along with the subsequent half of TMD11, TMD12 and the cytoplasmic tail,[1] which very likely affects transport activity. Other variations 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), and 1553C>T (Ser518Leu) are located in TMD3, TMD4, the short cytoplasmic loop between TMD4 and TMD5, and the large extracellular loop between TMD9 and TMD10, respectively.[1] Using the PolyPhen program (http://genetics.bwh.harvard.edu/pph/) to predict the functional effects of the four amino acid substitutions, three substitutions, Met104Lys, Met170Thr and Ser518Leu, were expected to alter the protein function based on the PSIC (position-specific independent count) score differences derived from multiple alignments. The functional significance of these 5 novel nonsynonymous variations should be clarified in the future. In addition, a novel variation at the splice acceptor site, IVS12-1 G>T, was detected at a 0.003 frequency. This variation might cause aberrant splicing of *SLCO1B1* pre-mRNA and thus influence the expression level of active protein. Furthermore, -3A>C might reduce translational efficiency since this purine-to-pyrimidine alteration results in a deviation from the Kozak motif, where the purine nucleotide at position -3 from the translational initiation codon is important.[14]

Four known variations, 388A>G (Asn130Asp), 452A>G (Asn151Ser), 521T>C (Val174Ala), and 1007C>G (Pro336Arg), were detected at 0.667, 0.034, 0.175, and 0.006 frequencies, respectively, which are similar to the Japanese data reported previously.[5,11] The allele frequencies of 521T>C (Val174Ala) in Japanese (0.11–0.18) are comparable to those in other Asian populations (0.04–0.25) and Caucasians (0.14–0.22), but higher than that in African-Americans (0.02).[10,15,16] The frequencies of 388A>G (Asn130Asp) in Japanese (0.63–0.67) are also similar to those in other Asians (0.57–0.88) and African-Americans (0.75), but higher than those in Caucasians (0.30–0.51).[10,15,16] Variations 452A>G (Asn151Ser) and 1007C>G (Pro336Arg) have not been reported in other ethnic populations. Analysis of these four known variations with PolyPhen program showed that only Val174Ala was expected to alter protein function, which is consistent with the previous functional analysis.[2,4,6] Variations 1454G>T (Cys485Phe) and 1628T>G (Leu543Trp) previously reported in Japanese were not detected in this study.[11,17] Hepatocyte nuclear factor 1$\alpha$ is known to transactivate *SLCO1B1* through binding to the promoter region (from -10432 to -10420 from the translational start codon);[18] however, no variation was found in this region.

Using -11187G>A, -3A>C, IVS12-1G>T and 9 nonsynonymous variations, diplotype configuration was estimated for each subject. The configuration was estimated with >0.99 probabilities for all but four subjects. The predicted haplotype frequencies for **1b* [harboring 388A>G (Asn130Asp)], **5* [harboring 521T>C (Val174Ala)], **15* [harboring 388A>G (Asn130Asp) and 521T>C (Val174Ala)] and **17* [harboring -11187G>A, 388A>G (Asn130Asp), and 521T>C (Val174Ala)] were 0.469, 0.000 (not detected), 0.037 and 0.133, respectively. The haplotype frequencies for **1b* and **5* are similar to those in the previous studies in Japanese.[5,11] The **17* frequency is higher than those in Chinese (0.085), Finnish Caucasians (0.069), Malay (0.029) and Indians (0.009).[15,16] It should be noted that 76% (n=47 alleles) of 521T>C (Val174Ala)-bearing haplotypes were assigned as **17*, and 21% (n=13) of them as **15*. The remaining two (3%) was estimated to exist with 1007C>G (Pro336Arg) and **17* variations [-11187G>A, 388A>G (Asn130Asp), and 521T>C (Val174Ala)] on the same chromosomes. The **17* ratio in 521T>C (Val174Ala)-bearing haplotypes is similar to that in Chinese (65%), but higher than those in Finnish Caucasians (34%), Malay (26%) and Indians (14%).[15,16] Variation 452A>G (Asn151Ser, n=12 alleles) or 1738C>T (Arg580Stop, n=3) were predicted to be on the **1a* background (no other variation).

In conclusion, 62 genetic variations were identified, including 28 novel ones, in *SLCO1B1*. One novel nonsynonymous variation results in a truncated protein and four novel nonsynonymous variations result in amino acids substitutions. In addition, novel variations IVS12-1 G>T at the splice acceptor site and -3A>C in the Kozak motif were detected. Approximately 76% of 521T>C (Val174Ala)-bearing haplotypes were assigned as **17* and the majority of the remaining haplotypes were **15*. This information would be useful for pharmacogenetic studies to investigate the associations of *SLCO1B1* variations with interindividual differences in drug disposition.

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ORIGINAL ARTICLE

# Impact of *CYP3A4* haplotypes on irinotecan pharmacokinetics in Japanese cancer patients

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**Abstract**
*Background and purpose* Cytochrome P450 3A4 (CYP3A4) converts an anticancer prodrug, irinotecan, to inactive metabolites such as APC. However, the contribution of *CYP3A4* genetic polymorphisms to irinotecan pharmacokinetics (PK) and pharmacodynamics (PD) is not fully elucidated. In paclitaxel-administered cancer patients, an association of *CYP3A4*16B* harboring the low activity allele **16* [554C > G (Thr185Ser)] has been shown with altered metabolite/paclitaxel area under the plasma concentration–time curve (AUC) ratios, suggesting a possible impact of **16B* on the PK of other drugs. In this study, the effects of *CYP3A4* haplotypes including **16B* on irinotecan PK/PD were investigated in irinotecan-administered patients.
*Methods* The *CYP3A4* genotypes for 177 Japanese cancer patients who received irinotecan were defined in terms of

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4 major haplotypes, i.e., *1A (wild type), *1G (IVS10 + 12G > A), *16B [554C > G (Thr185Ser) and IVS10 + 12G > A], and *18B [878T > C (Leu293Pro) and IVS10 + 12G > A]. Associations of *CYP3A4* genotypes with irinotecan PK and severe toxicities (grade 3 diarrhea and grade 3 or 4 neutropenia) were investigated.

*Results* Area under the concentration–time curve ratios of APC/irinotecan, an in vivo parameter for CYP3A4 activity, were significantly higher in females than in males. The male patients with *16B showed significantly decreased AUC ratios (APC/irinotecan) with 50% of the median value of the *non-*16B* male patients (no *16B-bearing female patients in this study), whereas no significant alteration in the AUC ratios was observed in the patients with *18B. A slight trend toward increasing AUC ratios (20%) was detected in both male and female patients bearing *1G. Multivariate analysis confirmed contributions of *CYP3A4*16B* (coefficient ± SE = −0.18 ± 0.077, $P = 0.021$) and *1G (0.047 ± 0.021, $P = 0.029$) to the AUC ratio. However, no significant association was observed between the *CYP3A4* genotypes and total clearance of irinotecan or toxicities (severe diarrhea and neutropenia).

*Conclusion* This study suggested that *CYP3A4*16B* was associated with decreased metabolism of irinotecan to APC. However, the clinical impact of *CYP3A4* genotypes on total clearance and irinotecan toxicities was not significant.

**Keywords** CYP3A4 · Haplotype · Irinotecan · Pharmacogenetics

## Introduction

Human cytochrome P450 3A4 (CYP3A4) is a major CYP enzyme, abundant in the liver and intestine, and is involved in the metabolism of endogenous substances, including steroid hormones, and a variety of exogenous compounds such as environmental chemicals and pharmaceuticals. Large inter-individual differences in liver and intestinal CYP3A4 expression levels are known and thought to be caused by multiple factors including genetic variations, disease status, and modulation by exogenous stimuli, such as smoking, diet, and drugs [5, 18, 31]. The tissue-specific CYP3A4 expression is regulated by constitutive and inducible mechanisms via activation of the nuclear receptors, pregnane X receptor (PXR), constitutive androstane receptor (CAR), and vitamin D receptor (VDR) [5, 18]. Since approximately half of clinical drugs currently in use are metabolized by CYP3A4 [5, 33], it is important to find suitable biomarkers, including genetic polymorphisms, which can reflect in vivo CYP3A4 activity and predict individual responses to CYP3A4-metabolized drugs. Recent progress in pharmacogenetic research has led to the accumulation of knowledge about *CYP3A4* genetic variations responsible for altered expression or function. To date, more than 30 *CYP3A4* variations have been identified (http://www.cypalleles.ki.se/cyp3a4.htm), and large ethnic differences in their frequencies have been recognized. *CYP3A4*1B* (−392A > G), a single nucleotide polymorphism (SNP) in the 5′-flanking region, is found in Caucasians (2–9.6%) and African–Americans (35–67%), but not in Asians [16]. As relatively frequent coding SNPs, *2 [664T > C (Ser222Pro)] (2.7%) and *17 [566T > C (Phe189Ser)] (2%) were detected in Caucasians; *10 [520G > C (Asp174His)] in Caucasians (0.24–2%) and Mexicans (5%); *15 [485G > A (Arg162Gln)] (2–4%) in African–Americans; *16 [554C > G (Thr185Ser)] in East Asians (1.4–5%) and Mexicans (5%); *18 [878T > C (Leu293Pro)] (2.3–10%) in East Asians [2, 4, 17, 24]. We previously identified 25 *CYP3A4* haplotypes in a Japanese population [4]. The haplotypes *6 [including 830_831insA (Glu277fsX8)] (0.1%), *11 [including 1088C > T (Thr363Met)] (0.2%), *16B [including 554C > G (Thr185Ser)] (1.4%), and *18B [including 878T > C (Leu293Pro)] (2.8%) were identified, but *1B (−392A > G) was not found. These findings indicate that ethnic-specific *CYP3A4* haplotypes must be taken into consideration in pharmacogenetic studies.

Irinotecan, an anticancer prodrug, is used for treatment of various cancers including lung and colon, and metabolized by CYP3A4 to produce inactive compounds such as APC (a major CYP3A4-mediated product) and NPC (a minor product) [6, 7]. An active metabolite SN-38 (a topoisomerase I inhibitor) is produced from the parent compound by carboxylesterases (CES) [28] and subsequently glucuronidated by UDP-glucuronosyltransferase 1As (UGT1As) to form inactive compound SN-38G [12] (Fig. 1). The parent compound and its metabolites are mainly excreted into the bile [29], where several ABC transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein 2 (MRP2) are involved in excretion [30]. The dose-limiting toxicities of irinotecan are severe diarrhea and neutropenia, and high plasma concentrations of SN-38 and/or its accumulation in tissues are thought to cause these toxicities [3, 30]. Recent extensive pharmacogenetic studies on irinotecan, mostly focusing on the *UGT1A1* genotypes, have revealed important roles for *UGT1A1*28* and *6 in reduced in vivo UGT activity and enhanced toxicities [1, 8, 9, 11, 13, 22, 26]. On the other hand, CYP3A4 can modulate irinotecan pharmacokinetics (PK). Co-administration of ketoconazole, a CYP3A4 inhibitor and also a potent UGT1A1 inhibitor [34], with irinotecan resulted in a decreased value of the area under the concentration–time curve (AUC) for APC and also increased AUC for SN-38 [14]; and vice versa, co-administration of St. John's Wort,

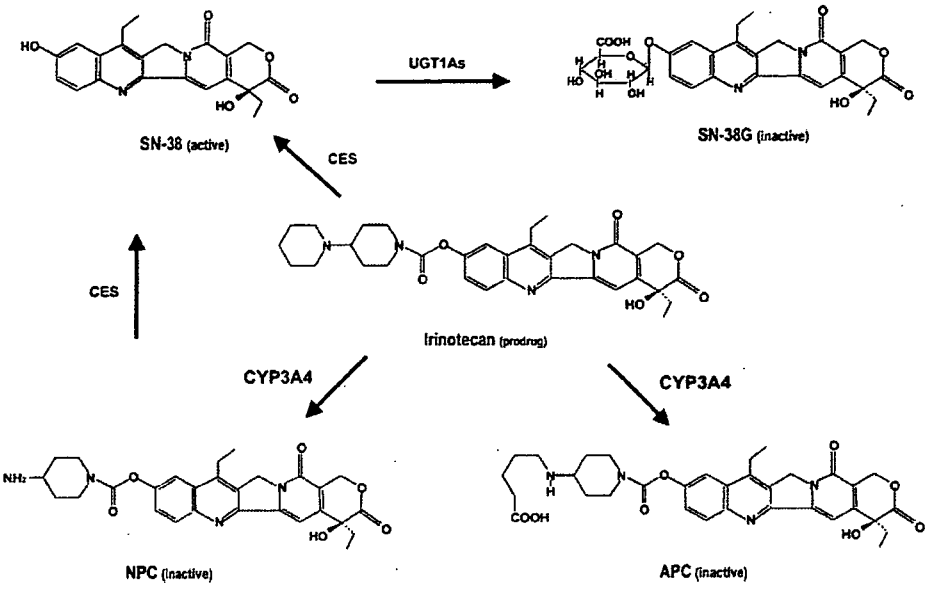



**Fig. 1** Irintoecan metabolism in human liver. CYP3A4 mediates oxidation of irinotecan to produce inactive compounds, such as APC (a major CYP3A4-mediated product) and NPC (a minor product)

a CYP3A4 inducer, decreased the AUC of SN-38 [19]. A close association was also reported between in vivo CYP3A4 phenotypes and irinotecan clearance [21]. To date, however, no clinical impact by *CYP3A4* polymorphisms, such as **1B* (−392A > G) and **3* [1334T > C (Met445Thr)], has been demonstrated on irinotecan PK in Caucasians [20]. We previously found that **16* [554C > G (Thr185Ser)] caused decreased in vitro CYP3A4 activities [23]. Furthermore, a significant association of **16B* [harboring 554C > G (Thr185Ser)] was demonstrated with decreased AUC ratios of metabolite/paclitaxel, an in vivo parameter of CYP3A4 activity, in paclitaxel-administered Japanese patients [24].

In this study, to determine the clinical impact of the *CYP3A4* polymorphisms on irinotecan therapy, we identified the *CYP3A4* diplotypes of 177 Japanese cancer patients who received irinotecan and analyzed associations of the *CYP3A4* genotypes with irinotecan PK and toxicities.

## Materials and methods

### Patients and irinotecan treatment

One hundred seventy-seven patients with cancers who started irinotecan-containing therapy from 2002 to 2004 at two National Cancer Center Hospitals (Tokyo and Kashiwa, Japan) were enrolled for this pharmacogenetic study on irinotecan. This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences, and written informed consent was obtained from all participants. No participant received irinotecan previously, and other eligibility criteria included: bilirubin < 2 mg/dl, aspartate aminotransferase (GOT) < 105 IU/l, alanine aminotransferase (GPT) < 120 IU/l, creatinine < 1.5 mg/dl, white blood cell count > 3000/μl, performance status of 0–2, and an interval of at least 4 weeks after the last session of chemotherapy (2 weeks after the last session of radiotherapy). Exclusion criteria were diarrhea, active infection, intestinal paralysis or obstruction, and interstitial pneumonitis. Irinotecan was administered as a single agent or in combination chemotherapy at the discretion of attending physicians. Doses and schedules were applied according to the approved treatment recommendations in Japan: intravenous 90-min infusion at a dose of 100 mg/m$^2$ weekly or 150 mg/m$^2$ biweekly for irinotecan-monotherapy, and 60 mg/m$^2$ weekly for combination therapy with cisplatin. Profiles of the patients and irinotecan regimens are summarized in Table 1.

### Genotyping of *UGT1A1* and *CYP3A4*

DNA was extracted from pretreatment whole-blood samples taken from 177 patients who received irinotecan. Data on *UGT1A1* genetic polymorphisms obtained from the same set of DNA samples have been published elsewhere [22]. The *CYP3A4* genotypes for 88 patients were previously determined [4]. Additional *CYP3A4* genotyping for the remaining 89 patients was conducted using the pyrosequencing method described previously [24], and the *CYP3A4* diplotypes/haplotypes [4] were inferred using an expectation-maximization-based program, LDSUPPORT [15].

### Pharmacokinetics and toxicities

Pharmacokinetic analysis for irinotecan in 176 patients (data on one patient was unavailable) was performed as

**Table 1** Profiles of Japanese cancer patients in this study

| | | | No. of patients |
|---|---|---|---|
| Patients for genotyping | | | 177 |
| (Male/female) | | | (135/42) |
| Age | | | |
| Mean/range | 60.5/26–78 | | |
| Performance status | 0/1/2 | | 84/89/4 |
| Combination therapy, tumor type and initial dose of irinotecan[a] | | | |
| Irinotecan monotherapy | Lung | 100 (60–100)/w | 21 |
| | Colon | 150 (120–150)/2w | 28 |
| | Others | 100 (100–150)/w | 7 |
| With platinum-containing drug[b] | Lung | 60 (50–90)/w | 58 |
| | Stomach | 70/2w | 9 |
| | Others | 60/w | 5 |
| With 5-fluorouracil (5-FU)/leucovorin (LV)[c] or tegafur/gimeracil/oteracil potassium[d] | Colon | 100 (90–180)/w or 150/2w | 34 |
| | Others | 90/w or 100/w | 2 |
| With mitomycin C (MMC)[e] | Stomach | 150/2w | 10 |
| | Colon | 150/2w | 1 |
| With amrubicin[f] | Lung | 60/w | 2 |

[a] The median value and range in the parentheses are shown. "/w" and "/2w" represent weekly and biweekly, respectively

[b] Mostly, cisplatin (60 or 80 mg/m$^2$) was administered after irinotecan treatment

[c] LV (10 mg/m$^2$) was administered right after irinotecan treatment and then followed by 5-FU treatment. (500 mg/m$^2$ injection); or LV (200 mg/m$^2$) was administered simultaneously with irinotecan and followed by 5-FU treatment (400 mg/m$^2$ bolus injection and 2.0–2.4 g/m$^2$ infusion)

[d] Tegafur (80 mg/m$^2$ per day)/gimeracil/oteracil potassium was administered twice (before irinotecan treatment and on the next day)

[e] MMC (5 mg/m$^2$) was administered just before irinotecan treatment

[f] Amrubicin (30 or 35 mg/m$^2$) was administered 24 h after irinotecan treatment

previously described [26]. Briefly, heparinized blood was collected before administration of irinotecan, and 0, 0.3, 1, 2, 4, 8, and 24 h after termination of the first infusion of irinotecan. Plasma concentrations of irinotecan and APC were determined by HPLC [25], and $AUC_{inf}$ and other PK parameters were calculated using the trapezoidal method of the 202 non-compartmental model for a constant infusion in WinNonlin ver. 4.01 (Pharsight Corporation, Mountain View, CA, USA). As for the co-administered anti-cancer and other drugs which were administered within 1 week before irinotecan-treatment, no drugs significantly affected the PK parameters related to CYP3A4 activity. Information on foods and drinks taken by the patients which might induce or inhibit CYP3A4 activity was not available.

A complete medical history and data on physical examinations were recorded prior to irinotecan therapy. Complete blood cell counts with differentials and platelet counts, as well as blood chemistry, were measured once a week during the first 2 months of irinotecan treatment. Toxicities were graded according to the Common Toxicity Criteria of National Cancer Institute version 2. Association of genetic factors with irinotecan toxicities was analyzed primarily in patients who received irinotecan as a single agent.

## Statistical analysis

Statistical analysis on the differences in PK parameters between sexes and among *CYP3A4* genotypes was performed using the Mann–Whitney test or Kruskal–Wallis test, and associations of *CYP3A4* genotypes with the irinotecan toxicities were assessed by the Chi-square test, using Prism version 4.0 (GraphPad Prism Software Inc. San Diego, CA, USA). $P = 0.05$ (two-tailed) was set as a significant level of difference. Multivariate analysis for the log-transformed AUC ratio (APC/irinotecan) was performed using age, sex, body surface area, dosage of irinotecan, history of smoking or drinking, performance status, co-administered drugs, serum biochemistry parameters at baseline, and genetic factors (including *CYP3A4* haplotypes and the *UGT1A1*6 or *28* haplotype obtained in our previous study [22]) as independent variables. Multivariate analysis on toxicities (grade 3 diarrhea or nadir of absolute neutrophil counts) was conducted for the patients who received irinotecan monotherapy, where the variables included dosing interval and the absolute neutrophil count at baseline, in addition to the other patient background and genetic factors described above. The variables in the final

models for both AUC ratio and toxicities were chosen by the forward and backward stepwise procedure at the significance level of 0.1 using JMP version 6.0.0 software (SAS Institute, Inc., Cary, NC, USA).

## Results

### Sex difference in PK parameters

Since hepatic CYP3A4 levels were reported to be significantly higher in females than in males [24, 32], we first analyzed the sex differences in the major PK parameters for irinotecan and APC, a major CYP3A4 metabolite (Table 2). As for irinotecan, lower total clearance and MRT, and higher AUC/dose were observed in females, but the differences (3, 5 and 3%, respectively) were not significant. A small but significant increase in $C_{max}$/dose for irinotecan was observed in females. This is attributable to the smaller distribution volume of females. On the other hand, the median values of AUC/dose and $C_{max}$/dose for APC of the females were significantly higher than those of the males (1.29- and 1.33-fold, respectively). The AUC ratio (APC/irinotecan), a parameter of in vivo CYP3A4 activity, was significantly higher (1.28-fold) in females than in males. These findings suggest that these differences may reflect the higher CYP3A4 activity in the females.

### *CYP3A4* genotypes

*CYP3A4* diplotypes/haplotypes in 177 Japanese cancer patients were determined according to the previous definition [4]. The *CYP3A4* haplotypes found in this population were **1A* (wild type), **1G* (IVS10 + 12G > A alone), **16B* [554C > G (Thr185Ser) and IVS10 + 12G > A], and **18B* [878T > C (Leu293Pro) and IVS10 + 12G > A]. In the current study, neither **6* [830_831insA (Glu277fsX8)] nor **11* [1088C > T (Thr363Met)] were found. The frequencies of **1G*, **16B*, and **18B* were 0.215, 0.014, and 0.020 (Table 3), and they were comparable to those obtained in previous reports [4, 24]. Note that the haplotypes **16B* and **18B* were detected only in male patients.

### Associations of *CYP3A4* genotypes with PK parameters

Considering the significant sex difference in APC levels, associations between the *CYP3A4* genotypes and PK parameters were analyzed for each sex separately. In male patients, no significant differences among the *CYP3A4* genotypes were observed for total clearance and MRT of irinotecan (Fig. 2a, b). In females, a slightly but significantly lower (10%) median value for MRT of irinotecan was observed in patients bearing **1G* compared with those carrying the wild type (**1A/*1A*) ($P = 0.022$, Mann–Whitney test) (Fig. 2b), whereas no significant **1G*-dependency was observed for total clearance (Fig. 2a). No significant

**Table 3** Frequencies of *CYP3A4* haplotypes (A) and diplotypes (B) for Japanese cancer patients in this study

| (A) Haplotype group[a] | No. of chromosomes ($N = 354$) | Frequency |
|---|---|---|
| **1A* | 266 | 0.751 |
| **1G* | 76 | 0.215 |
| **16B* | 5 | 0.014 |
| **18B* | 7 | 0.020 |
| **(B) Diplotype** | **No. of patients ($N = 177$)** | **Frequency** |
| **1A/*1A* | 100 | 0.565 |
| **1G/*1A* | 55 | 0.311 |
| **1G/*1G* | 10 | 0.056 |
| **16B/*1A* | 4 | 0.023 |
| **16B/*1G* | 1 | 0.006 |
| **18B/*1A* | 7 | 0.040 |

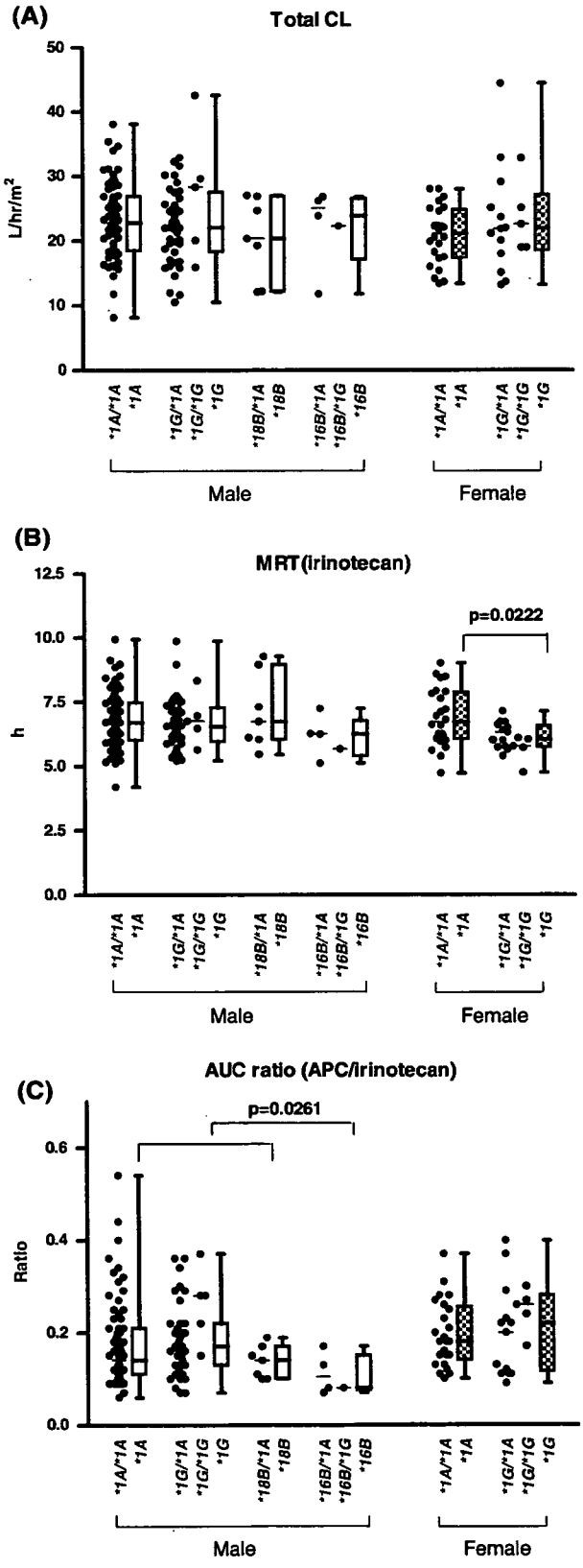
[a] Groups based on tagging SNPs of major haplotypes previously defined [4]; **1A* wild type, **1G* IVS10 + 12G > A; **16B* 554C > G (Thr185Ser) and IVS10 + 12G > A; **18B* 878T > C (Leu293Pro) and IVS10 + 12G > A

**Table 2** Pharmacokinetic parameters for irinotecan-administered Japanese patients and sex differences

| Parameters | Male ($N = 134$) Median (25–75‰) | Female ($N = 42$) Median (25–75‰) | $P$ value[a] |
|---|---|---|---|
| Irinotecan | | | |
| Total CL (l/h per $m^2$) | 22.6 (18.5–26.9) | 21.8 (17.8–25.1) | 0.242 |
| AUC/dose ($10^{-3}$ h $m^2$ per l) | 44.4 (37.3–54.1) | 45.8 (39.8–55.8) | 0.242 |
| $C_{max}$/dose ($10^{-3}$ $m^2$ per l) | 10.0 (8.96–11.3) | 11.4 (10.4–12.4) | 0.0003 |
| MRT (h) | 6.61 (6.01–7.40) | 6.29 (5.78–7.12) | 0.202 |
| APC | | | |
| AUC/dose (10 h $m^2$ per l) | 6.72 (5.23–9.49) | 8.66 (6.57–13.1) | 0.0071 |
| $C_{max}$/dose ($10^{-3}$ $m^2$ per l) | 0.560 (0.430–0.805) | 0.745 (0.610–1.14) | 0.0007 |
| AUC ratio (APC/irinotecan) | 0.151 (0.114–0.210) | 0.194 (0.132–0.266) | 0.0179 |

*CL* clearance; *MRT* mean residence time

[a] Mann–Whitney test




◀ **Fig. 2** Association of *CYP3A4* genotypes with irinotecan pharmacokinetics in Japanese cancer patients. The values of mean residence time (MRT) of irinotecan in female patients were significantly lower in those with **1G* than those with the wild-type (**1A/*1A*) ($P = 0.0222$, Mann–Whitney test). The levels of the AUC ratio (APC/irinotecan), a parameter of CYP3A4 activity, in male patients were significantly lower in those with **16B* than those without **16B* ($P = 0.0261$, Mann–Whitney test)

differences in $C_{max}$/dose for irinotecan among the genotypes were observed in both males and females (data not shown). Regarding the AUC ratio (APC/irinotecan) in males, a significantly lower median value (50%) was observed in patients with **16B* than patients without **16B* (i.e., *non-*16B* patients) ($P = 0.0261$, Mann–Whitney test) (Fig. 2c). In contrast, no significant changes in the AUC ratio (APC/irinotecan) were detected in the male **18B* heterozygotes. In both males and females, a higher median AUC ratio (20%), without statistical significance, was observed in **1G*-bearing patients (**1G/*1A* and **1G/*1G*) than wild-type patients (**1A/*1A*). As for $C_{max}$/dose of APC, similar trends were observed (without statistical significance): 35% decrease in the median value for **16B* compared with *non-*16B*; 10 and 20% increases in males and females, respectively, for **1G* compared with the wild type (data not shown).

Multivariate analysis of PK parameters

To further clarify contributions of the *CYP3A4* polymorphisms to APC generation, multivariate analysis was conducted on the AUC ratio (APC/irinotecan) data, where variables included patient backgrounds, irinotecan regimens, and *CYP3A4* (**1G, *16B* and **18B*) and *UGT1A1* (**6 or *28*) haplotypes. Significant contributions of *CYP3A4*16B* (coefficient ± SE = $-0.18 \pm 0.077$, $P = 0.021$) and **1G* ($0.047 \pm 0.021$, $P = 0.029$) to the AUC ratio (APC/irinotecan) were confirmed, in addition to the contributions of two patient background factors, sex (female) and hepatic function (serum GOT and ALP) (Table 4). No significant associations were observed between the *CYP3A4* polymorphisms and total clearance or MRT of irinotecan (data not shown).

Associations of *CYP3A4* genotypes with toxicities

Severe irinotecan toxicities, grade 3 diarrhea and grade 3 or 4 neutropenia, were monitored in 176 patients during 2 months after starting irinotecan therapy. Since incidences of severe toxicities depended on the irinotecan regimens used and a higher incidence of severe neutropenia with comedication was evident [22], associations of the *CYP3A4*

**Table 4** Multivariate analysis of AUC ratio (APC/irinotecan)

| Variable | Coefficient | SE | *P* value |
|---|---|---|---|
| Female | 0.040 | 0.016 | 0.0132 |
| Serum GOT and ALP[a] | 0.110 | 0.021 | <0.0001 |
| Serum creatinine[b] | 0.132 | 0.071 | 0.0651 |
| *CYP3A4*16B* | −0.180 | 0.077 | 0.0213 |
| *CYP3A4*1G* | 0.047 | 0.021 | 0.0291 |

The values after logarithmic conversion were used

$R^2$ 0.225; Intercept −0.794; *N* 176

[a] Grade 1 or greater scores in both serum GOT and ALP before irinotecan treatment

[b] The absolute value (mg/dl) before irinotecan treatment

haplotypes with toxicities were evaluated in patients who received irinotecan monotherapy. Because there was no sex difference in the incidences of severe toxicities, the patients with irinotecan monotherapy were not stratified by sex. Furthermore, significant contributions of *UGT1A1*6* and **28* to neutropenia were previously demonstrated [22]. Therefore, the incidence of severe neutropenia was also evaluated among the wild-type patients without *UGT1A1*6 or *28* (*UGT −/−*). No significant differences in the incidences of severe diarrhea and neutropenia were observed among the *CYP3A4* diplotypes of all or *UGT −/−* patients with irinotecan monotherapy (Table 5). It must be noted that the **16B*-bearing patient ($N = 1$) treated with irinotecan monotherapy did not experience either toxicity. Similarly, for **1G* and **18B*, no statistically significant change in the neutropenia or diarrhea incidence was observed. Multivariate analysis also revealed no significant contribution of the *CYP3A4* polymorphisms to severe diarrhea (logistic model) or absolute neutrophil count nadir (data not shown).

**Table 5** Association of *CYP3A4* genotypes with severe toxicities in irinotecan monotherapy

| Diplotype | Diarrhea[a]/total (%) | Neutropenia[b]/total (%) | |
|---|---|---|---|
| | All | All | UGT-/-[c] |
| **1A/*1A* | 3/27 (11.1) | 5/27 (18.5) | 2/11 (18.2) |
| **1G/*1A* | 2/20 (10.0) | 5/20 (25.0) | 1/9 (11.1) |
| **1G/*1G* | 0/3 (0.0) | 2/3 (66.7) | 0/0 (–) |
| **16B/*1A* | 0/1 (0.0) | 0/1 (0.0) | 0/0 (–) |
| **18B/*1A* | 1/4 (25.0) | 2/4 (50.0) | 0/1 (0.0) |
| *P* value[d] | 0.8571 | 0.289 | |

[a] Grade 3

[b] Grade 3 or 4

[c] Wild type without *UGT1A1 *6 or *28*

[d] Chi-square test

## Discussion

In the current study, the higher in vivo CYP3A4 activity in females than in males [24, 32] was suggested from the CYP3A4-mediated APC formation. Since correlations between in vivo CYP3A4 activity and irinotecan PK parameters have been reported [14, 19, 21], clinical impact of *CYP3A4* polymorphisms on irinotecan PK has been presumed. In this study, we demonstrated for the first time a role of *CYP3A4*16B* [554C > G (Thr185Ser) and IVS10 + 12G > A] in reduced APC generation (Fig. 2; Table 4). This finding is concordant with the findings of our previous studies showing a reduced in vitro activity of CYP3A4 by **16* [23] and altered AUC ratios of metabolite/paclitaxel in paclitaxel-administered Japanese patients bearing **16B* [24]. These findings indicate that *CYP3A4*16* could modulate pharmacokinetics of other drugs which are metabolized by CYP3A4. On the contrary, **18B* [878T > C (Leu293Pro) and IVS10 + 12G > A] did not alter the AUC ratios (APC/irinotecan) in irinotecan-administered patients. This also coincides with our previous finding that showed no clinical impact of **18B* on the metabolite/paclitaxel AUC ratio [24].

In the current study, an increasing trend in the AUC ratios (APC/irinotecan) by **1G* (IVS10 + 12G > A) was detected in both males and females, although their increases were small (20% in the median values). In accordance with this tendency, significant reduction in MRT of irinotecan by **1G* was observed in females, whereas this was not significant in males. At present, the reason of this sex-difference in MRT is not clear. Our previous haplotype analysis of the *CYP3A4* and *CYP3A5* regions revealed that *CYP3A4*1G* is mostly linked to *CYP3A5*1* but rarely to *CYP3A5*3* [3] which is a defective allele [10, 16, 17, 33]. Therefore, there is a possibility that *CYP3A5* polymorphisms rather than *CYP3A4*1G* contribute to irinotecan PK. However, this speculation is unlikely because CYP3A5 produces only a very minor metabolite of irinotecan, a de-ethylated product [27]. Since the effect of **1G* was relatively small and was not shown in case of paclitaxel [23], the clinical importance of **1G* should be further evaluated in pharmacogenetic studies on other drugs.

Contrary to the clear reduction in APC production, changes in the PK parameters for the parent compound, i.e., total clearance and Cmax of irinotecan, were not affected by the *CYP3A4* haplotypes. Furthermore, multivariate analysis revealed no associations of the *CYP3A4* haplotypes with the AUC ratio of (SN-38 + SN-38G)/irinotecan, an in vivo parameter for CES activity, and with the AUC ratio of SN-38 (SN-38/irinotecan) (data not shown). We previously observed that the total clearance of irinotecan was affected by other non-genetic factors, such as age, smoking, hepatic and renal functions, and co-administered drugs

(unpublished data), and that the plasma level of SN-38 was largely influenced by *UGT1A1*6* and **28* [22]. Therefore, it is likely that the contribution of CYP3A4 to irinotecan clearance is rather small as compared with other genetic and non-genetic factors.

In accordance with the above observations, no significant associations were observed between the *CYP3A4* haplotypes and severe toxicities (grade 3 diarrhea and grade 3 or 4 neutropenia) in the patients with irinotecan monotherapy (Table 5). Similarly, we observed no significant effect of the *CYP3A4* haplotypes on incidence of the severe toxicities in the patients treated with both irinotecan and cisplatin (data not shown), although the numbers of patients bearing **16B* and **18B* were small. Taken together, the current study indicates that the influence of the *CYP3A4* genotypes on the activation pathway of irinotecan (generation of SN-38) might be small.

In conclusion, the current study suggested that *CYP3A4*16B* was associated with decreased metabolism of irinotecan to APC. However, impact of the *CYP3A4* haplotypes on total clearance of irinotecan and severe toxicities was not significant.

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# Genetic variations and haplotypes of *ABCC2* encoding MRP2 in a Japanese population

Running title: *ABCC2* haplotypes in Japanese

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Footnotes

As of October 7, 2007, the novel variations reported here are not found in the database of Japanese

Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

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**Abstract**

The multidrug resistance-associated protein 2 (MRP2) encoded by the *ABCC2* gene is expressed in the liver, intestine and kidneys and preferentially exports organic anions or conjugates with glucuronide or glutathione. In this study, all 32 exons and the 5'-flanking region of *ABCC2* in 236 Japanese were resequenced, and 61 genetic variations including 5 novel nonsynonymous ones were detected. A total of 64 haplotypes were determined/inferred and classified into five *1 haplotype groups (*1A, *1B, *1C, *1G, and *1H) without nonsynonymous substitutions and *2 to *9 groups with nonsynonymous variations. Frequencies of the major 4 haplotype groups *1A (-1774delG), *1B (no common SNP), *1C (-24C>T and 3972C>T), and *2 [1249G>A (Val417Ile)] were 0.331, 0.292, 0.172, and 0.093, respectively. This study revealed that haplotype *1A, which has lowered activity, is quite common in Japanese, and that the frequency of *1C, another functional haplotype, was comparable to frequencies in Asians and Caucasians. In contrast, the haplotypes harboring 3972C>T but not -24C>T (*1G group), which are reportedly common in Caucasians, were minor in Japanese. Moreover, the allele 1446C>T (Thr482Thr), which has increased activity, was not detected in our Japanese population. These findings imply possible differences in MRP2-mediated drug responses between Asians and Caucasians.

**Key words:** *ABCC2*, MRP2, genetic variation, haplotype, amino acid change

**Introduction**

The multidrug resistance-associated protein 2 (MRP2) or canalicular multispecific organic anion transporter (cMOAT) is a 190-200 kDa transmembrane glycoprotein comprised of 1545 amino acids and belongs to the superfamily C of ATP-binding cassette (ABC) transporters. This transporter is expressed on hepatic canalicular membranes, intestinal apical membranes, luminal membranes of renal proximal tubules, placental epithelial cells, and the blood brain barrier [1]. MRP2 exports endogenous and exogenous substances, preferentially organic anions or conjugates with glucuronide, glutathione and sulfate [1-3]. This protein originally identified in cisplatin-resistant tumor cells [4] is shown to confer drug resistance to other anti-cancer drugs, such as vincristine and doxorubicin [5, 6].

MRP2 is encoded by the *ABCC2* gene located on chromosome 10q24 and consists of 32 exons (31 coding exons) and spans 69 kb. Several *ABCC2* genetic variations have been detected in patients with Dubin-Johnson syndrome (DJS), an autosomal recessive disease characterized by hyperbilirubinemia with conjugated bilirubin or increased coproporphyrin excretion in urine [2,7]. Recent studies on *ABCC2* have identified common single nucleotide polymorphisms (SNPs) such as -24C>T and -3972C>T (Ile1324Ile) among several ethnic populations, and several studies have suggested their association with altered MRP2 expression or function [8-17]. In more recent studies on *ABCC2* haplotypes covering an extended 5'-flanking region, close linkages were found among -1549A>G in the 5'-flanking region and two common SNPs -24C>T and -3972C>T (Ile1324Ile) [8]. In addition, as possible functional SNPs, -1774delG in Koreans [8] and -1019A>G in Caucasians [10] were reported. However, there is little information on detailed haplotype structures throughout the gene, and comprehensive haplotype analysis in Japanese has not yet been conducted.

We previously analyzed *ABCC2* genetic variations within all 32 exons and the proximal 5'-flanking region (approximately 800 bp upstream of the translation initiation site) using established cell lines derived from Japanese cancer patients to obtain preliminary information on *ABCC2* SNPs in Japanese [18]. In this study, to reveal *ABCC2* haplotype structures in Japanese, we resequenced the *ABCC2* gene including the distal 5'-upstream region (approximately 1.9 kb upstream from the

translation initiation site) as well as all 32 exons in 236 Japanese subjects and conducted haplotype analysis using the detected genetic polymorphisms.

## Materials and Methods

### Human DNA samples

Genomic DNA samples were obtained from blood leukocytes of 177 Japanese cancer patients at two National Cancer Center Hospitals (Tokyo and Chiba, Japan) and Epstein-Barr virus-transformed lymphoblastoid cells prepared from 59 healthy Japanese volunteers at the Tokyo Women's Medical University under the auspices of the Pharma SNP consortium (Tokyo, Japan). Written informed consent was obtained from all subjects. Ethical review boards of all participating organizations approved this study.

### PCR conditions for DNA sequencing

We sequenced all 32 exons of the *ABCC2* gene and approximately 800 bp upstream of the translation initiation codon (proximal 5'-flanking region) as described previously [18] and also extended the sequenced region to 1.9 kb upstream of the translation initiation site (distal 5'-flanking region). Briefly, for amplification of the proximal 5'-flanking region and 32 exons, 5 sets of multiplex PCR were performed from 200 ng of genomic DNA using 1.25 units of Z-taq (Takara Bio. Inc., Shiga, Japan) with 0.3 uM each of the mixed primers as shown in Table 1 [1st PCR]. The first PCR conditions consisted of 30 cycles of 98℃ for 5 sec, 55℃ for 5 sec, and 72℃ for 190 sec. Next, each exon was amplified separately using the 1st PCR product by Ex-Taq (0.625 units, Takara Bio. Inc.) with appropriate primers (0.3 uM) [Table 1, 2nd PCR]. The conditions for the second round PCR 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. For amplification of the distal 5'-flanking region, multiplex PCR was performed from 25 ng of genomic DNA using 1 unit of Ex-Taq (Takara Bio. Inc.) with 0.4 uM each of the 2 sets of primers as shown in Table 1 [PCR]. The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min.

Following the PCR, products were treated with a PCR Product Pre-Sequencing Kit (USB Co.,

Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in Table 1 (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All variations were confirmed by sequencing PCR products generated from new amplifications from genomic DNA. Genbank NT_030059.12 was used as the reference sequence.

**Linkage disequilibrium (LD) and haplotype analyses**

Hardy-Weinberg equilibrium and LD analyses were performed using SNPAlyze 3.1 software (Dynacom Co., Yokohama, Japan). Pairwise LDs were shown as rho square ($r^2$) and |D'| values in Figure 1. Diplotype configurations (haplotype combinations) were inferred by LDSUPPORT software, which determined the posterior probability distribution of diplotype configurations for each subject based on estimated haplotype frequencies [19].

**Results and Discussion**

In this study, sixty-one *ABCC2* genetic variations including 36 novel ones were detected in 236 Japanese subjects (Table 2). All detected variations were in Hardy-Weinberg equilibrium ($p>0.05$). Novel variations consisted of 5 non-synonymous and 4 synonymous variations in the coding region, 22 in the intronic regions, 3 in the 5'-flanking region, 1 in the 3'- flanking region, and 1 in the 3'-UTR. The novel non-synonymous variations were 1177C>T (Arg393Trp), 1202A>G (Tyr401Cys), 2358C>A (Asp786Glu), 2801G>A (Arg934Gln), and 3320T>G (Leu1107Arg), and their frequencies were 0.002. No statistically significant differences were found in the allele frequencies of all variations between 177 cancer patients and 59 healthy subjects ($P>0.05$, Fisher's exact test), although a larger number of subjects would be needed to conclude.

The frequency of the known common SNP -24C>T (0.173) was comparable to those reported in Asians (0.17-0.25) [8, 12, 20] and Caucasians (0.15-0.23) [9, 10, 14, 15, 21]. The allele frequency of another common SNP, 3972C>T (Ile1324Ile) (0.216), was also comparable to those in Asians (0.22-0.30) [8, 12, 20] but lower than those in Caucasians (0.32-0.37) [9, 10, 14, 15, 21]. The other major variations in the 5'-flanking region, -1774delG and -1549G>A, were found at frequencies of 0.343 and 0.203, respectively, and these values were similar to those obtained in Koreans (0.34 and 0.21, respectively) [8]. However, the relatively frequent SNPs 1446C>G (Thr482Thr) (allele frequency = 0.125), IVS15-28C>A (0.333) and IVS28+16G>A (0.167) in Caucasians [17] were not detected in our study.

The LD profile of the *ABCC* variations (no less than 3% allele frequency) is shown in Fig. 1. As assessed by $r^2$ values, close linkages were observed among -1774delG, -1023G>A and IVS29+154A>G, and among -1549G>A, -1019A>G, -24C>T, IVS3-49C>T, IVS12+148A>G, IVS15+169T>C, IVS16-105C>T, IVA23+56C>T, IVS27+124C>G, and 3972C>T (Ile1324Ile). It must be noted that complete linkage was observed between -1549G>A and -1019A>G in our population. In |D'| values, strong LD was also observed almost throughout the region analyzed. Overall, since close associations between the variations were observed throughout the entire *ABCC2*

gene, the region sequenced was analyzed as a single LD block for the haplotype inference.

The *ABCC2* haplotype structures were analyzed using 61 detected genetic variations and a total of 64 haplotypes were identified/inferred. Figure 2 summarizes the haplotypes and their grouping. Our nomenclature system is based on the recommendation of Nebert [22]. Haplotypes without any amino acid substitution were assigned as the *1 group and named with small alphabetical letters in descending frequency order (*1a to *1x). Haplotypes with nonsynonymous variations were assigned from *2 to *9 groups, and their subtypes were named with small alphabetical letters. The haplotypes (*7a to *9a) were inferred in only one patient and described with "?" due to their ambiguity. Also, ambiguous rare haplotypes in the *1 and *2 groups were classified as "Others" in Figure 2. The *1 haplotypes were further classified into the *1A, *1B, *1C, *1G and *1H groups (capital alphabetical letters of the most frequent haplotypes were used) according to the common tagging SNPs, such as -1774delG, -24C>T, 3972C>T (Ile1324Ile), and 2937G>A (Ser978Ser).

The most frequent *1 group, *1A, harbors the common SNPs -1774delG and -1023G>A in the 5'-flanking region and mostly IVS29+154A>G, and the frequency of *1A (0.331) is almost the same as that in healthy Koreans (0.323) reported by Choi *et al.* [8]. They have shown that -1774delG reduced promoter activity both at the basal level and after induction by chenodeoxycolic acid (CDCA), a component of bile acids, and that the haplotype bearing -1774delG is associated with chemical-induced hepatitis (cholestatis and mixed types) [8]. Therefore, it is possible that *1A can affect the pharmacokinetics or pharmacodynamics of MRP2-transported drugs.

The *1B group haplotypes (0.292 frequency) harbor no or any intronic or synonymous variations the functions of which are unknown. The functional significance of variations in the *1B group, including the most frequent SNP IVS24+25T>C, needs further confirmation.

The third group *1C (0.172 frequency) harbors the known common SNPs -1549G>A, -1019A>G, -24C>T, IVS3-49C>T, and 3972C>T (Ile1324Ile), except for one rare ambiguous haplotype lacking 3972C>T (Ile1324Ile). The *1C haplotypes also harbor IVS12+148A>G, IVS15+169T>C and IVS16-105C>T. The haplotypes bearing -1549G>A, -24C>T and 3972C>T