Table II Multiple Regression Analysis on the Relationships Between the *in Vivo* Indices of CYP2C19 (Omeprazole Hydroxylation Index) or CYP3A Activity ($CL_{cortisol\ 6\text{-}HC}$) and Patients' Covariates in Patients With Chronic Liver Diseases and Healthy Controls

	Versus Omeprazole Hydroxylation Index n = 61 R^2 = .7300			Versus $CL_{cortisol\ 6\text{-}HC}$ n = 61 R^2 = 3088		
Variable	Partial Regression Coefficient	*P*	Standardized Partial Regression Coefficient	Partial Regression Coefficient	*P*	Standardized Partial Regression Coefficient
Disease condition[a]	13.792	<.0001	0.612	−0.956	.0491	−0.420
Age	0.014	.8377	0.020	−0.012	.2761	−0.169
Sex	0.031	.9869	0.001	−0.277	.3673	−0.116
Albumin	−8.026	.0095	−0.344	−0.501	.3022	−0.213
Total bilirubin	0.641	.2718	0.115	−0.007	.9401	−0.013
AST	−0.090	.3175	−0.268	−0.013	.3589	−0.393
ALT	0.052	.3862	0.162	0.011	.2772	0.326
Serum creatinine	−2.870	.4739	−0.064	−0.405	.5310	−0.090
CYP2C19 genotype[b]	11.097	<.0001	0.403	0.083	.8171	0.030

AST, asparate aminotransferase; ALT, alanine aminotransferase.
a. Disease condition implies patients with chronic hepatitis (n = 9), patients with liver cirrhosis (n = 22), and healthy controls.
b. CYP2C19 genotype implies 3 genotypes: homozygous extensive, heterozygous extensive, and poor metabolizer.

DISCUSSION

The present study is the first to investigate the influence of CLD on the *in vivo* CYP2C19 activity in patients having different genotypes of CYP2C19 using the 3-hour omeprazole hydroxylation index (ie, 3-hour postdose plasma OPZ/5OH-OPZ concentration ratio). We demonstrated a dramatic reduction in this biomarker of *in vivo* CYP2C19 activity in CLD patients with the homozygous and heterozygous EM genotypes to the extent that the activity became comparable to the level of healthy subjects with the PM genotype (Figure 1). Because subjects with the PM genotypes for CYP2C19 have no functional CYP2C19 enzymes, our findings are compatible with the notion that the expression of CYP2C19 in the homozygous or heterozygous EM patients with CLD should have been suppressed to an almost null level. Because CYP2C19 is involved in the hepatic metabolism of many therapeutically important drugs other than omeprazole, patients with CLD may be susceptible to adverse reactions from taking drugs that depend on CYP2C19 activity for metabolism. In addition, CLD patients with the PM genotype had a significantly ($P < .05$) higher hydroxylation index than those with the homozygous EM genotype. Because the omeprazole hydroxylation index has a reciprocal relationship with the *in vivo* CYP2C19 activity, whether this statistically significant difference is associated with a clinically relevant difference in dose requirement remains to be studied.

Our data show a good agreement with the previous studies in patients with CLD[1,2] that demonstrated a preferential reduction (77% from the baseline) of CYP2C19 activity assessed by the hydroxylation of mephenytoin compared with that of CYP3A4 (28%) and CYP2D6 (4%) assessed by hydroxylation of dapsone and debrisoquine, respectively. In the present study, the hydroxylation index of omeprazole in CLD patients with the homozygous EM genotype increased more than 10-fold than that of the healthy subjects with the same genotype, indicating a marked reduction in CYP2C19 activity associated with CLD. It is of note that the majority of our CLD patients had only mild liver dysfunction (9 had chronic hepatitis, and 20 of 22 cirrhotic patients were Child-Pugh type A), indicating that compared with other CYP isoforms (eg, CYP3A4), the hepatic CYP2C19 activity is more susceptible to CLD. This degree of reduction is in agreement with the report of Andersson et al.[23] In contrast, the index of *in vivo* CYP3A activity, $CL_{cortisol\ 6\text{-}HC}$, obtained from CLD patients decreased to only a half of the control value.

Kimura et al[24] studied the omeprazole hydroxylation index in Japanese healthy subjects and patients with gastrointestinal disease and found that the hy-

droxylation indices obtained from 3 CLD patients with CYP2C19*1/*1 (n = 1) or CYP2C19*1/*2 (n = 2) were comparable with those observed in their healthy controls with the PM genotypes. Because they studied only 3 CLD patients under multiple dosing of the drug, they were unable to conclude whether their finding was attributable to a CLD-induced reduction of CYP2C19 activity or to partial saturation of the enzyme by multiple dosing. Our data confirm that the dramatic increase of the omeprazole hydroxylation index in CLD patients does represent a substantial reduction in the CYP2C19 activity in CLD patients. Nevertheless, the precise mechanism associated with the preferential reduction of this CYP isoform remains obscure at present.

We found that the omeprazole hydroxylation index obtained from CLD patients with the PM genotypes was further increased as compared with healthy subjects having the same genotype. Our data suggest that hepatic drug metabolizing enzyme(s) other than CYP2C19 may be involved in the *in vivo* 5-hydroxylation of omeprazole because both groups should have had no functional CYP2C19 enzyme. Our data are consistent with the study of Furuta et al,[10] demonstrating that the area under the plasma concentrations (AUC) ratio of OPZ/5OH-OPZ in healthy subjects with PM genotypes coadministered the CYP3A4 inhibitor, clarithromycin, was 4 times greater than the value obtained from the same subjects given omeprazole alone. In the present study, we observed that the mean value of a putative index of CYP3A activity, $CL_{cortisol\ 6\text{-}HC}$, was approximately 50% lower in CLD patients than in control subjects. The data of Furuta et al[10] and of our study collectively suggest that CYP3A4 may be involved in part in the 5-hydroxylation of omeprazole.

There are certain limitations in the present study. We assigned the phenotypes of CYP2C19 based solely on the genotyping of the 2 major mutations (CYP2C19*2 in exon 5 and CYP2C19*3 in exon 4). We did not genotype other rare variant alleles (eg, from CYP2C19*4 to CYP2C19*12[25,26]) that have been reported in non-Asian populations. However, because these 2 variant alleles have been shown to account for 98% of the PM phenotype in the Japanese population,[6,7] we consider our assignment of the CYP2C19 phenotype to be valid. In addition, we evaluated the in vivo CYP2C19 activity based on the plasma omeprazole hydroxylation index at 3-hour postdose after a single oral load of 20 mg. Therefore, we cannot totally deny the possibility that an interindividual variability in the intestinal drug absorption may have jeopardized an accurate assessment of the enzyme activity. However, Andersson et al[23] and Renetti et al[27] reported that time required to reach peak plasma concentration of the drug was less than 3 hours after oral administration. We therefore consider that the 3-hour postdose hydroxylation index would be a robust *in vivo* index of CYP2C19 activity.

On the other hand, omeprazole is also known to be metabolized to omeprazole sulfone *via* CYP3A, and omeprazole sulfone is further metabolized to 5-hydroxyomeprazole sulfone via CYP2C19.[28,29] In PMs of CYP2C19, the metabolic pathway of omeprazole to omeprazole sulfone is supposed to be enhanced, resulting in an accumulation of the sulfone metabolite. However, Ieiri et al[22] have demonstrated that PM status does not elevate the omeprazole to omeprazole sulfone ratio (1.8 to 2.5–3; not significant) or change the AUC ratio (1.8 to 0.8–0.9) compared with the EM status. Their results suggest that the change in omeprazole hydroxylation index is mostly because of a change in CYP2C19 activity, with a minor contribution from CYP3A4, even in those with PM status. Because we did not measure the sulfone level, it remains unknown how liver damage modifies the metabolism of omeprazole in PMs and heterozygous EMs.

Many factors including gender,[30,31] concomitant medication,[24,28,29] age,[32] liver disease,[23,24,27] and length of the therapy[33] have been reported to affect the *in vivo* metabolic activity of CYP2C19, thus confounding a simple relationship between genotypes and phenotypes. The multiple regression analysis performed in the present study revealed that age and gender were not associated with the reduction in *in vivo* CYP2C19 activity. However, caution has to be exercised in interpreting these results because the mean age of CLD patients was significantly higher than that of healthy controls (Table 1) and the relationship between age and CYP2C19 activity might not have been linear as assumed by the analysis. Several reports[34-36] have demonstrated a significant correlation between the *in vivo* CYP2C19 activity and certain biochemical parameters associated with liver function (albumin, transaminase, and prothrombin index). The multiple regression analysis in the present study revealed that only serum albumin level, disease condition (ie, presence or absence of CLD), and genotype may explain the decrease of *in vivo* CYP2C19 activity.

We found that the mean $CL_{cortisol\ 6\text{-}HC}$ obtained from CLD patients was decreased to 48% of that obtained from the healthy subjects. Because CYP3A isoforms (eg, CYP3A4 and CYP3A5) are primarily responsible for 6 -hydroxylation of the endogenous cortisol, $CL_{cortisol\ 6\text{-}HC}$ may be a clinically useful tool as an *in vivo*

parameter of CYP3A activity. We have already reported that a 3-hour $CL_{cortisol\ 6\text{-}HC}$ is a better clinical index of in vivo CYP3A activity than is the traditionally employed ratio of urinary 6 -HC to cortisol for the assessment of the inhibitory effect of clarithromycin on CYP3A activity in patients receiving *Helicobacter pylori* eradication therapy.[18] An oral administration of clarithromycin at 800 mg/d reduced $CL_{cortisol\ 6\text{-}HC}$ to approximately 50% of the baseline value. The present finding that the mean $CL_{cortisol\ 6\text{-}HC}$ of CLD patients was reduced by approximately 50% compared with healthy controls implies that the magnitude of reduction in *in vivo* CYP3A activity induced by CLD is largely comparable to that induced by clarithromycin. However, multiple regression analysis detected no significant correlation between $CL_{cortisol\ 6\text{-}HC}$ and biochemical parameters associated with liver function. Thus, there is controversy of whether the 6 -hydroxylation of cortisol is a reliable index of the *in vivo* hepatic CYP3A activity. It has been suggested that a certain amount of cortisol is secreted into the gut and is metabolized by the epithelial CYP3A on reabsorption. It is possible that CYP3A5 is expressed in the kidneys. Thus, the overall catalytic activity of cortisol 6 -hydroxylation may be attributed not only to the hepatic CYP3A activity but also to certain extrahepatic tissues (eg, the kidney and the small intestine).[37,38] This may be a reason we did not obtain any significant correlation between $CL_{cortisol\ 6\text{-}HC}$ and the biochemical parameters of hepatic function. In addition, there is a concern that omeprazole administered to CLD patients and control subjects might have affected the CYP3A4 activity measured by $CL_{cortisol\ 6\text{-}HC}$. However, Furuta et al[39] reported in their *in vitro* study performed with human liver microsomes that omeprazole is 50 times weaker as an inhibitor for CYP3A4 than for CYP2C19, and Tateishi et al[40] demonstrated that omeprazole did not affect *in vivo* erythromycin breath test, an established index of CYP3A activity, in healthy subjects. In this context, we consider that the administration of omeprazole did not interfere with $CL_{cortisol\ 6\text{-}HC}$.

In conclusion, the present study demonstrated that the impaired CYP2C19 and CYP3A activity in CLD patients may be reflected by an increase in the omeprazole hydroxylation index and a decrease in $CL_{cortisol\ 6\text{-}HC}$, respectively. It is interesting that CLD appears to induce preferential reduction in CYP2C19 activity compared with CYP3A. The omeprazole hydroxylation index of CLD patients with the EM genotype of CYP2C19 was largely comparable to that of control subjects with the PM genotype. Further studies are required to assess whether the *in vivo* index of CYP2C19 may be useful for dosage adjustment of drugs that are eliminated mainly via CYP2C19-mediated hepatic metabolism.

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Interaction magnitude, pharmacokinetics and pharmacodynamics of ticlopidine in relation to *CYP2C19* genotypic status

Ichiro Ieiri[a], Miyuki Kimura[b], Shin Irie[b], Akinori Urae[b], Kenji Otsubo[a] and Takashi Ishizaki[c]

Objectives The aim of this study was to investigate the impact of *CYP2C19* polymorphism on the extent of the interaction and on the pharmacokinetics and pharmacodynamics of ticlopidine.

Methods Homozygous (hmEMs) and heterozygous extensive metabolizers (htEMs), and poor metabolizers (PMs, $n=6$ each) took an oral dose (20 mg) of omeprazole. After a 1-week washout period, each subject received ticlopidine (200 mg) for 8 days, and ticlopidine pharmacokinetics were studied on days 1 and 7. On day 8, omeprazole was given again and its kinetic disposition was compared with that in the first dose. ADP-induced platelet aggregation was measured as a pharmacodynamic index.

Results In contrast to the PMs, whose mean kinetic parameters were not altered by the repeated dosings of ticlopidine, an eight- to 10-fold increase in the mean AUC ratio of omeprazole to 5-hydroxyomeprazole was observed in both the EM groups. No significant intergenotypic differences in the pharmacokinetic parameters of ticlopidine were observed, although the accumulation ratio tended to be greater in hmEMs than in PMs (2.4 ± 0.2 versus 1.7 ± 0.2). A significantly positive correlation ($P=0.031$) was observed between the individual percent inhibition of platelet aggregation and AUC_{0-24} of ticlopidine regardless of the *CYP2C19* polymorphism.

Conclusions Ticlopidine is a potent inhibitor for CYP2C19 and may be associated with the phenocopy when CYPC19 substrates are co-administered to EMs. Whether and to what extent CYP2C19 would be involved in the metabolism of ticlopidine remain unanswered from the present in-vivo study. *Pharmacogenetics and Genomics* 15:851–859

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[a]Department of Hospital Pharmacy, Faculty of Medicine, Tottori University, Yonago, Japan, [b]Kyushu Clinical Pharmacology Research Clinic, Fukuoka, Japan and [c]Department of Clinical Pharmacology and Pharmacy, Teikyo Heisei University School of Pharmaceutical Science, Ichihara, Japan.

Correspondence and requests for reprints to Ichiro Ieiri, PhD, Department of Hospital Pharmacy, Faculty of Medicine, Tottori University, Nishi-machi 36-1, Yonago, 683-8504, Japan.
Fax: +81-859-34-8087; e-mail: ieiri-ttr@umin.ac.jp

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Introduction

Ticlopidine is the first of the thienopyridine antiplatelet agents, known as adenosine diphosphate (ADP) receptor antagonists [1]. Studies have demonstrated that ticlopidine reduces the risk of thrombotic events in patients with stroke [2,3], and is effective for the treatment of unstable angina [4], myocardial infarction [5], and intermittent claudication [6]. Furthermore, in combination with aspirin, ticlopidine reduces thrombotic complications following coronary stent placement [7–9].

The pharmacokinetic behavior of ticlopidine has been mainly investigated in Caucasian subjects [10,11]. Although up to 90% of an oral dose is absorbed, ticlopidine is extensively metabolized in the liver, resulting in the formation of more than 13 metabolites, of which the 2-keto derivative is reported to inhibit platelet aggregation in rats [10]. Plasma ticlopidine concentrations [e.g., the peak concentration (C_{max}) and the area under the plasma concentration–time curve (AUC)] increase by approximately three-fold on repeated twice-daily dosings over 2 to 3 weeks [10,11], suggesting that an accumulation or saturation in the metabolism may occur during a repeated administration.

Studies *in vitro* and *in vivo* have documented that ticlopidine is an inhibitor of cytochrome P450 (CYP) enzymes (e.g., CYP2B6, 2C19, 2D6 and/or 2C9) [12–17]. Ko *et al.* [15] and Ha-Duong *et al.* [16] have reported that ticlopidine is an inhibitor of human CYP2C19, whereas Lopez-Garcia *et al.* [17] have reported that thiophene derivatives such as tienilic acid are mechanism-based inhibitors of yeast-expressed human liver CYP2C9. A more recent study by Richter *et al.* [13] has indicated that ticlopidine is a mechanism-based inhibitor of CYP2B6, as well as shows an inhibitory effect against CYP2C19.

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Tateishi *et al.* [18] have shown that ticlopidine inhibits the in-vivo activity of CYP2C19 using omeprazole as a model substrate in small number ($n = 6$) of *CYP2C19*-genotyped Japanese extensive, but not poor, metabolizers. In addition, two in-vitro studies using recombinant human liver CYPs have demonstrated that CYP2C19 and CYP3A4 are involved in the metabolism of ticlopidine [16,19].

With this background in mind, we designed the present study (1) to confirm the in-vivo inhibitory effect of ticlopidine on the metabolism of omeprazole mediated via CYP2C19 (i.e., omeprazole 5-hydroxylation) in 18 Japanese subjects including six poor metabolizers, and (2) to assess the pharmacokinetics and dynamics of ticlopidine administered in single and multiple doses in the same 18 *CYP2C19*-genotyped subjects. These two study aims are justified because it is possible that ticlopidine itself is a substrate of CYP2C19 and CYP3A4 as it undergoes extensive hepatic metabolism [10,16,19], although it is reasonable to suspect that ticlopidine inhibits multiple CYP isoforms including CYP2B6, 2C19, 2C9, 1A2 and 3A [12–18].

Methods

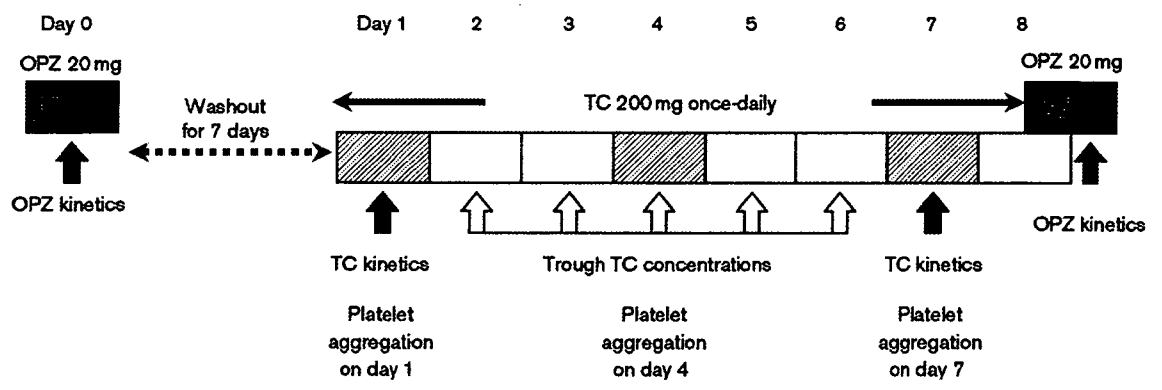
Subjects

Eighteen unrelated healthy volunteers identified as Japanese by lineage (age, 20~33 years; weight, 52.5~75.9 kg; body mass index; 18.5~24.9 kg/m^2) were enrolled. Subjects were genotypically classified into the following three groups on the basis of a PCR–restriction fragment length polymorphism analysis for *CYP2C19*: homozygous (*CYP2C19*1/*1*) extensive metabolizers (hmEMs, $n = 6$), heterozygous (*CYP2C19*1/*2*) extensive metabolizers (htEMs, $n = 6$), and poor metabolizers (PMs, *CYP2C19*2/*2*, $n = 4$ and *CYP2C19*2/*3*, $n = 2$). None had taken any drugs for at least 1 week before the study. Each subject was physically normal and had no antecedent history of significant medical illness or hypersensitivity to any drugs. The subjects' health status was judged to be normal on the basis of a physical examination with hepatic function screening, a complete blood cell count, serum creatinine analysis, urinalysis, a platelet aggregation test, and an electrocardiogram before the study. The study subjects included nine nonsmokers and nine smokers (< 10 cigarettes per day). Smokers were not allowed to smoke during the period of hospitalization. All subjects were required to abstain from alcohol 2 days before the drug administration and during the period of hospitalization. The subjects were served standard meals on the study days. To assess the safety of the drugs studied, we evaluated spontaneous adverse events reports and conducted electrocardiogram recordings, laboratory safety evaluations (hematology, blood chemistry, and urinalysis), and immunological tests (antinuclear antibody, and anti-liver–kidney microsome antibody type 1 and type 2), before and after the trial phases. The study protocol had been approved in advance by the ethics review board of Kyushu Clinical Pharmacology Research Clinic, Fukuoka, Japan, and each subject gave their written informed consent before the study.

Study protocol

The protocol is summarized in Fig. 1. The participants came to the clinical research site on the day before the study, and after an overnight fast, each subject was administered a single oral 20-mg dose of omeprazole (Omepral, AstraZeneca Co. Ltd., Osaka, Japan) with 150 ml of water (day 0). Food was given 4 h after the ingestion of omeprazole. After a wash-out period of 1 week, each subject was administered a once-daily 200-mg dose of ticlopidine (two tablets of 100 mg Panaldine, Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan) with 150 ml of water at 8 am after a standard breakfast on day 1

Fig. 1



Schema of the study design. Subjects received a single dose of omeprazole (OPZ). After a 7-day washout period, they received a once-daily 200-mg dose of ticlopidine (TC) for 8 days. On day 8, an oral 20-mg dose of omeprazole was administered again at 1 h after the administration of the oral 200-mg dose of ticlopidine.

through day 8. Ticlopidine pharmacokinetics were studied on days 1 and 7. On day 8, an oral 20-mg dose of omeprazole was administered at 1 hour after the administration of the oral 200-mg dose of ticlopidine. Venous blood samples (4 ml each) for determining omeprazole, 5-hydroxyomeprazole and ticlopidine concentrations were obtained just before and 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after the administration. In order to assess the accumulation of ticlopidine, trough concentrations were measured just before the ticlopidine administration on day 2 through day 8. The plasma samples were immediately separated after centrifugation and stored at −20°C until analyzed.

Platelet aggregation

Blood samples (4.5 ml each) were obtained with a 21-gauge needle by direct venepuncture and drawn into vacuum tubes containing sodium citrate for measuring platelet aggregation. The vacuum tube was filled to capacity and gently inverted five times to ensure complete mixing of the anticoagulant, and then centrifuged at 90*g* for 10 minutes at 22°C in order to obtain platelet-rich plasma (PRP). The rest of the samples were centrifuged at 1630*g* for 10 minutes at 22°C in order to obtain platelet-poor plasma (PPP). Platelet aggregation was determined five times during the study period; at the screening visit, on days 1, 4 and 7 of the ticlopidine dosings, and at 14 to 20 days after discharge from the clinic for the monitoring of platelet recovery. All samples were taken immediately before lunch. Platelet aggregation was measured according to the method of Born [20] with a PA-200 instrument (Kowa Co. Ltd., Tokyo, Japan). Adenosine diphosphate (ADP, MC Medical Co. Ltd., Tokyo, Japan) was used as an aggregating agent. The extent of platelet aggregation was evaluated by measuring the maximal extension of the aggregation curve 5 min after the addition of 5 μM ADP.

Analytical methods

Plasma concentrations of ticlopidine, omeprazole, and 5-hydroxyomeprazole were measured using the respective validated liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) methods as described below.

For the ticlopidine analysis 10 μl of methanol and 20 μl of internal standard (mianserin, 1000 ng/ml in methanol) were added to 0.1 ml of plasma. The analytes were extracted into *n*-hexane (6 ml) from an alkaline pH (0.2 ml of 1 mol/l NaOH), then back-extracted into 0.1 M HCl (0.5 ml). Aliquots (10 μl) were injected into the LC/MS/MS system which consisted of an API3000 system (AB/MDS Sciex, Foster City, California, USA) and Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, California, USA).

For the omeprazole and 5-hydroxyomeprazole analyses, 0.1 ml of plasma was mixed with 10 μl of methanol, 10 μl of a methanolic solution with 1000 ng/ml of phenacetin as an internal standard and 0.1 M phosphate buffer (pH 7.5, 0.6 ml), and then applied to a solid-phase extraction cartridge (Oasis HLB, 30 mg/ml, Waters, Milford, Massachusetts, USA). These cartridges were then washed with 1.0 ml of 15% methanol, and the sample was eluted with 3 ml of methanol. The eluate was evaporated to dryness under a gentle stream of nitrogen at 40°C, and the residue was reconstituted in 0.2 ml of a high pressure liquid chromatography mobile phase consisting of 10 mM ammonium formate (pH 8.5)/acetonitrile (75:25 [vol/vol]). After sonication and centrifugation, the supernatant was filtered through Ultrafree (0.45 μm, PTFE, Millipore, Bedford, Massachusetts, USA), and an aliquot of the filtrate (5 μl) was injected into the LC/MS/MS system.

Chromatographic separation of ticlopidine was performed on a Luna C18(2) column (3 μm, 2.0 50 mm; Phenomenex, Torrance, California, USA) with a Security Guard C18(ODS) column (2.0 4 mm, Phenomenex) at 30°C using a mobile phase consisting of 0.1% acetic acid/acetonitrile (80:20 [vol/vol]) at a flow rate of 0.20 ml/min. Omeprazole and 5-hydroxyomeprazole were separated on a Xterra MS C18 column (3.5 μm, 2.1 50 mm; Waters) at 40°C using a mobile phase consisting of 10 mM ammonium formate (pH 8.5)/acetonitrile (75:25 [vol/vol]) at a flow rate of 0.2 ml/min.

Detection was carried out in the electrospray ionization (TurboIonSpray) mode with multiple reaction monitoring using the API3000 system. The monitor ions were *m/z* 264→154 (Q1→Q3) [−25 eV] for ticlopidine, *m/z* 265→208 (Q1→Q3) [−29 eV] for mianserin, *m/z* 346→198 (Q1→Q3) [−17 eV] for omeprazole, *m/z* 362→214 (Q1→Q3) [−15 eV] for 5-hydroxyomeprazole, and *m/z* 180→138 (Q1→Q3) [−23 eV] for phenacetin. Calibration was performed with blank plasma samples spiked with the respective standard substances. Calibration curves were constituted from the ratio of peak area relative to the internal standard using Analyst version 1.1 (AB/MDS Sciex) with weighted (1/*x*) linear regression in a range of 0.5–500 ng/ml for ticlopidine and with weighted (1/*x*) quadratic regression in a range of 5–500 ng/ml for omeprazole and 5-hydroxyomeprazole. The accuracy in four of six quality control samples prepared using blank human plasma was within ±20%. The plasma samples exceeding the quantitation range were diluted 10-fold with blank human plasma.

Pharmacokinetic analysis

Peak concentrations (C_{max}) and time of maximum concentration (T_{max}) were obtained directly from the data. Pharmacokinetic analysis was performed in a model-independent manner, and non-compartmental kinetic parameters were calculated using standard methods. The area under the observed concentration–time curve from

time 0 to 24 h (AUC_{0-24}) was calculated with the linear trapezoidal rule. The elimination rate constant (k_e) was estimated using the least regression analysis from the terminal post-distribution phase of the concentration-time curve. The terminal half-life ($t_{1/2}$) was calculated by dividing 0.693 by the elimination rate constant. The AUC from 0 h to infinity ($AUC_{0-\infty}$) was calculated as follows: $(AUC_{0-\infty}) = (AUC_{0-24}) + C_t/k_e$, in which C_t represents the last measured time point concentration. To assess the possibility of accumulation of ticlopidine with multiple dosings, the accumulation ratio, the AUC_{0-24} ratio of day 7 to day 1, was calculated.

Statistical analysis

The numerical values are given as the mean ± SE. Statistical differences in mean pharmacokinetic parameters for omeprazole, 5-hydroxyomeprazole and ticlopidine among the three *CYP2C19* genotype groups were determined by use of one-way ANOVA followed by the Scheffé multiple comparison test. Statistically significant differences in pharmacodynamic parameters among the three genotype groups were determined by use of the Mann–Whitney *U* test. Statistical differences in mean pharmacokinetic parameters of ticlopidine observed on days 1 and 7, and in the omeprazole/5-hydroxyomeprazole ratio before and after the multiple ticlopidine dosings were evaluated with the paired *t*-test. For correlation between % inhibition of platelet aggregation and AUC of ticlopidine, the Spearman rank correlation test was applied. All *P* values were two-sided; $P < 0.05$ was considered statistically significant.

Results

No clinically undesirable signs and symptoms possibly attributed to the administration of ticlopidine and/or omeprazole were recognizable throughout the study. According to the protocol, all subjects completed the study successfully.

Omeprazole pharmacokinetics with and without ticlopidine co-administration in relation to *CYP2C19* genotypic status

Plasma concentrations of omeprazole on day 0 were higher and those of 5-hydroxyomeprazole were lower in PMs compared with those in the two EM groups (Fig. 2a, b). However, these intergenotypic differences almost disappeared on the eighth day of ticlopidine administration (Fig. 2c, d). As observed from the change in AUC and in elimination half-life, the inhibition of omeprazole metabolism was significant in both the EM groups (Table 1). After the administration of ticlopidine for 1 week, the AUC values of omeprazole and 5-hydroxyomeprazole in the EMs were increased by three to six times and reduced by approximately 40%, respectively, resulting in the values similar to those in the PMs (Table 1). The $AUC_{0-\infty}$ ratio of omeprazole to 5-hydroxyomeprazole showed a significant reduction of 5-hydroxyomeprazole production in both the EM groups; in contrast, no significant changes were observed in PMs (Fig. 3).

Ticlopidine pharmacokinetics in relation to *CYP2C19* genotypic status

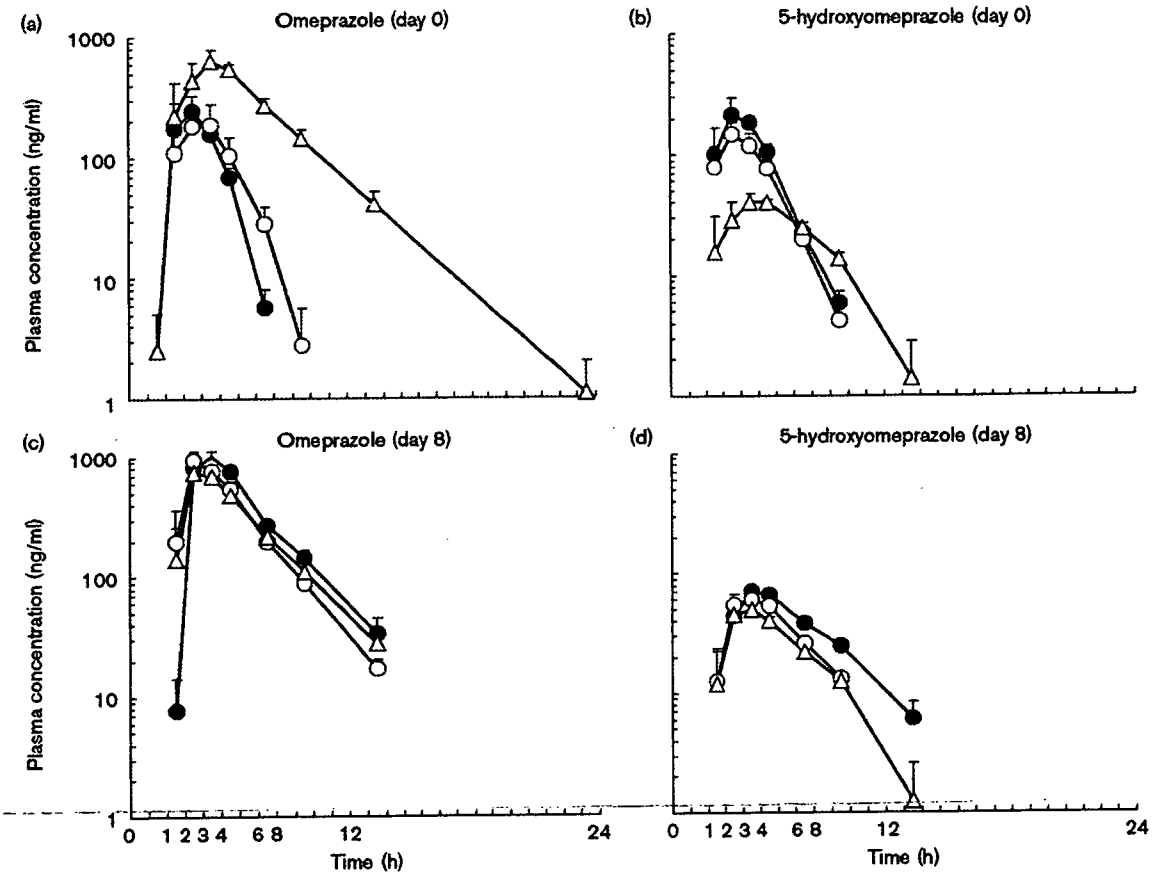
The mean plasma concentration–time curves of ticlopidine in relation to *CYP2C19* status on the first and seventh days of ticlopidine dosings is shown in Fig. 4. The pharmacokinetic parameters of ticlopidine are summarized in Table 2. Statistically significant intergenotypic differences in the mean pharmacokinetic parameters of ticlopidine were not observed on either of the study days. However, the mean (± SE) accumulation ratio of ticlopidine, the AUC_{0-24} ratio of day 7 to day 1, in the hmEMs, htEMs, and PMs was 2.4 ± 0.2, 2.0 ± 0.3, and 1.7 ± 0.1, respectively. Although the difference did not reach the level of significance, the accumulation ratio tended to be greater in the hmEM subjects compared with the htEM or PM subjects. The mean trough concentration data of ticlopidine from day 2 to day 8 are also shown in Fig. 4. Similar to the accumulation ratio, the mean trough levels tended to be higher in the hmEM subjects compared with the PM subjects, and the htEM subjects had the values between those in hmEM and PM subjects throughout the study period (see the inset of Fig. 4).

Pharmacodynamics of ticlopidine versus *CYP2C19* status

As a pharmacodynamic assessment of ticlopidine, ADP-induced platelet aggregation was measured on the first, fourth and seventh days of the 8-day dosing period and compared with the baseline values in the different *CYP2C19* genotypic groups. The mean maximum percentage values for ADP-induced platelet aggregation relative to the baseline values on the first, fourth and seventh post-dose days were: 77.3 ± 11.5, 55.0 ± 15.3 and 46.5 ± 14.3 in the hmEMs, 75.8 ± 8.7, 50.0 ± 7.7 and 42.5 ± 5.5 in the htEMs, and 77.0 ± 14.4, 59.8 ± 15.4 and 55.0 ± 17.2 in the PMs, respectively. Thus, the mean percentage inhibition of ADP-induced platelet aggregation on the first versus the fourth and seventh post-dose days increased with the repeated doses, irrespective of *CYP2C19* status: 29.5 ± 12.4% and 40.4 ± 12.4% in the hmEMs, 33.7 ± 10.6% and 43.7 ± 6.8% in the htEMs, and 21.3 ± 18.0% and 28.4 ± 17.2% in the PMs. The mean maximum and percentage inhibition values for ADP-induced platelet aggregation observed throughout the treatment period did not differ significantly among the genotypic groups.

The relationship between the individual percent inhibition of platelet aggregation and AUC_{0-24} values observed on the seventh day of ticlopidine dosing is shown in Fig. 5. The AUC_{0-24} of ticlopidine is significantly ($P = 0.031$) correlated with the percent inhibition of

Fig. 2



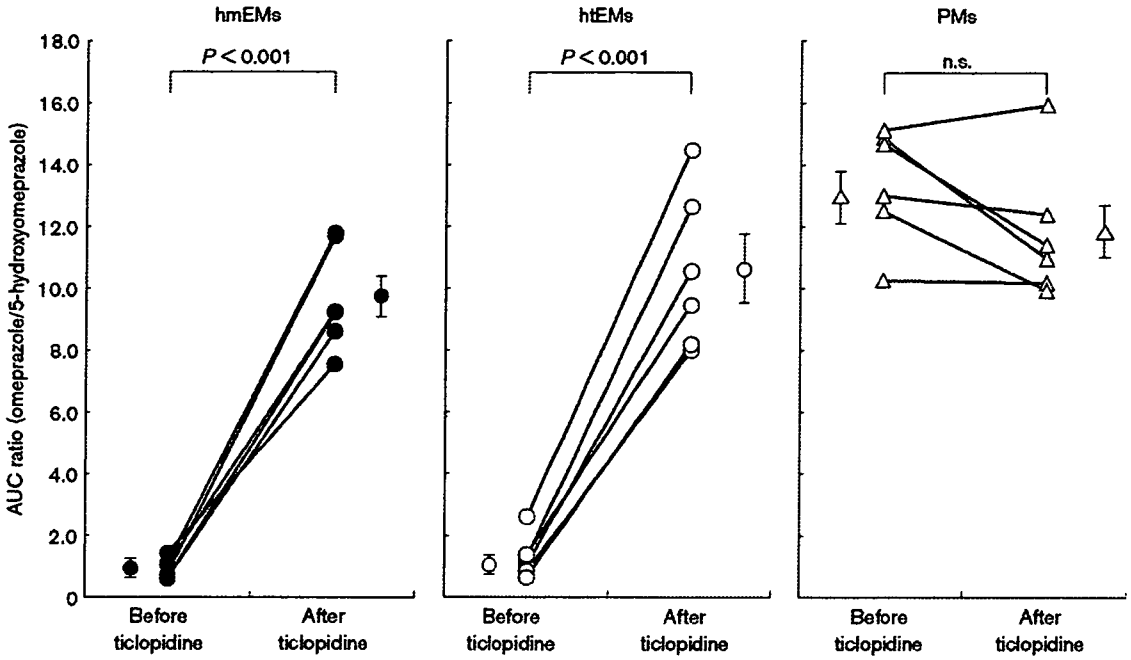
The mean plasma concentrations of omeprazole and 5-hydroxyomeprazole among homozygous extensive metabolizers (hmEMs, closed circles), heterozygous extensive metabolizers (htEMs, open circles) and poor metabolizers (PMs, triangles) after a single oral 20-mg dose of omeprazole before (day 0) and after (day 8) the concomitant intake of 200 mg/day ticlopidine.

Table 1 Pharmacokinetic data for omeprazole and 5-hydroxyomeprazole in the *CYP2C19* genotypic groups

	Before ticlopidine (day 0)			After ticlopidine (day 8)		
	Homozygous EMs ($n=6$)	Heterozygous EMs ($n=6$)	PMs ($n=6$)	Homozygous EMs ($n=6$)	Heterozygous EMs ($n=6$)	PMs ($n=6$)
Omeprazole						
C_{max} (ng/ml)	383.0±67.7	289.8±71.0	889.0±71.7**	1143.3±100.9††	978.1±62.1††	839.1±60.9
T_{max} (h)	2.0±0.4	2.3±0.4	2.7±0.4	2.8±0.3	2.7±0.3	2.5±0.2
$T_{1/2}$ (h)	0.7±0.1	0.7±0.1	2.1±0.2**	1.8±0.2†	1.6±0.1	1.9±0.2
$AUC_{0-\infty}$ (ng h/ml)	646.7±110.8	653.5±159.5	3284.4±288.8***	4023.2±255.2†††	3271.8±300.5†††	3208.9±252.6
5-hydroxyomeprazole						
C_{max} (ng/ml)	325.7±43.5	183.4±21.6	53.5±7.7***	76.3±6.6†††	71.5±2.7**	54.1±3.9
T_{max} (h)	2.2±0.3	2.3±0.4	3.2±0.7	3.0±0.4	2.7±0.3	2.3±0.3
$T_{1/2}$ (h)	1.0±0.1	1.0±0.1	2.4±0.2**	2.8±0.4††	2.1±0.1††	2.5±0.3
$AUC_{0-\infty}$ (ng h/ml)	692.4±40.5	494.5±62.5	248.6±18.9**	419.6±22.9††	312.5±14.3†	271.7±7.8
AUC ratio*	0.9±0.1	1.3±0.3	13.4±0.7	9.7±0.7†††	10.6±1.0†††	11.8±0.9

Data are the mean±SE.
AUC ratio: $AUC_{0-\infty}$ ratio of omeprazole to 5-hydroxyomeprazole (i.e., omeprazole hydroxylation index).
$^{*}P<0.05$ (versus EMs); $^{**}P<0.01$ (versus EMs); $^{***}P<0.001$ (versus EMs); $^{\dagger}P<0.05$ (versus before ticlopidine); $^{\dagger\dagger}P<0.01$ (versus before ticlopidine); $^{\dagger\dagger\dagger}P<0.001$ (versus before ticlopidine).

Fig. 3



CYP2C19 genotype-dependent changes in the AUC ratio (omeprazole/5-hydroxyomeprazole) by the repeated 8-day ticlopidine administration.

Table 2 Pharmacokinetic parameters of ticlopidine following the first (day 1) and seventh (day 7) doses in relation to *CYP2C19* genotypic status

	Homozygous EMs ($n=6$)	Heterozygous EMs ($n=6$)	PMs ($n=6$)
Day 1			
C_{max} (ng/ml)	496.5±70.3	642.6±146.3	513.9±96.5
T_{max} (h)	2.2±0.5	1.5±0.2	1.5±0.2
$T_{1/2}$ (h)	10.3±1.1	12.7±2.4	13.3±1.4
AUC_{0-24} (ng h/ml)	1698.5±259.5	1847.3±289.1	1426.6±347.9
Day 7			
C_{max} (ng/ml)	1059.8±123.7**	816.4±87.5	614.4±166.8
T_{max} (h)	1.8±0.2	2.0±0.4	1.7±0.5
$T_{1/2}$ (h)	12.9±0.8	17.3±5.7	15.8±3.0
AUC_{0-24} (ng h/ml)	3933.3±503.6**	3346.2±279.7*	2506.9±800.9*
Ratio of day 7 to day 1 data			
C_{max} (ng/ml)	2.3±0.3	1.7±0.6	1.1±0.1
T_{max} (h)	1.0±0.2	1.6±0.4	1.2±0.3
$T_{1/2}$ (h)	1.3±0.1	1.3±0.2	1.2±0.3
AUC_{0-24} (ng h/ml)	2.4±0.2	2.0±0.3	1.7±0.1

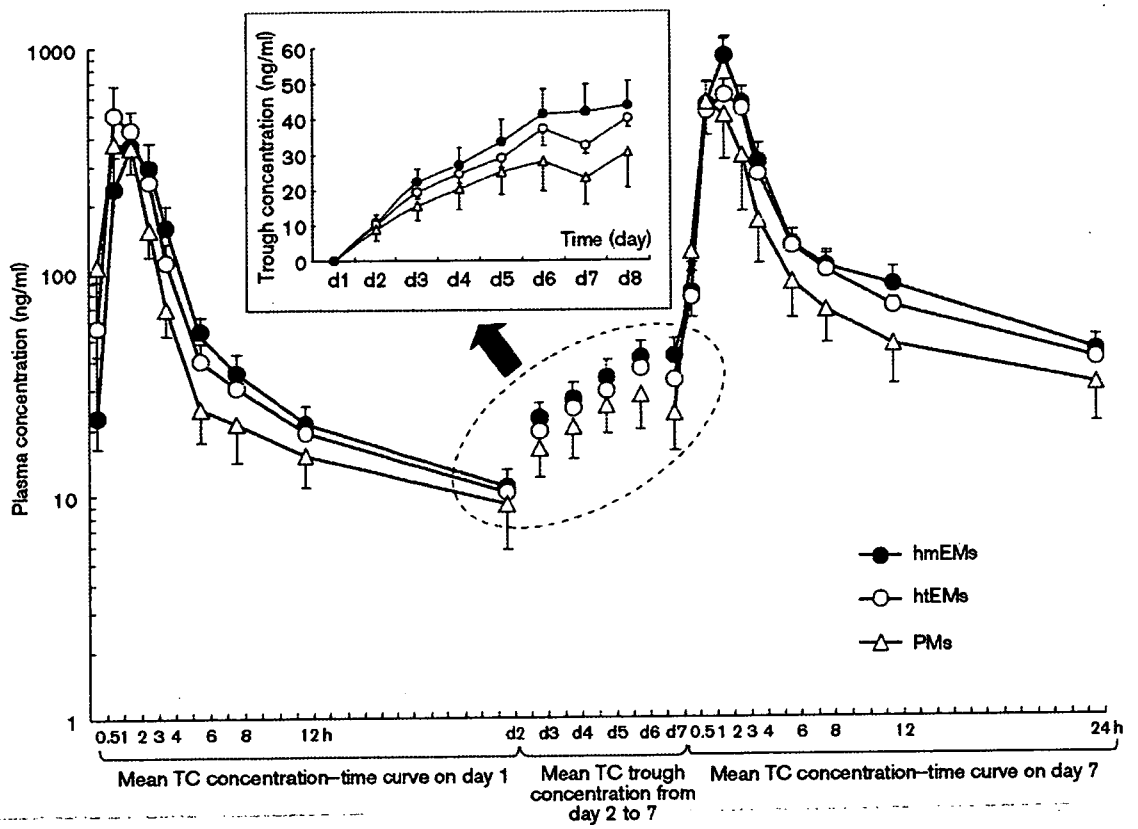
*$P<0.01$ (versus day 1 data); **$P<0.001$ (versus day 1 data).

platelet aggregation. This relationship appeared to exist regardless of *CYP2C19* genotypic status.

Discussion

The primary objective of this study was to evaluate the pharmacokinetics and pharmacodynamics of ticlopidine in relation to genetically determined CYP2C19 polymorphism. The most important findings were that: (1) a significant intergenotypic change in CYP2C19 activity (i.e., omeprazole 5-hydroxylation capability) was observed after multiple doses of ticlopidine; (2) although statistically significant intergenotypic differences in the pharmacokinetic parameters of ticlopidine were not observed, the accumulation ratio of ticlopidine [i.e., the AUC_{0-24} ratio of day 7 to day 1] and trough concentrations at all observed points tended to be greater in the hmEM subjects; and (3) the mean percentage inhibition of

Fig. 4



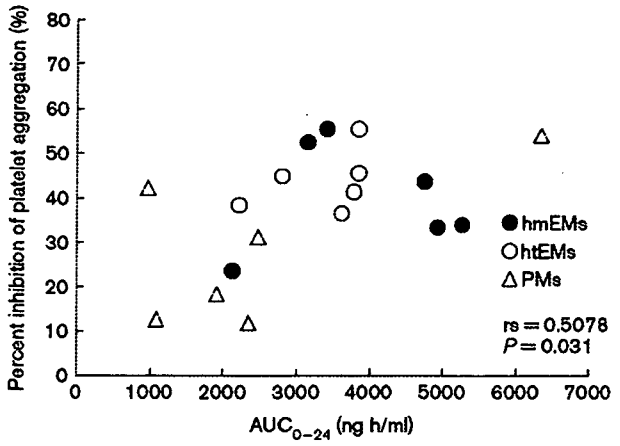
Mean plasma concentrations of ticlopidine (TC) in relation to *CYP2C19* genotypic status during the study period. The mean trough concentrations of ticlopidine from day 2 to day 8 are also indicated in the inset.

ADP-induced platelet aggregation increased with the repeated doses, but irrespective of CYP2C19 genotypic status. These results indicate that ticlopidine is a potent inhibitor *in vivo* and a less prominent substrate for CYP2C19 and that the *CYP2C19* polymorphic genes are likely important in terms of the interaction profiles of ticlopidine, but not in terms of platelet aggregation.

This study describes a substantial decrease in the CYP2C19 activity after repeated doses of ticlopidine in both the groups of EMs, but not in PM subjects. Our results confirm previous human studies showing that ticlopidine is a potent inhibitor of CYP2C19 [18,21]. Importantly, the inhibition of the CYP2C19 activity occurred only in the EM subjects, in whom hepatic CYP2C19 is believed to be sufficiently expressed. Similar genotype-dependent interactions have been reported for certain drug combinations (CYP2C19 substrate plus inhibitors) such as omeprazole plus moclobemide [22] and fluvoxamine plus chloroguanide [23]. In all cases, the pharmacokinetic parameters of substrates for CYP2C19 and their metabolites in the EM subjects changed to the values similar to those in the PM subjects. Thus, multiple doses of ticlopidine may be associated with a change in phenotypic status from extensive to poor metabolizers, so-called 'phenocopying' [24,25], when substrates for CYP2C19 are co-administered to EM subjects.

Despite a long history of clinical use, there is limited published information about the pharmacokinetics of ticlopidine. As shown in Fig. 4, the mean trough concentrations continued to rise over the seventh day of dosing, suggesting that an accumulation or saturation in the metabolism may occur during repeated dosings. These results are well consistent with the earlier reports that plasma ticlopidine concentrations (e.g., C_{max} and AUC) increase by three- to four-fold on repeated twice-daily dosings over 2 to 3 weeks [10,11]. In the present study, there was a trend toward the observation that the accumulation of ticlopidine seemed to be influenced by *CYP2C19* polymorphism: the accumulation ratio tended to be greater in the hmEM subjects. Similar to the ratio, the mean trough levels also tended to be higher in the hmEM subjects compared with the PM subjects, and

Fig. 5



Correlation between individual % inhibition of platelet aggregation and the AUC_{0-24} of ticlopidine on the 7th day of dosing. Individual *CYP2C19* genotypes are also indicated.

the htEM subjects had the values between those in hmEM and PM subjects throughout the study period. Ticlopidine is rapidly oxidized by recombinant CYP2C19 with the formation of two major metabolites, the keto tautomer of 2-hydroxyticlopidine and dimers of ticlopidine *S*-oxide [16]. The former has actually been detected as a metabolite of ticlopidine *in vivo* [26]. Recently, Ha-Duong *et al.* [16] demonstrated that this CYP2C19-catalyzed oxidation of ticlopidine occurs in parallel with an inactivation of CYP2C19. Since the ticlopidine-mediated inactivation of CYP2C19 is due to the covalent binding of the reactive ticlopidine *S*-oxide to the CYP2C19 active site, accumulation or saturation would be expected to more discernibly occur in the EM subjects, especially in the hmEM subjects. However, in addition to CYP2C19, other CYP enzymes, such as CYP2D6, CYP3A4 [16,19] and CYP2B6 (our unpublished microsomal experiments), may be involved in the oxidation of ticlopidine. Furthermore, the current in-vitro studies examined ticlopidine to undergo the principal routes of metabolism (i.e., *N*-dealkylation, *N*-oxidation and oxidation of the thiophene ring) by peroxidases and monoamine exidase [19]. Thus, this multiple enzyme-mediated metabolism may explain why there were no significant differences in the accumulation ratio among the three *CYP2C19* genotypic groups.

In this study we used the AUC_{0-24} of ticlopidine on day 7 for the calculation of the accumulation index. For a better understanding of the potential effects of genetic variation on the pharmacokinetics of ticlopidine, sufficient time to reach the steady state (i.e., 14 days) and long duration of sampling time (i.e., 96 h) is needed. For these limitations, the pharmacokinetics data on ticlopidine presented here should be viewed as the apparent values obtained during the pseudo-steady state. Furthermore, whether and to what extent CYP2C19 would be involved in the overall metabolism of ticlopidine remain unanswered from this in-vivo human study.

Despite its clinical usefulness, chronic administration of ticlopidine results in a relatively high incidence of hepatotoxicity [27–29]. Although the mechanisms involved in the ticlopidine-induced hepatic injury remain unknown, immune mechanisms and drug hypersensitivity have been proposed. Previous studies indicated that tienilic acid (a thiophene derivative and a substrate of CYP2C9) and ticlopidine act as a selective suicide substrate of CYP2C9 [17] and CYP2C19 [16], respectively. It is well known that tienilic acid sometimes induces immunoallergic hepatitis in a subset of patients who produce anti-liver–kidney microsome antibody type 2 (LKM-2) autoantibodies [30–33]. Since LKM autoantibodies are observed in autoimmune hepatitis, in some patients with drug-induced hepatitis, they are markers of autoimmune hepatitis. In contrast to LKM-2 antibodies, LKM-1 antibodies mostly target CYP2D6 [34]. In this regard, we monitored LKM-1 and LKM-2 in the present study. However, no changes in these antibody markers were observed during the present short study period (data not shown).

Although a significant positive correlation was observed between the individual percent inhibition of platelet aggregation and the AUC_{0-24} of ticlopidine on the seventh day of dosing, a large interindividual difference in platelet aggregation could not be explained by the CYP2C19 polymorphism. Antiaggregant effects were noted at 24 to 48 h with a maximal effect observed after 3 to 5 days of dosings [35,36] or after 6 to 10 days in a Chinese study [37]. Thus, the AUC_{0-24} observed on day 7 may be as a pharmacokinetic parameter obtained at the appropriate time of the maximal antiaggregant effect. Ticlopidine does not inhibit ADP-induced platelet aggregation *in vitro*, and hepatic conversion into active metabolite(s) is required for drug action [38]. More than thirteen metabolites have been described to date, but those responsible for the antiplatelet effect have not yet been well documented. Very recently, Yoneda *et al.* [39] identified a metabolite with a potent antiplatelet activity, UR-4501, which was generated after incubation of 2-oxo-ticlopidine with phenobarbital-induced rat liver homogenate. They indicated that UR-4501 produced a concentration-dependent inhibition of ADP-induced human platelet aggregation, whereas 2-oxo-ticlopidine did not elicit inhibitory responses. Although the major CYP enzyme(s) responsible for the formation of UR-4501 has yet to be identified, our results suggest that it is not CYP2C19. An in-vitro study is definitely required for screening CYP enzyme(s) responsible for the formation of the pharmacologically active metabolite(s) such as UR-4501.

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SNP Communication

Genetic Variations and Haplotypes of UGT1A4 in a Japanese Population

Mayumi SAEKI[1], Yoshiro SAITO[1,2,*], Hideto JINNO[1,3], Kimie SAI[1,4], Akiko HACHISUKA[2], Nahoko KANIWA[1,5], Shogo OZAWA[1,6], Manabu KAWAMOTO[7], Naoyuki KAMATANI[7], Kuniaki SHIRAO[8], Hironobu MINAMI[9], Atsushi OHTSU[10], Teruhiko YOSHIDA[11], Nagahiro SAIJO[12], Kazuo KOMAMURA[13,14], Takeshi KOTAKE[15], Hideki MORISHITA[15], Shiro KAMAKURA[13], Masafumi KITAKAZE[13], Hitonobu TOMOIKE[13] and Jun-ichi SAWADA[1,2]

[1]*Project Team for Pharmacogenetics,* [2]*Division of Biochemistry and Immunochemistry,* [3]*Division of Environmental Chemistry,* [4]*Division of Xenobiotic Metabolism and Disposition,* [5]*Division of Medicinal Safety Science,* [6]*Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan* [7]*Division of Genomic Medicine, Department of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan* [8]*Division of Internal Medicine, National Cancer Center Hospital,* [11]*Genetics Division, National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan* [9]*Division of Oncology/Hematology,* [10]*Division of GI Oncology/Digestive Endoscopy,* [12]*Deputy Director, National Cancer Center Hospital East, Chiba, Japan* [13]*Division of Cardiology,* [14]*Department of Cardiovascular Dynamics Research Institute,* [15]*Department of Pharmacy, National Cardiovascular Center, Osaka, Japan*

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Summary: Nineteen genetic variations, including 11 novel ones, were found in exon 1 and its flanking region of the UDP-glucuronosyltransferase (UGT) 1A4 gene from 256 Japanese subjects, consisting of 60 healthy volunteers, 88 cancer patients and 108 arrhythmic patients. These variations include −217T>G and −36G>A in the 5′-flanking region, 30G>A (P10P), 127delA (43fsX22; frame-shift from codon 43 resulting in the termination at the 22nd codon, codon 65), 175delG (59fsX6), 271C>T (R91C), 325A>G (R109G), and 357T>C (N119N) in exon 1, and IVS1+1G>T, IVS1+98A>G and IVS1+101G>T in the following intron. Among them, 127delA and 175delG can confer early termination of translation, resulting in an immature protein that probably lacks enzymatic activity. Variation IVS1+1G>T is located at a splice donor site and thus may lead to aberrant splicing. Since we did not find any significant differences in the frequencies of all the variations among the three subject groups, the data were analyzed as one group. The allele frequencies of the novel variations were 0.006 for IVS1+101G>T, 0.004 for 30G>A (P10P) and 357T>C (N119N), and 0.002 for the 8 other variations. In addition, the two known nonsynonymous single nucleotide polymorphisms (SNPs), 31C>T (R11W) and 142T>G (L48V), were found at 0.012 and 0.129 frequencies, respectively. The SNP 70C>A (P24T), mostly linked with 142T>G (L48V) in German Caucasians, was not detected in this study. Sixteen haplotypes were identified or inferred, and some haplotypes were confirmed by cloning and sequencing. It was shown that most of 142T>G (L48V) was linked with −219C>T, −163G>A, 448T>C (L150L), 804G>A (P268P), and IVS1+43C>T, comprising haplotype **3a*; haplotype **4a* harbors 31C>T (R11W); 127delA (43fsX22) and 142T>G (L48V) were linked (haplotype **5a*); 175delG (59fsX6) was linked with 325A>G (R109G) (**6a* haplotype); and −219C>T, −163G>A, 142T>G (L48V), 271C>T (R91C), 448T>C (L150L), 804G>A (P268P), and IVS1+43C>T comprised haplotype **7a*. Our results provide fundamental and useful information for genotyping *UGT1A4* in the Japanese and probably Asian populations.

Key words: UGT1A4; amino acid alteration; frameshift; splice donor site; drug metabolism

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To whom correspondence should be addressed: Yoshiro SAITO, Ph.D., *Project Team for Pharmacogenetics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel. +81-3-3700-9453, Fax. +81-3-3707-6950, E-mail: yoshiro@nihs.go.jp*

Table 1. Primers utilized for *UGT1A4* amplification and sequencing

	Direction	Primer Name	Sequences	Location[a]
Amplification	forward	UGT1A4-1stF	TTAACAAAGTAGAAGGCAGTG	135092
	reverse	UGT1A4-1stR	TGAAAACTTGAAATACACTAGGC	136460
Sequencing	forward	UGT1A4-1stF	TTAACAAAGTAGAAGGCAGTG	135092
	forward	UGT1A4seqF2	GGGCTGAGAGTGGAAAGGT	135502
	forward	UGT1A4seqF3	TCCTTCCTCCTATATTCCTAAGTT	135995
	reverse	UGT1A4seqR1-2	ATCAAATTCCTTCTGGGTCC	135698
	reverse	UGT1A4seqR2	AAGGGGCAGAAAAAGTATGG	136119
	reverse	UGT1A4-1stR	TGAAAACTTGAAATACACTAGGC	136460

[a]The 5′-end of each primer on AF297093.1.

On December 2, 2004, these variations were not found on the UDP Glucuronosyltransferase home page (http://som.flinders.edu.au/FUSA/ClinPharm/UGT/), the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB (http://www.pharmgkb.org/do/) databases.

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Introduction

As phase II enzymes, the UDP-glucuronosyltransferase enzymes (UGTs) play crucial roles in the detoxification and elimination of a large number of endogenous and exogenous compounds.[1] Of the UGT1 and UGT2 subfamilies expressed in humans, the genes encoding UGT1As have a unique genetic structure consisting of at least 13 different exon 1's, including four inactive ones, and the common exons 2 to 5 clustered on chromosome 2q37.[2] One of the exon 1's can be spliced on to the common exons. The *N*-terminal domains (encoded by the exon 1's) of the UGT1A proteins determine their substrate-binding specificity, and the common *C*-terminal domain (encoded by exons 2 to 5) is important for UDP-glucuronic acid binding.[3]

UGT1A4 is expressed in the liver, bile ducts, colon, small intestine, and pancreas.[1,4,5] UGT1A4 catalyzes the conjugation of exogenous amines and alcohols, including nicotine, sapogenins, imipramine, trifluoperazine, and tamoxifen.[1,6-9] In addition, many androgens and progestins are reported as endogenous substrates of UGT1A4.[6] Several genetic polymorphisms of *UGT1A4* were reported in the public databases. Among them, two nonsynonymous single nucleotide polymorphisms (SNPs), 70C>A (P24T) and 142T>G (L48V), were found in German Caucasians, and they were shown to be closely associated.[10] The variant enzymes (24T and 48V) had reduced *in vitro* activities for β-naphtylamine, benzidine, *trans*-androsterone, and dihydrotestosterone in a substrate-specific manner.[10]

In spite of the clinical importance of UGT1A4, there is no report on the comprehensive sequencing analysis for the genetic polymorphisms of *UGT1A4* in Asian populations, including the Japanese. In the present study, *UGT1A4* exon 1 was sequenced from 256 Japanese subjects. Eleven novel genetic variations were identified, including 4 nonsynonymous ones.

Materials and Methods

Human genomic DNA samples: DNA was obtained from the blood leukocytes of 88 Japanese cancer patients and 108 Japanese arrhythmic patients. Written informed consent was obtained from all participating patients. DNA was also extracted from Epstein-Barr virus-transformed lymphoblastoid cells, for which blood samples were collected from 60 healthy Japanese volunteers at the Tokyo Women's Medical University under the auspices of the Pharma SNP Consortium (Tokyo, Japan). Informed consent was also obtained from all healthy subjects. The ethical review boards of all the participating organizations approved this study.

PCR conditions for DNA sequencing: First, exon 1 of *UGT1A4* was amplified from genomic DNA (100 ng) using 0.625 units of *Ex*-Taq (Takara Bio. Inc., Shiga, Japan) with 0.2 μM of amplification primers designed in the introns (**Table 1**). The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. These PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and were directly sequenced on both strands using an ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) (see **Table 1** for sequencing primers). The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All variations were confirmed by repeating

Table 2. Summary of *UGT1A4* polymorphisms detected in a Japanese population

SNP ID					Position				Number of subjects			Frequency			
This Study	dbSNP-NCBI database	JSNP database	Pharm GKB atabase[b]	Location	AF297093.1	From the translational initiation site or from the end of exon 1 (IVS1+)	Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Wild-type	Hetero-zygote	Homo-zygote	Total (n=256)	Healthy volunteers (n=60)	Cancer patients (n=88)	Arrhythmic patients (n=108)
MPJ6_U1A081	rs3732219	IMS-JST085729	O	5′-flanking	135210	−219	GGGTCAGATGAGC/TTTTTCAAGATAG		195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A082[a]				5′-flanking	135212	−217	GTCAGATGAGCTT/GTTCAAGATAGGC		255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A083	rs3732218	IMS-JST085728	O	5′-flanking	135266	−163	TAACGAAAGGCAG/ATTATAGATTAAT		195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A084[a]				5′-flanking	135393	−36	CAGGCACAGCGTG/AGGGTGGACAGTC		255	1	0	0.002	0.000	0.006	0.000
MPJ6_U1A085[a]				Exon 1	135458	30	GGTTCCCCTGCCG/ACGGCTGGCCACA	P10P	254	2	0	0.004	0.000	0.000	0.009
MPJ6_U1A086	rs3892221		O	Exon 1	135459	31	GTTCCCCTGCCGC/TGGCTGGCCACAG	R11W	250	6	0	0.012	0.025	0.011	0.005
MPJ6_U1A087[a]				Exon 1	135555	127	AGCCCCTGGCTCA/-GCATGCGGGAGG	43fsX22	255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A088	rs2011425		O	Exon 1	135570	142	ATGCGGGAGGCCT/GTGCGGGAGCTCC	L48V	197	52	7	0.129	0.133	0.148	0.111
MPJ6_U1A089[a]				Exon 1	135603	175	GGCCACCAGGCGG/-TGGTCCTCACCC	59fsX6	255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A090[a]				Exon 1	135699	271	AAGGAATTTGATC/TGCGTTACGCTGG	R91C	255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A091[a]				Exon 1	135753	325	CATCTTCTGAAGA/GGATATTCTAGAA	R109G	255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A092[a]				Exon 1	135785	357	AATTATGAACAAT/CGTATCTTTGGCC	N119N	254	2	0	0.004	0.008	0.006	0.000
MPJ6_U1A093	rs12468274		O	Exon 1	135876	448	TTTGATGTGGTTT/CTAACAGACCCCG	L150L	195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A094	rs2011404		O	Exon 1	135899	471	CGTTAACCTCTGC/T[c]GGGGCGGTGCTG	C157C	251	5	0	0.010	0.008	0.011	0.009
MPJ6_U1A095	rs3732217	IMS-JST085727	O	Exon 1	136232	804	CTACCCCAGGCCG/AATCATGCCCAAC	P268P	195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A096[a]				Intron 1	136296	IVS1+1	CCACTATCTCAGG/TTCTGTATTGGTG		255	1	0	0.002	0.000	0.000	0.005
MPJ6_U1A097	rs2011219	IMS-JST085726	O	Intron 1	136338	IVS1+43	TTCCAGGCAAAAC/TACTTTTTAAAAA		195	54	7	0.133	0.133	0.142	0.125
MPJ6_U1A098[a]				Intron 1	136393	IVS1+98	ACTTATCTTTCCA/GAAGATTTTATTT		255	1	0	0.002	0.000	0.006	0.000
MPJ6_U1A099[a]				Intron 1	136396	IVS1+101	TATCTTTCCAAAG/TATTTTATTTTGG		253	3	0	0.006	0.008	0.006	0.005

[a]Novel variations detected in this study.
[b]The SNPs included in the PharmGKB database was shown as "O".
[c]T in the reference sequence.

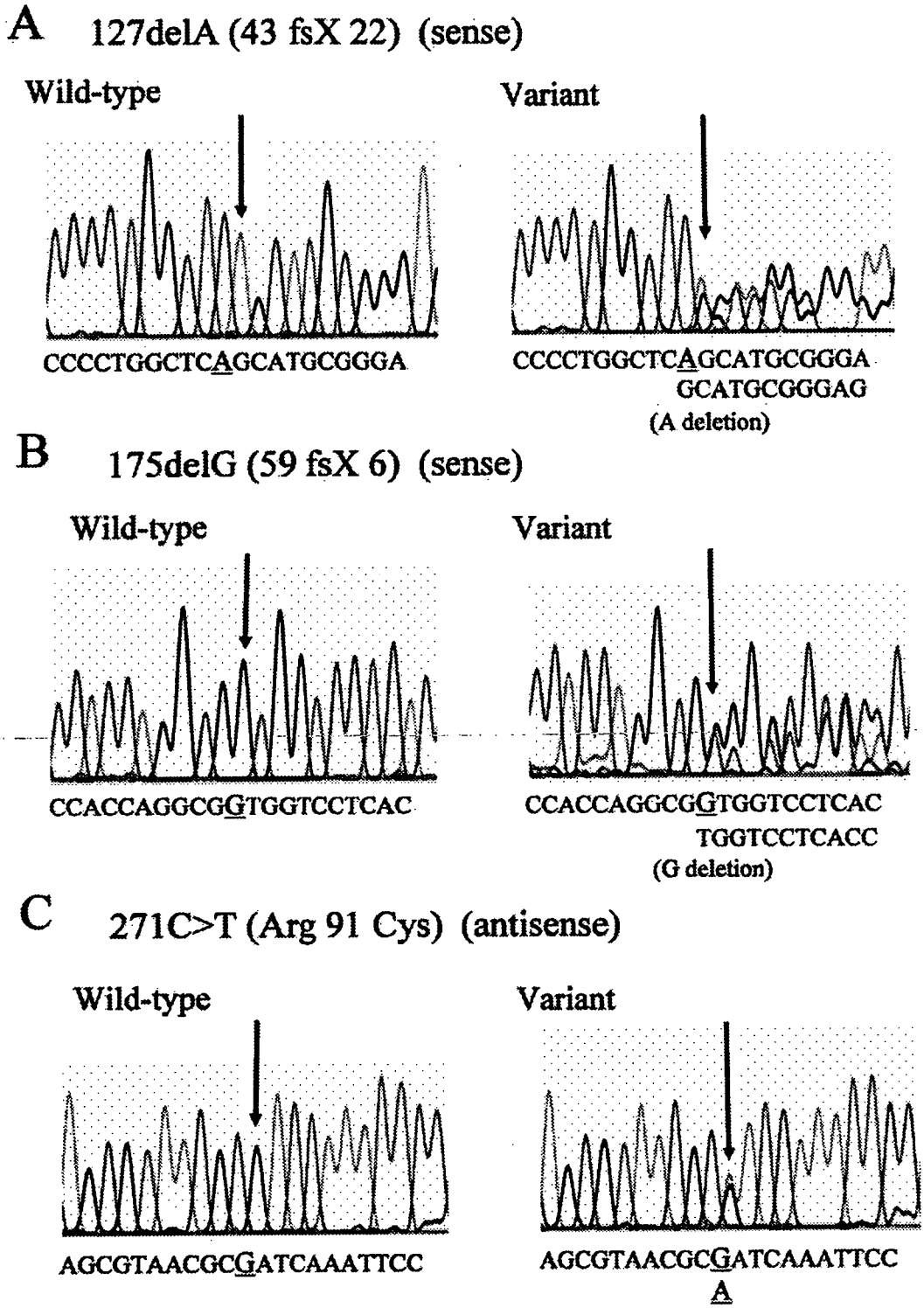
A
127delA (43 fsX 22) (sense)
Wild-type
Variant
CCCCTGGCTCAGCATGCGGGA
CCCCTGGCTCAGCATGCGGGA
GCATGCGGGAG
(A deletion)
B
175delG (59 fsX 6) (sense)
Wild-type
Variant
CCACCAGGCGGTGGTCCTCAC
CCACCAGGCGGTGGTCCTCAC
TGGTCCTCACC
(G deletion)
C
271C>T (Arg 91 Cys) (antisense)
Wild-type
Variant
AGCGTAACGCGATCAAATTCC
AGCGTAACGCGATCAAATTCC
A

Fig. 1

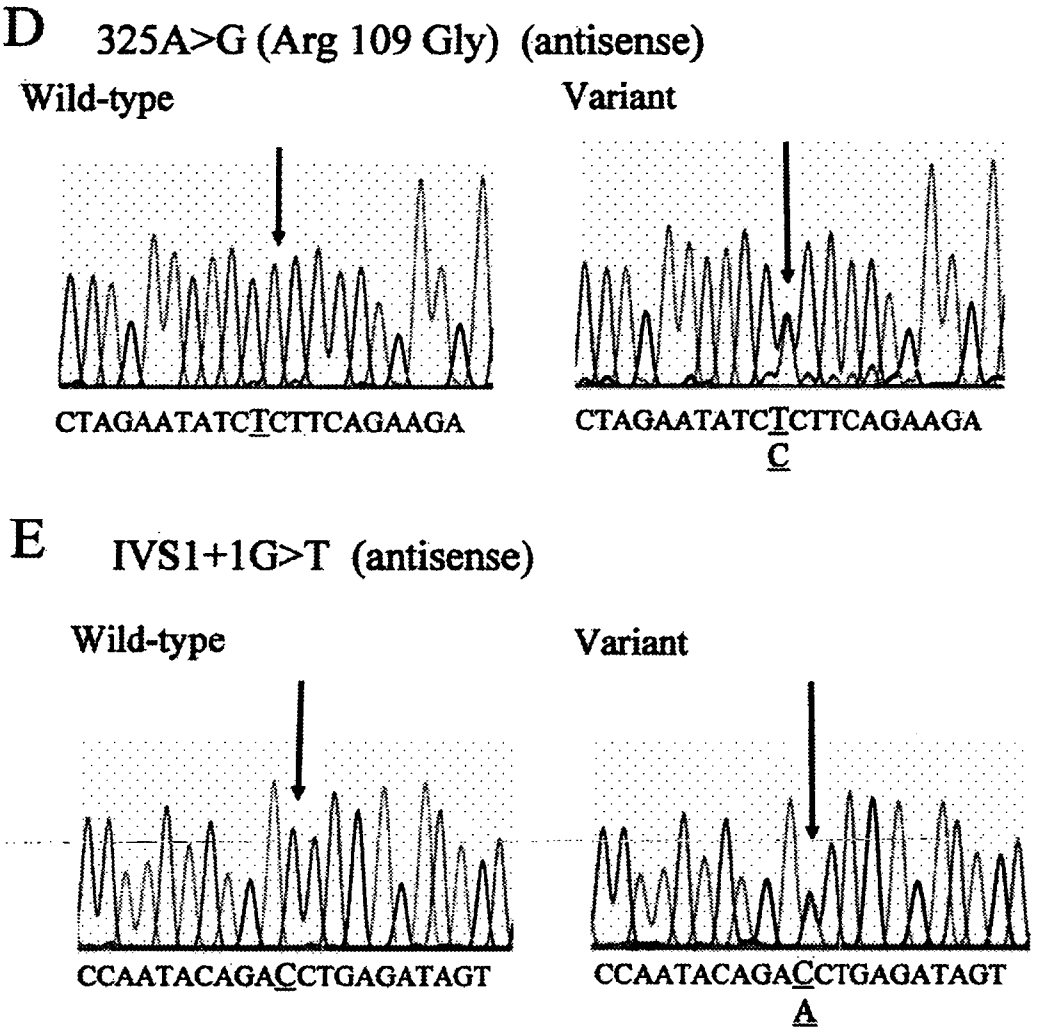

Fig. 1. The 4 novel genetic variations with amino acid substitutions and 1 splice donor site variation of human *UGT1A4*. (A) MPJ6_U1A087 (wild-type, 127A/A; variant, 127A/-). (B) MPJ6_U1A089 (wild-type, 175G/G; variant, 175G/-). (C) MPJ6_U1A090 (wild-type, 271C/C; variant, 271C/T). (D) MPJ6_U1A091 (wild-type, 325A/A; variant, 325A/G). (E) MPJ6_U1A096 (wild-type, IVS1+1G/G; variant, IVS1+1G/T). Arrows indicate the positions of the nucleotide changes.

the PCR on genomic DNA and sequencing the newly generated PCR products. Furthermore, the rare variations found in only one subject were confirmed by sequencing the PCR fragments produced by amplification with a high fidelity DNA polymerase KOD-Plus- (TOYOBO, Tokyo, Japan).

Linkage disequilibrium (LD) and haplotype analysis: Hardy-Weinberg equilibrium analysis and LD analysis were performed by SNPAlyze software (Dynacom Co., Yokohama, Japan). Pairwise LDs were shown in rho square (r^2) values. Some of the haplotypes were unambiguous from the subjects with homozygous SNPs at all sites or a heterozygous SNP at only one site. Separately, the diplotype configurations (a combination of haplotypes) were inferred by LDSUPPORT software, which determines the posterior probability distribution of the diplotype configuration for each subject based on the estimated haplotype frequencies.[11] The haplotypes were described as a number plus a small alphabetical letter.

Results and Discussion

UGT1A4 exon 1 and its flanking regions (from −286 bases upstream of the translational start site to 112 bases downstream of the end of exon 1) were sequenced from 256 Japanese subjects. Genbank accession number AF297093.1 was utilized for the reference sequence. Nineteen polymorphisms were detected, including 11 novel ones (2 were in the 5′-flanking region, 6 in exon 1, and 3 in the following intron) (**Table 2**). All of the allelic frequencies were in Hardy-Weinberg equilibrium ($p=$

0.13 or over). Since we did not find any significant differences in the frequencies of all the variations among three subject groups ($p>0.25$ by χ^2 test) and between two of the three groups ($p>0.13$ by χ^2 test or Fisher's exact test), the data for all subjects were analyzed as one group.

We found two novel nonsynonymous variations, 271C>T (R91C) and 325A>G (R109G), and two novel deletions, 127delA (43fsX22) and 175delG (59fsX6), as individual heterozygotes at a 0.002 frequency. Among them, 127delA (43fsX22) and 175delG (59fsX6) are the frameshift variations starting from codon 43 and 59, respectively, resulting in early stop codons at the 22nd (*i.e.* codon 65) and the 6th (*i.e.* codon 65) codons, respectively. It is most likely that these variations generate an immature protein that probably has null activity. The functional significance of 271C>T (R91C) and 325A>G (R109G) is currently unknown. Additionally, IVS1+1G>T, which was found at a frequency of 0.002, was located at a splice donor site and thus may lead to aberrant splicing (**Fig. 1**).

We also detected two known nonsynonymous SNPs, 31C>T (R11W) and 142T>G (L48V), at 0.012 and 0.129 frequencies, respectively. The frequency of 142T>G (L48V) was almost comparable to that of German Caucasians (0.09).[10] L48V was reported to lead to a partial decrease in glucuronidation of β-naphthylamine and benzidine, a marked decrease in the activity to *trans*-androsterone, and no activity toward dihydrotestosterone *in vitro*.[10] The functional significance of SNP 31C>T (R11W) has not been reported yet.

High linkage disequilibrium ($r^2 \geqq 0.89$) was observed among −219C>T, −163G>A, 142T>G (L48V), 448T>C (L150L), 804G>A (P268P), and IVS1+43C>T. A perfect linkage ($r^2=1$) was found between 175delG and 325A>G (R109G), but found in only one subject. The r^2 values were below 0.014 between the other pairs of polymorphisms. The SNP 70C>A (P24T), mostly linked with 142T>G (L48V) in German Caucasians,[10] was not detected in this study. Thus, it must be clarified whether the differences in the linkage of those SNPs may lead to the ethnic differences in the enzymatic activities of UGT1A4. A similar kind of ethnic difference has been found in the **1B* haplotype, which harbors the three linked SNPs in the 3′-untranslated region of UGT1A common exon 5 found in a Japanese population.[12] In Caucasian and African-American populations, this linkage of the 3 SNPs was not complete, especially in African-Americans.[13]

Using the detected SNPs, haplotype analysis was then performed (**Table 3**). Since *UGT1A4*2* [70C>A (P24T)] and **3* [142T>G (L48V)] were defined in AF465196 and AF465197 (Genbank accession numbers), respectively, the novel haplotypes with amino acid changes, frameshift variations, or splice donor site

Table 3. *UGT1A4* haplotypes in a Japanese population

Haplotype[a]	Amino acid change	Nucleotide change[b]	-219 C>T	-217 T>G	-163 G>A	-36 G>A	30 G>A	31 C>T	127 delA	142 T>G	175 delG	271 C>T	325 A>G	357 T>C	448 T>C	471 C>T	804 G>A	IVS1 +1 G>T	IVS1 +43 C>T	IVS1 +98 A>G	IVS1 +101 G>T	Frequency
							P10P	R11W	43fsX22	L48V	59fsX6	R91C	R109G	N119N	L150L	C157C	P268P					
*1		*1a																				0.818
		*1b																				0.010
		*1c																				0.068
		*1d																				0.006
		*1e																				0.004
		*1f																				0.004
		*1g																				0.002
		*1h																				0.002
		*1i																				0.002
																						0.856
*3		*3a																				0.123
		*3b																				0.002
*4		*4a																				0.013
*5		*5a																				0.002
*6		*6a																				0.002
*7		*7a																				0.002
*8		*8a																				0.002
																						0.125
																						0.012
																						0.002
																						0.002
																						0.002
																						0.002
																						0.002

[a] A of the translational start codon of *UGT1A4* is numbered 1. AF297093.1 was used as the reference sequence.
[b] The haplotypes were described as a number plus a small alphabetical letter.

variation, were assigned as haplotypes **4* to **8*. Several haplotypes were first unambiguously assigned by homozygous SNPs at all sites (**1a* and **3a*) or a heterozygous SNP at only one site (**1b*, **1d*-**1i*, **3b*, **4a*, and **8a*). Separately, we estimated the diplotype configuration (a combination of haplotypes) for each subject by LDSUPPORT software. The diplotype configurations of 256 subjects were inferred with probabilities (certainty) of 0.9998 or over, except for one subject. The additionally inferred haplotypes were **1c*, **5a*, **6a*, and **7a*. As for one subject with a low probability (who had heterozygous SNPs of −219C>T, −163 G>A, 31C>T, 142T>G, 448T>C, 804G>A, and IVS1+43C>T), the diplotype was determined by the cloning and sequencing of DNA fragments. One chromosome had haplotype **3a* (consisting of −219C>T, −163 G>A, 142T>G, 448T>C, 804G>A, and IVS1+43C>T) and the other had haplotype **4a* (31C>T). Moreover, the data obtained by cloning and sequencing analysis confirmed the presence of haplotypes **5a* [127delA (43fsX22) and 142T>G (L48V)], **6a* [175delG (59fsX6) and 325A>G (R109G)], and **7a* [−219C>T, −163G>A, 142T>G (L48V), 271C>T (R91C), 448T>C (L150L), 804G>A (P268P), and IVS1+43C>T] (**Table 3**). The most frequent haplotype was **1a* (frequency: 0.818), followed by **3a* (0.123), **4a* (0.012) and **1b* (0.010). The frequencies of the other haplotypes were less than 0.01. Since 325A>G (R109G) was linked with 175delG (59fsX6), the enzymatic activity of this haplotype (**6a*) is probably null. The other SNP, 271C>T confers the R91C substitution. In human UGT1A4, eight cysteine residues were located in the lumenal domain.[3,14] Though the disulfide-bond formation and its significance are not clear in the UGT1A4, it has been reported that the reduction of disulfide-bonds of rat UGT1A6 with dithiothreitol increases its enzymatic activity in the liver microsomes.[15] On the other hand, the alterations of several lumenal cysteines into serine residues seem to reduce the UGT1A6 activity when the mutant enzymes were expressed in COS cells.[15] The effect of additional cysteine residue at codon 91 in the UGT1A4 should be determined in the future.

In conclusion, we detected 19 polymorphisms, including 11 novel ones, in *UGT1A4* from a Japanese population. Using the detected polymorphisms, 16 haplotypes were identified. Our results provide fundamental and useful information for genotyping *UGT1A4* in the Japanese, and probably Asian populations.

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