD SYBR qPCR Mix (Toyobo) with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The quantitative PCR was carried out in 20 µl reaction volume containing 0.3 µM of each primer, SYBR qPCR Mix and 100 ng of cDNA. The specific primers used for amplification are also listed in Table 1. PCR conditions consisted of initial denaturation at 95 °C for 30 s, followed by denaturation at 95 °C for 5 s, annealing at 58 °C for 20 s and extension at 72 °C for 30 s. The results are expressed as a relative value after normalization to the *RPL13A* gene expression.

Luciferase assay

BeWo and MCF-7 cells were plated in 24-well plates (1.0 × 10⁶ and 5.0 × 10⁵ cells per well, respectively) and were transiently co-transfected with reporter constructs of pGL-*STS* Wd or mutants (0.75 µg per well in BeWo cells and 0.25 µg per well in MCF-7 cells) and renilla reniformis pGL4.74 vector (Promega) (15.0 ng per well in BeWo cells and 5.0 ng per well in MCF-7 cells) using TransFast Transfection Reagent (Promega) according to the manufacturer's recommendations. The firefly and renilla luciferase activities were determined 42 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) and Gene-Light 2000 (Microtec Co., Chiba, Japan).

Expression of *STS* protein and western blot analysis

COS-1 cells were plated in a 60-mm dish and co-transfected with *STS* expression plasmids (pALTER-*STS* Wd or V476M) and pGL4.74 vector as an internal control at 80% confluency. After incubation for 48 h and transfection efficiency was measured, transfected cells were collected and suspended in an extraction buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 10 mM EDTA, 0.5% Triton X-100 and 0.5% sodium cholate. After freezing and thawing twice, suspended cells were centrifuged at 13000 g. The

Figure 1 Position of the SNPs discovered in the *STS* gene in healthy Japanese subjects. Arrows indicate the position of SNPs found in our previous study.²⁹ Black and white boxes represent coding exons and noncoding exons, respectively.

Table 1 Sequences of primers and size for amplified products

Number ^a	Primer name	Forward primer (5'–3') ^b	Reverse primer (5'–3') ^b	Amplified region	Size (bp)
1	Luc-STS	CGGggtaccCTGCAACTCCATTGGTCCAAAAG	CCGctcgagCTCCAGCTTGTGATCCTGTG	5019479–5016156 ^c	3344
2	STS cDNA	CCGgaattcATGCCTTTAAGGAAGATGAAGATCCCTTTTC	ACGCgtcgacTGCCACATGCGTCTGTCTG	249–2031 ^d	1803
3	V476M-1	CCGgaattcATGCCTTTAAGGAAGATGAAGATCCCTTTTC	GTTGGAACCCATGGGGTTG	249–1683 ^d	1444
4	V476M-2	CAACCCCATGGGTCCAAC	ACGCgtcgacTGCCACATGCGTCTGTCTG	1665–2031 ^d	377
5	STS mRNA	AGTCTACGGGGATGCTGTTG	CCATTACTTCGCCATGAA	1163–1330 ^d	168
6	RPL13A	CCTGGAGGAGAAGAGAAAGAGA	TTGAGGACCTCTGTGTAATTGTCAA	542–666 ^e	125

^a1, for construction of plasmid for reporter gene assay; 2–4, for construction of plasmid to express *STS* protein; 5–6, for detection of *STS* and ribosomal protein L13A mRNA by quantitative real-time PCR.

^bSmall letters and underlined bars represent recognition sites of restriction enzyme and a mismatched nucleotide, respectively.

^cThe reference sequence from GenBank is NT_167197.1.

^dThe reference sequence from GenBank is NM_000351.

^eThe reference sequence from GenBank is NM_012423.

supernatant was separated by 9.0 (w/v)% sodium dodecyl sulfate polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. After blocking with 3 (w/v)% BSA in Tris-buffered saline for 1 h at room temperature, the membrane was cut into two pieces to detect STS (61 kDa) and the housekeeping gene β -actin (42 kDa). Upper and lower piece of the membrane were incubated overnight at 4 °C with polyclonal rabbit anti-human STS antibody (Abcam, Cambridge, MA, USA) and polyclonal rabbit anti-human β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Wako) for 1 h at room temperature. Protein bands were visualized by using an ECL Western Blotting Detection Reagent (GE Healthcare, Tokyo, Japan) and LAS-1000 plus (Fujifilm, Tokyo, Japan). Protein concentrations were determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The band intensities were quantified using ImageJ 1.46 (<http://rsb.info.nih.gov/ij/>).

Preparation of microsomes and assay of STS activity

STS protein was expressed in cells cultured with a 100-mm dish, and microsomal protein was prepared. Cells were sonicated in 100 mM potassium phosphate buffer (pH 7.4) with 0.25 M sucrose and centrifuged at 2400 g for 20 min at 4 °C. The supernatant was recentrifuged at 105 000 g for 1 h at 4 °C. The resultant microsomal pellet was suspended in 50 mM potassium phosphate buffer (pH 7.4) with 20% (v/v) glycerol. A new method to determine DHEA-S desulfatase activities by using reverse-phase high-performance liquid chromatography (HPLC) was developed in this study. Reaction mixtures (each 500 μ l) containing various concentrations of DHEA-S (Sigma-Aldrich Japan, Tokyo, Japan) in 100 mM potassium phosphate buffer (pH 7.4) were incubated with microsomes at 37 °C for 25 min. After the reaction had been stopped by vigorous vortexing followed by chilling on ice for 3 min, 5 μ l of 1 mM 11 β -hydroxyandrosterone was added as an internal standard. Then the mixture was applied onto Sep-Pak Plus QMA (Waters, Milford, MA, USA) and washed with 5.0 ml of water. After shaking the eluate with 6 ml of ethyl acetate for 5 min and centrifuging at 3000 r.p.m. for 5 min, the organic layer was evaporated *in vacuo*. The residue was dissolved in 200 μ l of 0.5% trichloroacetic acid/benzene solution, and 100 μ l of 0.2% dansyl hydrazine (Sigma-Aldrich Japan)/methanol solution was added. This mixture was incubated at 70 °C for 25 min and was then evaporated under reduced pressure. The residue dissolved in 2 ml of dichloromethane and 1 ml of 0.2 M sodium hydroxide solution was vortexed and centrifuged at 3000 r.p.m. for 5 min. After the aqueous layer had been removed, 1 ml of 0.2 M sodium hydroxide solution was added. The mixture was vortexed and centrifuged at 3000 r.p.m. for 5 min again. The organic layer was evaporated under reduced pressure, and the residue was redissolved in 400 μ l of methanol. An aliquot of this sample was injected into an HPLC system.

Instrument and HPLC conditions

The HPLC system comprised an L-2100 pump, an L-2200 autosampler, an L-2300 column oven, an L-2485 fluorescence detector (Hitachi, Tokyo, Japan) and an Inertsil ODS-4 column (5 μ m, 4.6 $\times$ 150 mm²; GL Sciences, Tokyo, Japan). The analyte was separated under linear gradient GL with the mobile phase consisting of methanol/water (65:35 v/v, component A) and methanol (component B). Flow rate of the mobile phase was 1.0 ml min⁻¹ and column temperature was 35 °C. The elution program was as follows: 0–10 min = 100% A, 10–45 min = 20% A and 80% B and 45–50 = 100% B. The elution was monitored at 365 nm for excitation and at 520 nm for emission.

Data analysis

Data from the reporter gene assay were analyzed by Dunnett's test. Kinetic parameters (K_m and V_{max}) obtained from the enzyme assay were determined by using nonlinear regression analysis in Prism Version 5.01 (GraphPad Software, La Jolla, CA, USA). Curves were fitted to the Michaelis–Menten equation $V = V_{max} [S] / (K_m + [S])$. Comparison of expression levels and enzyme activities of STS between two groups were carried out by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Expression of STS mRNA in each cell line

Quantitative real-time PCR analysis was carried out to compare the expression levels of STS mRNA in BeWo, MCF-7 and COS-1 cells (Figure 2). It has been reported that STS was highly expressed in placenta and breast cancer cells, but the expression of STS was negligible in COS-1, which was used as host cells for heterologous expression of human STS.³⁰ As expected, STS mRNA was highly expressed in both BeWo and MCF-7 cells, but the expression level was higher in BeWo cells (% of the expression level in BeWo cells $\pm$ s.d., 100.0 $\pm$ 1.06% in BeWo cells and 42.2 $\pm$ 0.17% in MCF-7 cells). On the other hand, STS mRNA was almost the level of detection limit in COS-1 cells (% of the expression level in BeWo cells $\pm$ s.d., 0.754 $\pm$ 0.05%). Consistent with previous reports, our results confirmed that STS is expressed at high levels in not only placental cells but also breast cancer cells. From these results, we decided to use both BeWo and MCF-7 cells for reporter gene assays and COS-1 cells for heterologous expression of human STS protein in further studies.

Effects of SNPs in the 5'-flanking region of the STS gene on transcriptional activity

In our previous study, five SNPs of the 5'-flanking region (–2837G>A, –2427G>A, –1588T>C, –1117T>C and –21G>A) and one SNP of the 5' untranslated region of noncoding exon 1 (155G>A) were detected in healthy Japanese subjects.²⁹ To determine effects of these SNPs on transcriptional activity of the STS gene, reporter gene assays were conducted in both BeWo and MCF-7 cells. In BeWo cells, 155A tended to decrease luciferase activity (% of Wd, mean $\pm$ s.e.: 86.91 $\pm$ 4.04; Figure 3a), whereas –1588C and –2837A tended to increase luciferase activity (% of Wd, mean $\pm$ s.e.: 109.95 $\pm$ 5.44 for –1588C and 109.53 $\pm$ 6.20 for –2837A). However, there was no significant difference in transcriptional activity between Wd and any of the SNPs in BeWo cells. On the other hand, 155A significantly decreased luciferase activity in MCF-7 cells (% of Wd, mean $\pm$ s.e.: 73.75 $\pm$ 0.85; Figure 3b). In addition, –2837A and –1588C showed significant ($P < 0.001$ and $P < 0.05$ comparing –2837A and –1588C with Wd, respectively) increases in transcriptional activity (% of Wd, mean $\pm$ s.e.: 134.82 $\pm$ 2.06 for –2837A and

Figure 2 Quantitative real-time PCR for the expression of STS mRNA in three cell lines, placental-derived BeWo, breast cancer-derived MCF-7 and monkey kidney-derived COS-1 cells. The amount of STS mRNA was normalized by the amount of the housekeeping *RPL13A* gene. These data represent % of the expression level in BeWo cells $\pm$ s.d. for three replicates.

Figure 3 Effects of SNPs in 5'-flanking region and 5' untranslated region of the STS gene on transcriptional activity of STS. Plasmids containing 3 kb of STS 5'-flanking region (Wd, -2837A, -2427A, -1588C, -1117C, -21A and 155A) were transiently transfected into (a) BeWo cells and (b) MCF-7 cells. Firefly luciferase activity was normalized by renilla luciferase activity. Mock cells transfected with vacant pGL4.10 vector. N. D., not detectable. These data represent % of control (constructs of pGL-STS Wd) and mean $\pm$ s.e. for six replicates. * P <0.05 and *** P <0.001 compared with Wd (Dunnett's test).

Figure 4 Expression of human STS protein in COS-1 cells (a) and a typical HPLC chromatogram for detection of DHEA (b). Immunoblot analysis for expression levels of Wd and V476M STS protein in subcellular fraction from transfected COS-1 cells (30 μ g protein) using STS and β -actin antibody. The expression levels of STS of Wd and V476M are expressed as a relative value after normalization to the β -actin expression (% of Wd expression $\pm$ s.d. for three replicates). Mock, fraction from COS-1 cells transfected with pALTER-MAX Vector; Wd, fraction from COS-1 cells transfected with pALTER-STS; V476M, fraction from COS-1 cells transfected with pALTER-V476M; I.S., internal standard.

124.81 ± 4.99 for -1588C). The other three SNPs did not affect promoter activity in MCF-7 cells.

Effects of a nonsynonymous SNP on expression and catalytic activities of STS

To clarify the effects of the nonsynonymous SNP found in exon 10 (1647G>A, V476M) on expression and catalytic activities of STS, human STS was heterologously expressed in COS-1 cells. As shown in Figure 4a, there was no difference in the expression level of STS protein between Wd and variant (V476M) enzymes in COS-1 cells. As described in the Materials and methods section, we have developed a simple method to determine DHEA-S desulfatase activity, because no reverse-phase HPLC method for detecting DHEA has been reported to date. A typical HPLC chromatogram is shown in Figure 4b.

The chromatogram shows good separation of internal standard (11 β -hydroxyandrosterone) and DHEA, and retention times for internal standard and DHEA were 16 and 22 min, respectively. The standard curve of DHEA was linear ($R^2 = 0.9964$) in the range of 0.1–10.0 nM.

As shown in Figure 5, kinetics of DHEA-S desulfatase activity catalyzed by both Wd and variant enzymes followed the Michaelis-Menten equation. The K_m and V_{max} values (mean $\pm$ s.e.) of STS were $24.83 \pm 3.62 \mu\text{M}$ and $346.5 \pm 14.9 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (95% confidence intervals: 17.17–32.50 μM and 314.8–378.1 $\mu\text{mol min}^{-1} \text{mg}^{-1}$), respectively, for the Wd enzyme, and $20.66 \pm 2.76 \mu\text{M}$ and $354.4 \pm 13.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (95% confidence intervals: 14.81–26.51 μM and 326.7–382.1 $\mu\text{mol min}^{-1} \text{mg}^{-1}$), respectively, for the variant enzyme. The V_{max}/K_m values were $14.0 \pm 2.1 \text{ min}^{-1} \text{mg}^{-1}$

Figure 5 Comparison of DHEA-S desulfatase activity between Wd and V476M of STS. (a) Substrate concentration-velocity plots and (b) Eadie-Hofstee plots. A substrate concentration ranging 12.5–200.0 μM was employed. Open circles, Wd; closed circles, V476M. These data represent mean ± s.e. for three replicates.

and $17.2 \pm 2.4 \text{ min}^{-1} \text{ mg}^{-1}$ for Wd and variant enzymes, respectively. No significant difference in DHEA-S desulfatase activity between Wd and variant enzymes was observed.

DISCUSSION

In our previous study, seven SNPs including a novel nonsynonymous one (V476M), which was located in the region encoding the C-terminal domain of STS, were found in STS genes isolated from Japanese subjects. It is clear that none of the seven SNPs detected in our study are causal mutations of XLI, because these SNPs were detected in apparently healthy subjects. However, these SNPs may affect activation of estrogens by either changing the expression levels of STS protein or catalytic activities. Therefore, we investigated the functional significance of these seven SNPs in the present study.

The proposed negative regulatory region and the minimum sequences required for promoter activity of the STS gene exist in about 200 (from –1237 to –1032) and 80 bp (from –80 to –1) upstream from the transcription start site, respectively.³¹ Although two SNPs (–1117T>C and –21G>A) were found in these regions, these SNPs did not have significant effects on transcriptional activity in either BeWo or MCF-7 cells. It is reported that there are splicing variants of STS.^{32–35} The STS gene has at least nine alternate first exons and eight tissue-specific transcripts, suggesting that the expressions of STS in each type of cells are regulated in part by alternative splicing. The proximal promoter of placental STS does not have a TATA box, which is not GC-rich and lacks binding sites for Sp1 or other known transcription factors. On the other hand, upstream region of the transcriptional initiation site for some first exons has GC-rich sequences. These first exons are either noncoding sequences or in region encoding signal peptides, indicating that the tissue-specific regulation of STS may affect enzyme activity via mRNA expression level or interaction with membrane. In our study, one SNP (155G>A) found in the 5' untranslated region of noncoding exon 1 significantly decreased transcriptional activity in MCF-7 cells but not in BeWo cells. On the other hand, –2837A and –1588C significantly increased transcriptional activity in MCF-7 cells but not in BeWo cells (Figure 3). One of the reasons of the difference in transcriptional activities between BeWo and MCF-7 cells may be explained in part by the tissue-specific regulation mechanisms of STS expression. If –2837A and –1588C increase transcriptional activity of the STS gene also *in vivo*, it may be of interest to investigate relationship between the prevalence of these SNPs and breast cancer risk.

Several point mutations in the STS gene have been discovered from XLI patients, and these mutations are known to cause deficiency of

enzyme activity. Most of the point mutations were found in the region from exon 7 to 10, and they change amino-acid residues located in the C-terminal half polypeptide, which is thought to be important region for substrate binding.³⁰ On the other hand, Liao *et al.*¹⁹ and Winge *et al.*²⁰ have reported that mutations of the STS gene in the area of exons 3 and 5 encoding N-terminal half polypeptide also cause XLI. In these mutations, T165I is particularly important, because T165 is an amino-acid residue highly conserved in mammalian aryl sulfatases. The nonsynonymous SNP characterized in this study was found in exon 10 and is located in the C-terminal half polypeptide of STS. However, there was no significant difference in DHEA-S desulfatase activity between Wd and variant (V476M) enzymes. Therefore, this SNP (1647G>A) may have no effect at least on substrate recognition of DHEA-S. It would be of interest to investigate in a future study whether this nonsynonymous mutation has some effects on the metabolism of other substrates.

In conclusion, we clarified the effects of seven SNPs, including six novel ones, of the STS gene on transcriptional and catalytic activities of STS *in vitro*, and this is the first study to investigate the effects of SNPs of the STS gene found in healthy subjects.

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