

$$P_{sp} = \frac{\sum_i (1-x_i)(1-p_i)}{\sum_i (1-x_i)},$$

(4)

and

$$P_{ac} = \frac{\sum_i q_i}{n} = \frac{\left( \sum_i x_i p_i + \sum_i (1-x_i)(1-p_i) \right)}{n}$$

(5)

These measures are also consistent with sensitivity, specificity and accuracy which are based upon the number of tested chemicals, when each  $p_i$  becomes 1 or 0.

Using  $V(p_i)$ , we can also calculate the variance for the proposed measures,  $V(P_{sn})$ ,  $V(P_{sp})$  and  $V(P_{ac})$  (Appendix B). Furthermore we can construct the 95% confidence interval as follows;

$$P_{sn} \pm 1.96 \times \sqrt{V(P_{sn})},$$

$$P_{sp} \pm 1.96 \times \sqrt{V(P_{sp})}, \text{ and}$$

$$P_{ac} \pm 1.96 \times \sqrt{V(P_{ac})}.$$

Note that the proposed measures only depend on the set of chemicals in the validation study but not on the factor of laboratory.

### Results

Table 3 displays a 2 by 2 table of the summarized data of Table 2, in which "Equivocal" for the alternative test is assigned to "Positive". Based on Table 3, sensitivity, specificity and accuracy become 93.7% (15/16), 40.0% (8/20) and 63.9% (23/36) respectively. When "Equivocal" is eliminated from the numerator, sensitivity and accuracy are 62.5%(10/16) and 50%(8/16) respectively.

Table 4 shows  $y_{ij}$ ,  $x_i$ ,  $m_i$ ,  $p_i$ ,  $q_i$  and  $V(p_i)$  corresponding to Table 2. According to the table, the proposed measure,  $P_{sn}$ ,  $P_{sp}$  and  $P_{ac}$  are obtained as 78.1% (3.38/4), 47.5% (2.37/5) and 61.1%(5.75/9) respectively. The 95% confidence intervals of the proposed measures become

Table 3. The 2 by 2 table produced from Table 2. P: "Positive", E: "Equivocal".

		Animal test	
		Positive	Negative
Alternative Test	Positive	15 (P:10, E:5)	12 (P:9, E:3)
	Negative	1	8
		16	20

Table 4. Scores for data in Table 2, and  $p_i$ ,  $q_i$  and  $V(p_i)$ .

Chemical	$x_i$ (In vivo)	$y_{ij}$ (Laboratory)						$m_i$	$p_i$	$q_i$	$V(p_i)$
		a	b	c	d	e	f				
A	1	1	0.5	0.5	1			4	0.75	0.75	0.25
B	1	1	0	1	0.5			4	0.625	0.625	0.688
C	0	1	1	1	1			4	1	0	0
D	1	1	0.5			0.5	1	4	0.75	0.75	0.25
E	0	1	1			1	1	4	1	0	0
F	0	0	1			0	0	4	0.25	0.75	0.75
G	1			1	1	1	1	4	1	1	0
H	0			0	0.5	0.5	0.5	4	0.375	0.625	0.188
I	0			0	0	0	0	4	0	1	0

24.7 to 100%, 9.5 to 85.5% and 31.2 to 91.1%, respectively.

When we set 0.5 for "Equivocal" and calculate the values of sensitivity, specificity and accuracy, these are consistent with  $P_{sn}$ ,  $P_{sp}$  and  $P_{ac}$ , respectively. Though both measures are identical when the number of laboratories doing experiments for all the chemicals is the same, generally these return different values.

### Discussion

There are several recommended statistical methods for data analysis when alternative tests are assessed (Festing, 2001). However, it is seldom that statistical methods for the inter-laboratory validation study have been developed. The proposed measures in this paper are for the inter-laboratory validation study.

Sensitivity, specificity and accuracy are commonly used in studies on diagnostic test studies in medicine (Altman, 1994). In these, the results of diagnostic tests between separate groups of patients with and without a target disease are summarized by a 2 by 2 table. One of the biggest differences compared to the situation for evaluating an alternative test is that the researcher is able to control the values of these measures by selecting the set of chemicals for the validation study. Our proposal measures also have the same feature.

This feature also affects the construction of the confidence intervals. Though the way to constructing of the confidence intervals for these measures is also well known (Altman, 2000b), it is assumed that each observation is followed by an independent and identical distribution. It would be difficult to make the assumption that experimental results from different chemicals have the same distribution because toxicity for chemicals selected in a study is usually widespread. However, the proposed measures solved the problem and we can construct their confidence intervals.

The confidence interval is an index to show the precision of measures, and usually it is affected by the number of data. Thus,  $V(p_i)$  depends on the number of laboratories on the  $i$ th chemical,  $m_i$ ;  $V(P_{sn})$ ,  $V(P_{sp})$  and  $V(P_{ac})$  also depend on the number of chemicals,  $n$  beside  $m_i$ . Therefore, if a validation study with a few laboratories is conducted, the confidence intervals of the proposed measures,  $P_{sn}$ ,  $P_{sp}$  and  $P_{ac}$ , may become wider. Though this feature is a limitation of the proposed measure, we could obtain more reliable estimates

if there are replications for a chemical in a laboratory.

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## Appendix A

Suppose  $y_{ij}$  is a score of the  $i$  th chemical in the  $j$  th laboratory, which takes 1 for "Positive", 0 for "Negative" and 0.5 for "Equivocal";  $r_i^{(N)}$ ,  $r_i^{(E)}$  and  $r_i^{(P)}$  are frequencies for "Negative", "Equivocal" and "Positive" of the  $i$  th chemical;  $m_i$  is the number of laboratories which experimented the  $i$  th chemical. We assume that  $(r_i^{(N)}, r_i^{(E)}, r_i^{(P)})$  follows a trinomial distribution with parameters,  $m_i$ ,  $\pi_i^{(N)}$ ,  $\pi_i^{(E)}$  and  $\pi_i^{(P)}$ :

$$(r_i^{(N)}, r_i^{(E)}, r_i^{(P)}) \sim \text{tri}(m_i, \pi_i^{(N)}, \pi_i^{(E)}, \pi_i^{(P)}), \quad (\text{A1})$$

where  $\pi_i^{(N)} + \pi_i^{(E)} + \pi_i^{(P)} = 1$ .

The sum of  $y_{ij}$  for the  $i$  th chemical can write use  $(r_i^{(N)}, r_i^{(E)}, r_i^{(P)})$  is:

$$\sum_j y_{ij} = 0 \times r_i^{(N)} + 0.5 \times r_i^{(E)} + 1 \times r_i^{(P)}. \quad (\text{A2})$$

Therefore, the expected value of  $\sum_j y_{ij} / m_i$  is:

$$\begin{aligned} E\left[\sum_j y_{ij} / m_i\right] &= 0 \times \pi_i^{(N)} + .5 \times \pi_i^{(E)} + 1 \times \pi_i^{(P)} \\ &= .5 \times \pi_i^{(E)} + 1 \times \pi_i^{(P)}. \end{aligned} \quad (\text{A3})$$

The variance is:

$$\begin{aligned} V\left[\sum_j y_{ij} / m_i\right] &= 0 \times \frac{\pi_i^{(N)}(1 - \pi_i^{(N)})}{m_i} + .5 \times \frac{\pi_i^{(E)}(1 - \pi_i^{(E)})}{m_i} + 1 \times \frac{\pi_i^{(P)}(1 - \pi_i^{(P)})}{m_i} \\ &\quad - 2 \times \left(0 \times .5 \times \frac{\pi_i^{(N)}(1 - \pi_i^{(E)})}{m_i}\right) - 2 \times \left(.5 \times 1 \times \frac{\pi_i^{(E)}(1 - \pi_i^{(P)})}{m_i}\right) - 2 \times \left(0 \times 1 \times \frac{\pi_i^{(N)}(1 - \pi_i^{(P)})}{m_i}\right) \\ &= (.5)^2 \times \frac{\pi_i^{(E)}(1 - \pi_i^{(E)})}{m_i} + \frac{\pi_i^{(P)}(1 - \pi_i^{(P)})}{m_i} - \frac{\pi_i^{(E)}(1 - \pi_i^{(P)})}{m_i}. \end{aligned} \quad (\text{A4})$$

Since we can't know the values of  $\pi_i^{(N)}$ ,  $\pi_i^{(E)}$  and  $\pi_i^{(P)}$ , we need to estimate these values as  $\hat{\pi}_i^{(N)}$ ,  $\hat{\pi}_i^{(E)}$  and  $\hat{\pi}_i^{(P)}$ . When the maximum likelihood method is used, the estimates are  $\hat{\pi}_i^{(N)} = r_i^{(N)} / m_i$ ,  $\hat{\pi}_i^{(E)} = r_i^{(E)} / m_i$  and  $\hat{\pi}_i^{(P)} = r_i^{(P)} / m_i$ . Then, by applying the estimates to (A3) and (A4), the values of  $p_i$  and  $V(p_i)$  are:

$$p_i = .5 \times r_i^{(E)} / m_i + 1 \times r_i^{(P)} / m_i = \sum_j y_{ij} / m_i, \quad (\text{A5})$$

$$V(p_i) = \frac{1}{4} \times \frac{r_i^{(E)}(m_i - r_i^{(E)})}{m_i} + \frac{r_i^{(P)}(m_i - r_i^{(P)})}{m_i} - \frac{r_i^{(E)}(m_i - r_i^{(P)})}{m_i}. \quad (\text{A6})$$

Appendix B

Since  $p_i$  are independent for each other, using  $V(p_i)$  the variances of Psn, Psp and Pac,  $V(Psn)$ ,  $V(Psp)$  and  $V(Pac)$ , are obtained as:

$$\begin{aligned}
 V(Psn) &= V\left(\sum_i x_i p_i / \sum_i x_i\right) \\
 &= \sum_i x_i^2 V(p_i) / \left(\sum_i x_i\right)^2 \\
 &= \sum_i x_i V(p_i) / \left(\sum_i x_i\right)^2, \tag{B1}
 \end{aligned}$$

$$\begin{aligned}
 V(Psn) &= V\left(\sum_i (1-x_i)(1-p_i) / \sum_i (1-x_i)\right) \\
 &= \sum_i (1-x_i)^2 V(1-p_i) / \left(\sum_i (1-x_i)\right)^2 \\
 &= \sum_i (1-x_i) V(p_i) / \left(\sum_i (1-x_i)\right)^2, \text{ and} \tag{B2}
 \end{aligned}$$

$$\begin{aligned}
 V(Pac) &= V\left(\sum_i x_i p_i + \sum_i (1-x_i)(1-p_i) / n\right) \\
 &= \left\{ \sum_i x_i V(p_i) + \sum_i (1-x_i) V(p_i) \right\} / n^2 \\
 &= \sum_i V(p_i) / n^2. \tag{B3}
 \end{aligned}$$

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## Frequency distribution of phenol sulfotransferase 1A1 activity in platelet cells from healthy Japanese subjects

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### Abbreviations used:

CYP: cytochrome P450, OHT: *trans*-4-hydroxytamoxifen, PAPS: 3'-phosphoadenosine 5'-phosphosulfate, PhIP: 2-amino-1-methyl-6-phenylimidazo (4,5-*b*) pyridine, SULT: sulfotransferase, TAM: tamoxifen.

### Keywords:

genetic polymorphism, Japanese, sulfating activity, SULT1A1, *SULT1A1*\*2, *trans*-4-hydroxytamoxifen.

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## SUMMARY

*Aims:* To determine the distribution of sulfotransferase1A1 (SULT1A1) activities, we used *trans*-4-hydroxytamoxifen (OHT) as a substrate to test samples from a Japanese population to examine whether the *SULT1A1* \*2 allele can account for the wide distribution of OHT sulfating activity. We also studied genetic mutations other than the *SULT1A1* \*2 allele to determine the cause of differences in SULT1A1 protein expression and activity.

*Methods:* The subjects were 103 healthy Japanese adults. Identification of SULT1A1 genotypes was performed using a polymerase chain reaction-restriction fragment length polymorphism method. SULT1A1 activity in platelet cytosol was assayed using OHT as a substrate. SULT1A1 protein was detected using Western blotting analysis. Mutations other than *SULT1A1* \*2 in the *SULT1A1* gene were detected using sequencing analysis.

*Results:* *SULT1A1* \*2 allele frequency was found to be 16.5%, while SULT1A1 activity ranged from 63 to 1860 pmol/hr/mg protein ( $260 \pm 241$  pmol/hr/mg protein, median  $\pm$  S.D.) using OHT as a substrate. The median values in subjects with *SULT*\*1/\*2 ( $221 \pm 113$  pmol/hr/mg protein, range 63-442, n=26) and *SULT*\*2/\*2 ( $124 \pm 66$  pmol/hr/mg protein, range 74-231, n=4) were significantly lower than that in subjects with *SULT*\*1/\*1 ( $303 \pm 267$  pmol/hr/mg protein, range 97-1859, n=73). A novel G148C mutation was found in 1 subject, who showed the lowest OHT sulfating activity, for a frequency of 0.95%.

*Conclusion:* There was wide variety of OHT sulfating activities found among the present healthy Japanese subjects. The *SULT1A1* \*2 allele was found to be a common mutant allele and was associated with decreased OHT sulfating activity. These observations may be related to inter-individual variations of OHT pharmacokinetics and the pharmacologic effects of tamoxifen seen in Japanese patients with breast cancer.

## INTRODUCTION

SULT1A1 is a Phase II conjugating enzyme. Using 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a sulfate donor, the enzyme has been shown to sulfate lipophilic compounds hydroxylated mainly by cytochrome P450 (CYP) isozymes<sup>1)</sup>. Since SULT1A1 activity in the liver is correlated well with its levels in platelet cytosol, platelets have proven to be useful for evaluating inter-individual differences in SULT1A1 activity<sup>2)</sup>.

R Price et al., RB Raftogianis et al., A Brittelli et al., and S Nowell et al., each studied the distribution of SULT1A1 activity using *p*-nitrophenol as a substrate and found wide individual variations<sup>3-6)</sup>. SULT1A1 is important not only for catalyzing the sulfating reaction of carcinogen, but also for metabolic detoxification and activation of therapeutic drugs. For example, this enzyme is involved in the metabolic pathways of acetaminophen<sup>7, 8)</sup>, *trans*-4-hydroxytamoxifen (OHT: detoxification)<sup>9-11)</sup>, and minoxidil (activation)<sup>7, 12)</sup>. Platelet SULT1A1 activity correlates well with the urinary excretion of acetaminophen sulfate<sup>13)</sup>. In addition, OHT concentrations in plasma and breast cancer tissue vary among breast cancer patients<sup>14, 15)</sup>, and the level of SULT1A1 activity is considered to have an effect on the effectiveness of therapeutic drugs.

OHT is one of the active metabolites of tamoxifen (TAM) that is mainly produced by CYP2D6<sup>16)</sup>, and thereafter catalyzed by SULT1A1 and excreted<sup>9-11, 17)</sup>. Although OHT has been identified as one of the major active metabolites in human serum with a circulation level of less than 10% of that attained by the parent drug, it is approximately 100 times more interactive with human estrogen receptors than TAM<sup>15, 18, 19)</sup>. Therefore, we considered that establishment of a method for measuring OHT sulfating activity would be useful.

The *SULT1A1* gene encodes 7 allozymes, *SULT1A1*\*1 to \*7<sup>20)</sup>. *SULT1A1*\*2 has been reported to be associated with low levels of 2-amino-1-methyl-6-phenylimidazo (4,5-*b*)pyridine (PhIP) sulfating activity, which lowers the risk of bladder cancer by suppressing metabolic activation of PhIP<sup>21)</sup>. Likewise, various SULT1A1 activities or the *SULT1A1*\*2 allele have roles in OHT pharmacokinetics and TAM therapeutic effects. However, the effects of *SULT1A1*\*2 sulfating activity have been reported to be different according to the substrate<sup>7)</sup>. Therefore, the sulfating activity of *p*-nitrophenol might not necessarily reflect that of other substrates.

The *SULT1A1*\*2 allele has been found in Japanese subjects and shown to lower

*p*-nitrophenol sulfating activity by using the recombinant SULT1A1 protein expressed in *Escherichia coli* (*E. Coli*)<sup>7)</sup>. However, the distribution of SULT1A1 activity in a Japanese population has not been reported. In the present study, we investigated the distribution of SULT1A1 activities in Japanese subjects using OHT as a substrate. Our results clarified the influence of *SULT1A1*\*2 allele toward OHT sulfating activity using human platelet cytosol as the SULT1A1 enzyme source and a recombinant SULT1A1 protein expressed in *E. Coli*. Further, we analyzed OHT sulfating activity on the basis of the specific amount of SULT1A1 protein and mutations other than \*2 in the *SULT1A1* gene.



## MATERIALS AND METHODS

### *Subjects and blood samples*

One hundred three unrelated healthy Japanese subjects (45 males, 58 females) were recruited from the Sekino Clinical Pharmacology Clinic (Tokyo, Japan). The mean  $\pm$  S.D. age of the subjects was  $28 \pm 10$  years old (range, 20-66 years). Clinical examination test results (*i.e.*, white blood cells, red blood cells, platelets, hemoglobin, alanine aminotransferase, aspartate aminotransferase, triglyceride, and blood sugar) showed no abnormalities and none of the subjects were receiving concurrent medication. Venous blood sampling was always performed at 10:00 AM. The subjects refrained from smoking and consuming alcohol for at least 24 hours prior, and also fasted, except for water, at least 6 hours prior to providing blood samples. Gender, age, and smoking habits were examined by questionnaire. All subjects gave written consent for participation in the study, after having been informed both verbally and in writing of the experimental procedure and purpose of the study. The protocol of the study was approved by the Ethics Committee of Sekino Clinical Pharmacology Clinic (Tokyo, Japan).

### *Platelet isolation and homogenization*

Blood samples (7 ml) were collected in Vacutainer<sup>®</sup> tubes (containing EDTA-2Na as an anticoagulant, TERUMO, Tokyo, Japan) and centrifuged at  $200 \times g$  for 10 minutes. Each supernatant was centrifuged at  $16\,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$ , after which the platelet pellets were isolated and homogenized as described previously<sup>22, 23</sup>. The total protein concentration in the platelet homogenates was measured with a BCA protein assay kit (PIERCE, Illinois, USA) using bovine serum albumin as the standard. The platelet homogenates were stored at below  $-80^{\circ}\text{C}$  until performing the enzyme reaction assays and Western blotting procedures.

### *SULT1A1 genotyping procedures*

Genomic DNA was isolated using an SX-8G (Precision System Science, Chiba, Japan). Genotyping for <sup>213</sup>Arg/His (\*2) and <sup>223</sup>Met/Val (\*3) of the *SULT1A1* gene was performed using a modified method previously described<sup>24</sup>.

### *Sulfating assay*

A sulfating assay with *trans*-4-hydroxytamoxifen (OHT; Sigma-Aldrich Inc., Missouri, USA) was performed for 90 minutes at 37°C in a total volume of 300 µl in the presence of a platelet homogenate sample (5-10 µg), 0.5 µM of [<sup>35</sup>S] 3'-phosphoadenosine 5'-phosphosulfate (PAPS; Perkin Elmer, Massachusetts, USA), and 25 µM of OHT. Following pre-incubation of the mixture without the substrate for 10 minutes at 37°C, the reaction was started by the addition of the substrate dissolved in dimethyl sulfoxide and was terminated by placement of the reaction vessel into an ice bath. Next, a barium precipitation method was performed as described previously<sup>23, 25</sup>. Sulfating activity was expressed as pmol of the substrate sulfated/hour (hr)/mg of protein. All assays were performed in triplicate and the results are expressed as the median ± S.D.

#### *Western blotting*

Recombinant SULT1A1\*1 and \*2 proteins were expressed in *E. coli* and purified as described previously<sup>7</sup>. The platelet homogenate and recombinant SULT1A1\*1 protein were separated using SDS-PAGE (11% polyacrylamide gel) and transferred to PVDF membranes. The membranes were probed with SULT1A1 specific rabbit anti-sera (ST1A3, 410, ME-4) and immuno-reactive proteins were visualized according to ECL<sup>TM</sup> Western blotting protocols (Amersham Bioscience, New Jersey, USA). The intensity of the immuno-detectable protein was analyzed using an LAS3000mini and MultiGauge software (FUJIFILM, Tokyo, Japan). Individual SULT1A1 quantities in a specific amount of platelet protein were standardized by the intensity of the SULT1A1\*1 protein (1 µg).

#### *Kinetics studies of OHT sulfation*

One microgram each of recombinant SULT1A1\*1 and \*2 proteins was used for an enzyme reaction that was performed for 30 minutes at 37°C in the presence of 0.5 µM [<sup>35</sup>S] of PAPS and 25 µM of OHT. Other sulfating activity assay procedures were performed as described in the *Sulfating assay* section. The enzyme kinetics of OHT were characterized using an Eadie-Hofstee plot. The mean apparent  $K_m$ s and  $V_{max}$ s values for OHT sulfation catalyzed by the allozymes were determined in the presence of 6 concentrations of OHT (5.0-50 µM).

*SULT1A1 sequencing analysis*

The promoter region containing the Sp1 and Ets binding sites (within 112 bases upstream of the transcriptional start site) and the entire coding region of the *SULT1A1* DNA sequence were analyzed using an ABI PRISM<sup>®</sup> 377 DNA Sequencer and sequencing analysis software<sup>™</sup> (Applied Biosystems, Tokyo, Japan). Sequence analysis was performed using samples with sulfating activity lower than 114.4 or higher than 608.3 pmol/hr/mg protein.

*Statistical analysis*

Statistical analysis was performed using a Mann-Whitney *U*-test or Kruskal-Wallis *H*-test followed by Bonferroni inequality to evaluate differences among the *SULT1A1* genotypes (*SULT1A1*\*1/\*1, \*1/\*2, and \*2/\*2). Statistical significance of the correlations was determined with a Spearman-rank correlation test. A *p* value of less than 0.05 was considered statistically significant. The software used for the statistical analysis was Microsoft Excel XP (Microsoft, Tokyo, Japan).

## RESULTS

The <sup>213</sup>His allele (\*2) was found infrequently (16.5%) and none of our Japanese subjects had the <sup>223</sup>Val allele (\*3). The genotype frequencies of *SULT1A1*\*1/\*1, \*1/\*2, and \*2/\*2 were 70.9%, 25.2%, and 3.9%, respectively.

*SULT1A1* activity was evaluated using OHT as a substrate and found to range from 63 to 1860 pmol/hr/mg protein, a difference of approximately 30-fold. The median  $\pm$  S.D. of *SULT1A1* activity in the 103 subjects was  $260 \pm 241$  pmol/hr/mg protein [Fig. 1]. A comparison of OHT sulfating activity among different *SULT1A1* genotypes showed that the values in subjects with *SULT*\*1/\*2 ( $221 \pm 113$  pmol/hr/mg protein, range 63-442, n=26) and *SULT*\*2/\*2 ( $124 \pm 66$  pmol/hr/mg protein, range 74-231, n=4) were significantly lower (Kruskal-Wallis *H*-test followed by Bonferroni inequality:  $P < 0.05$ ) as compared to subjects with *SULT*\*1/\*1 ( $303 \pm 267$  pmol/hr/mg protein, range 97-1859, n=73) [Fig. 2]. There were no significant differences in OHT sulfating activities in regards to sex, age, or smoking habit among the groups (Mann-Whitney *U*-test: data not shown).

The *SULT1A1* protein content in platelet cytosol was  $2.03 \pm 0.92$  (mean  $\pm$  S.D.,  $\times 10^{-4}$  arbitrary units/mg platelet protein, n=19), a variation of 7.1-fold. Further, a significant correlation was found between *SULT1A1* protein contents and OHT sulfating activities (Spearman-rank test: coefficient 0.49,  $R^2=0.37$ ) [Fig. 3]. A comparison of *SULT1A1* protein contents in platelet cytosol showed that there were no significant differences among the *SULT1A1* genotypes (*SULT*\*1/\*2,  $2.05 \pm 0.88$ , 1.18-3.59, n=7; *SULT*\*2/\*2,  $2.62 \pm 1.38$ , 0.795-3.81, n=4) and *SULT*\*1/\*1 ( $2.03 \pm 0.92$  median  $\pm$  S.D.,  $\times 10^{-4}$  arbitrary units/mg platelet protein, 0.54-3.49, n=19, Kruskal-Wallis *H*-test). However, a comparison of OHT sulfating activities between the different *SULT1A1* genotypes showed that the median  $\pm$  S.D values in subjects with *SULT*\*1/\*2 ( $148 \pm 94$ , 69-350, n=7,  $P=0.09$ ) and *SULT*\*2/\*2 ( $49 \pm 65$ , 30-168, n=4,  $P=0.003$ ) were lower (Kruskal-Wallis *H*-test followed by Bonferroni inequality) than that in subjects with *SULT*\*1/\*1 ( $208 \pm 147 \times 10^{-4}$  arbitrary units/mg platelet protein, 43-564, n=19) [Fig. 4].

$V_{max}/K_m$  values (0.15:  $K_m$  6.62,  $V_{max}$  0.98) for OHT sulfation by the recombinant *SULT1A1*\*2 protein decreased to about half as compared to those by the recombinant *SULT1A1*\*1 protein (0.30:  $K_m$  4.58,  $V_{max}$  1.37). Further, the *SULT1A1*\*2 allele was shown to decrease *SULT1A1* activity toward OHT using a recombinant protein. When considering *SULT1A1* protein content, OHT sulfating activity in subjects with the

\*1/\*2 and \*2/\*2 genotypes was found to decrease to 71% and 24%, respectively, as compared to subjects with the \*1/\*1 genotype.

Sequence analysis for the Sp1 and Ets binding sites (from -108 to -62) as well as the coding region in *SULT1A1* was performed for samples from 20 subjects in whom OHT sulfating activity was lower than 114.4 or higher than 608.3 pmol/hr/mg protein. A novel G148C mutation (heterozygous) was found in 1 of the subjects, who had the lowest level of OHT sulfating activity. The frequency of this mutation was 0.95% (n=2, heterozygous) in 105 Japanese volunteers.

## DISCUSSION

The *SULT1A1*\*2 and \*3 allele frequencies found in the present subjects were consistent with a previous report (16.8% and 0.0%, respectively)<sup>7</sup>. These frequencies in Japanese are lower than those reported for Caucasian (33.2% and 1.2%, respectively) and African-American (29.4% and 22.9%, respectively) subjects, while the frequency of the \*2 allele was higher than reported for Chinese subjects (8.0%)<sup>26</sup>.

SULT1A1 activity was evaluated using OHT as a substrate and found to range from 63 to 1860 pmol/hr/mg protein [260 ± 241 pmol/hr/mg protein (median ± S.D.)], which was a difference of approximately 30-fold and 15-fold, respectively, in 1 patient with an extremely high level of OHT sulfating activity. The individual differences in SULT1A1 activity were 50-fold greater between Caucasians and African-Americans (n=132)<sup>4,6</sup>, as compared to the present Japanese subjects (n=103). Sulfating activity in our subjects with the *SULT*\*1/\*2 and \*2/\*2 genotypes were 73% (P=0.002) and 41% (P=0.03), respectively, which were lower than that in the subjects with the \*1/\*1 genotype. Therefore, we considered *SULT1A1*\*2 to be a common mutant allele that causes a decrease in OHT sulfating activity.

The *SULT1A1*\*2 allele was found to significantly decrease OHT sulfating activity, which was similar to the result when *p*-nitrophenol was used as the substrate<sup>4,7</sup>. The  $K_m$  and  $V_{max}/K_m$  values of SULT1A1\*2 were shown to decrease to nearly half those of SULT1A1\*1. Further, the thermal stability of the SULT1A1\*2 protein is known to be low, based on the results of both *in vitro* and *ex vivo* experiments<sup>4,7</sup>. In the present study, the \*2 allele was shown to also be a factor that caused individual variations of OHT sulfating activity in our Japanese population. These observations may be related to the inter-individual variations seen regarding the pharmacologic effects of TAM in Japanese patients with breast cancer.

The SULT1A1 protein content in platelet cytosol was not different among *SULT1A1* genotypes. OHT sulfating activity in subjects with the *SULT1A1*\*1/\*2 and \*2/\*2 genotypes on the basis of SULT1A1 protein content were 71% (P=0.09) and 24% (P=0.003), respectively, which were lower when compared to subjects with the \*1/\*1 genotype. As a result, *SULT1A1*\*2 was shown to be an allele that has no effect on SULT1A1 expression levels, while it decreases the OHT sulfating activity of the SULT1A1 protein. These findings were consistent with the results when we used recombinant SULT1A1\*1 and \*2 proteins, as the  $V_{max}/K_m$  values for OHT sulfation by

SULT1A1\*2 decreased to nearly half as compared to that by SULT1A1\*1. Therefore, individual variations of OHT sulfating activity were determined by both SULT1A1 protein expression level and SULT1A1 activity.

SULT1A1 protein expression levels are not likely affected by a nuclear receptor agonist such as dexamethasone<sup>27, 28</sup>), thus the individual variations in SULT1A1 expression level were considered to be due to genetic factors rather than environmental. Even in the same genetic group SULT1A1\*1/\*1 group, SULT1A1 protein expression in platelet cytosol varied widely. We then attempted to find another *SULT1A1* genetic mutation that caused the wide difference in SULT1A1 protein expression level. Previously, it was reported that the Sp1 and Ets binding sites (from -108 to -62) are especially important for SULT1A1 promoter activity<sup>27, 28</sup>). In addition, because SULT1A1 activity also varied widely in the *SULT1A1\*1* genotype group, we analyzed genetic mutations that may have had an effect on SULT1A1 activity. We performed sequencing analysis with samples from 20 participants with the lowest (n=10) and highest (n=10) levels of sulfating activity. We found a G148C (Gly50Ser) novel mutation in an open reading frame of the SULT1A1 gene in 1 subject, who had the lowest level of OHT sulfating activity, on the basis of the SULT1A1 protein. Since this novel mutation existed in the sequence considered important for PAPS binding<sup>29,30</sup>), it might be a factor involved with decreased SULT1A1 activity, and further investigation is required to determine whether this mutation is related to decreased sulfating activity for OHT or PAPS.

The results of the present study clarified the following 2 points. First, there was a wide variety of OHT sulfating activity in our Japanese population. Second, the *SULT1A1\*2* allele was found to be a common mutant allele that decreased OHT sulfating activity. Since OHT is sulfated mainly by SULT1A1, analysis of OHT sulfating activity is considered useful for describing the relationship of OHT sulfating activity with TAM therapeutic effects. However, SULT1A1 protein expression was widely different in the same SULT1A1 genotype group, which may have been due to genetic background factors other than the *SULT1A1\*2* allele. We considered that the wide differences in SULT1A1 expression and activity beside those caused by the *SULT1A1\*2* allele require further study.

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## Figure legends

Fig. 1. Frequency distribution of *trans*-4-hydroxytamoxifen (OHT) sulfating activity in platelet cytosol from 103 Japanese subjects. The OHT sulfating activities ranged from 63 to 1860 pmol/hr/mg protein. *SULT1A1*\*1/\*1, \*1/\*2, and \*2/\*2 subjects are indicated as open, striped, and filled columns, respectively.

Fig. 2. Comparison of OHT sulfating activities (pmol/hr/mg protein) in different *SULT1A1* genotypes.

$P=0.007$  by Kruskal-Wallis *H*-test. \* $P=0.03$  and \*\* $P=0.002$  followed by Bonferroni test.

Fig. 3. Correlation between OHT sulfating activities and SULT1A1 protein contents in platelet cytosol ( $\times 10^{-4}$  arbitrary units/mg platelet protein). Each of the samples contained 50  $\mu\text{g}$  of platelet cytosol. All of them have *SULT1A1*\*1/\*1 genotype ( $n=19$ ). Individual SULT1A1 quantities in a specific amount of platelet protein were standardized by the intensity of the SULT1A1\*1 protein (1  $\mu\text{g}$ ).

Spearman's correlation coefficient 0.49,  $R^2=0.37$

Fig. 4. Comparison of OHT sulfating activities (pmol/hr/SULT1A1 arbitrary units) in different *SULT1A1* genotypes.

$P=0.04$  by Kruskal-Wallis *H*-test. \* $P=0.09$  and \*\* $P=0.003$  followed by Bonferroni test.