category "probable phototoxicity" as "Equivocal" was set (Peters and Holzhütter, 2002). Sugiyama, et al (1994) proposed a red blood cell hemolysis assay to predict phototoxicity of chemicals, and they classified photohemolysis into three categories, +, ± and -.

In this paper, we discuss the above two points for the measures, sensitivity, specificity and accuracy, and propose new measures for evaluating the relevance of an inter-laboratory validation study. We also construct an equation for their confidence intervals, which measure their precision of them (Altman, 2000a).

Methods

Definition for sensitivity, specificity and concordance

Table 1 shows a 2 by 2 table. Sensitivity is defined as the proportion of chemicals judged as positive by an alternative test in which the chemicals are identified as positive by an animal test. When data is summarized as in table 1, sensitivity is calculated by a / (a + b). Specificity is defined as the proportion of chemicals judged as negative by the alternative test in which the chemicals are identified as negative by the animal test. The measure is d / (c + d). Accuracy is defined as the proportion of a corresponding number of chemicals by the judgment of the alternative test in which all the chemicals are identified by the animal test. The measure is obtained as (a + d) / (a + b + c + d).

It is rarely noted that the values of these measures depend on the selected set of chemicals. If the toxicity of the selected chemicals in a validation study has only the strongest classes and the weakest classes, the values of these measures would be expected to be higher when the assessed alternative test has a good correlation to the targeted animal test. If the researchers conducting the validation study can select test chemicals before the experiments on the alternative test, they can control the measures. On the other hand, if they choose many middle class chemicals in the study, the measures may show an inferior result compared to our expectation. Even if the chemicals are selected by an external person not directly involved in the study, the values of these are dependent on the selected chemicals. Thus, we should interpret the values of these as conditional proportions dependent on the set of selected chemicals in the study.

Motivated data

Table 2 shows a typical form of data from a validation study. The symbols "P", "E" and "N" in the Table mean "Positive", "Equivocal" and "Negative" to be judged by *In vivo* test or the al-

Table 1. The 2 by 2 table.

		Animal test	
		Positive	Negative
Alternative test	Positive	a	c
	Negative	b	d
		a+b	c+d

Table 2. A motivated example of a inter-laboratory validation study.

Chemical	In vivo	Laboratory					
		a	b	c	d	e	f
A	P	P	E	E	P		
B	P	P	N	P	E		
C	N	P	P	P	P		
D	P	P	E			E	P
E	N	P	P			P	P
F	N	N	P			N	N
G	P			P	P	P	P
H	N			N	E	E	E
I	N			N	N	N	N

Symbols: P, positive; E, equivocal; N, negative

ternative test. This data is from an actual validation study conducted in Japan which has not been published yet. In the study, nine chemicals were tested by six laboratories. In order to meet an increasing demand for assessing test chemicals, the laboratories used the alternative test for as many chemicals as possible. However, due to time and financial constraints, all the laboratories did not experiment applying the alternative test for all the chemicals. In view of animal welfare, data from animal tests is usually obtained from some published articles and/or databases including data from past experiments; animal tests are rarely conducted in validation studies. Therefore there is usually only one result for each chemical. On the other hand, some results for each chemical in an alternative test are obtained from the inter-laboratory study.

When the measures, sensitivity, specificity and accuracy, are calculated, data, as in Table 2, is summarized by a 2 by 2 table, in which a result from a chemical in a laboratory for an alternative test corresponds to a result from using the same chemical in an animal test; total for four cells in the 2 by 2 table is 36 as is the case in Table.

Consideration of two points

Furthermore, in addition to the fact that the measures are a conditional proportion of a set of chemicals, we also have to consider that these depend on the number of laboratories conducting inter-laboratory validation studies. However, when data is summarized by a 2 by 2 table, as in Table 2, distinguishing between the two factors, the set of selected chemicals and the number of participant laboratories is overlooked. Then the interpretation of the value is difficult. For instance, the sensitivity from a laboratory which has examined ten positive chemicals is 100% when all the chemicals are judged positive. The sensitivity from the ten laboratories which examined a positive chemical is also 100% when all laboratories judge positive for the chemical. Should we regard both sensitivities as the same? Some people often use only the values of these measures from different validation studies without taking into consideration these factors, when they compare the alternatives.

The presence of an "Equivocal" category is another difficulty involved in interpreting the measures. Since these measures are based on the assumption that the results of both tests are expressed as binary categories, often data for "Equivocal" is artificially changed: these are eliminated from the numerator; data for "Equivocal" is relabeled as "Positive" (e.g. Sugiyama et al., 1994). The value of the measures depends on which treatment is used.

Proposed methods

We propose similar measures to sensitivity, specificity and accuracy, which take into consideration and deal with the previous two points.

Firstly, we consider the relationship between two factors; chemical and laboratory. Since several laboratories experiment using the alternative test for a same chemical in the inter-laboratory validation study, data from the validation study has a hierarchical structure between two factors. In the proposed methods, the factor of chemical becomes a basic unit.

Suppose y_{ij} is a variable to explain the result from an alternative test, and x_i is a variable to explain the result from an animal test, where subscript i and j mean the i th chemical (i = 1, 2, ..., n) and the j th laboratory (j = 1, 2, ..., m_i) respectively. The variable y_{ij} take 1 for the "Positive" result, 0 for the "Negative" and 0.5 for the "Equivocal", when the alternative test is experimented for the i th chemical in the j th laboratory. The variable x_i is 1 for the "Positive" result of the targeted animal test, and 0 for the "Negative" result. We initially define p_i as a proportion for the number of positive results in the i th chemical for the alternative test, that is

$$p_i = \sum_j y_{ij} / m_i . \qquad (1)$$

As shown the appendix A, we can calculate the variance, $V(p_i)$, based on the assumption of trinomial distribution.

Using p_i, we also define q_i as

$$q_i = x_i p_i + (1 - x_i)(1 - p_i). \qquad (2)$$

Note that q_i is a measure for the reliability of the i th chemical. The alternative test shows good reliability when the value of q_i is close to 1.

Finally, we define three measures which correspond to sensitivity, specificity and accuracy, using p_i, and call these measures *Psn*, *Psp* and *Pac*, respectively;

$$Psn = \sum_i x_i p_i / \sum_i x_i \qquad (3)$$

$$Psp = \sum_i (1-x_i)(1-p_i) / \sum_i (1-x_i),$$

and (4)

$$Pac = \sum_i q_i / n = \left(\sum_i x_i p_i + \sum_i (1-x_i)(1-p_i) \right) / n \quad (5)$$

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(Psn)*, *V(Psp)* and *V(Pac)* (Appendix B). Furthermore we can construct the 95% confidence interval as follows;

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

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

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

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%(18/36) 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, *Psn*, *Psp* and *Pac* 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 *Psn*, *Psp* and *Pac*, 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(Psn)*, *V(Psp)* and *V(Pac)* 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, *Psn*, *Psp* and Pac, 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 $\left(r_i^{(N)}, r_i^{(E)}, r_i^{(P)}\right)$ follows a trinomial distribution with parameters, m_i, $\pi_i^{(N)}$, $\pi_i^{(E)}$ and $\pi_i^{(P)}$:

$$\left(r_i^{(N)}, r_i^{(E)}, r_i^{(P)}\right) \sim tri\left(m_i, \pi_i^{(N)}, \pi_i^{(E)}, \pi_i^{(P)}\right), \tag{A1}$$

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

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

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

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

$$\begin{aligned} E\left[\sum_i y_i / m_i\right] &= 0 \times \pi_i^{(N)} + .5 \times \pi_i^{(E)} + 1 \times \pi_i^{(P)} \\ &= .5 \times \pi_i^{(E)} + 1 \times \pi_i^{(P)}. \end{aligned} \tag{A3}$$

The variance is:

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

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:

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

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

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:

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

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

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

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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.

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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 ± 241 pmol/hr/mg protein, median ± S.D.) using OHT as a substrate. The median values in subjects with *SULT*1/*2* (221 ± 113 pmol/hr/mg protein, range 63-442, n=26) and *SULT*2/*2* (124 ± 66 pmol/hr/mg protein, range 74-231, n=4) were significantly lower than that in subjects with *SULT*1/*1* (303 ± 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[1]. 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[2].

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[3-6]. 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[7, 8], *trans*-4-hydroxytamoxifen (OHT: detoxification)[9-11], and minoxidil (activation)[7, 12]. Platelet SULT1A1 activity correlates well with the urinary excretion of acetaminophen sulfate[13]. In addition, OHT concentrations in plasma and breast cancer tissue vary among breast cancer patients[14, 15], 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[16], and thereafter catalyzed by SULT1A1 and excreted[9-11, 17]. 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[15, 18, 19]. 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*[20]. *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[21]. 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[7]. 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*)[7]. 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 ± S.D. age of the subjects was 28 ± 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® tubes (containing EDTA-2Na as an anticoagulant, TERUMO, Tokyo, Japan) and centrifuged at 200 x *g* for 10 minutes. Each supernatant was centrifuged at 16 000 x *g* for 10 minutes at 4°C, after which the platelet pellets were isolated and homogenized as described previously[22, 23]. 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°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 ^{213}Arg/His (**2*) and ^{223}Met/Val (**3*) of the *SULT1A1* gene was performed using a modified method previously described[24].

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 [^{35}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[23, 25]. Sulfating activity was expressed as *p*mol 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[7]. 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™ 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 [^{35}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_ms 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® 377 DNA Sequencer and sequencing analysis software™ (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 ^{213}His allele (*2) was found infrequently (16.5%) and none of our Japanese subjects had the ^{223}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 ± S.D. of SULT1A1 activity in the 103 subjects was 260 ± 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 ± 113 pmol/hr/mg protein, range 63-442, n=26) and *SULT*2/*2* (124 ± 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 ± 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 ± 0.92 (mean ± S.D., x10^{-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 ± 0.88, 1.18-3.59, n=7; *SULT*2/*2*, 2.62 ± 1.38, 0.795-3.81, n=4) and *SULT*1/*1* (2.03 ± 0.92 median ± S.D., x10^{-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 ± S.D values in subjects with *SULT*1/*2* (148 ± 94, 69-350, n=7, P=0.09) and *SULT*2/*2* (49 ± 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 ± 147 x10^{-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)[7]. 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%)[26].

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)[4, 6], 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[4, 7]. 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[4, 7]. 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[27, 28], 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[27, 28]. 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[29, 30], 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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