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Influence of common variants in the pharmacokinetic genes (*OATP-C*, *UGT1A1*, and *MRP2*) on serum bilirubin levels in healthy subjects

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Abstract

To assess the contribution of OATP-C to the hepatobiliary transport of bilirubin, a pharmacogenomic evaluation with regard to polymorphisms of three candidate genes, *OATP-C*, *MRP2*, and *UGT1A1*, was performed. Serum total and direct (conjugated) bilirubin levels were used as phenotypic indexes. Pharmacokinetic variables of pravastatin, a typical substrate for OATP-C, were obtained from our previous study. Among 23 volunteers, two variants (Val417Ile and Ser789Phe) were observed in the *MRP2* gene. While there was no apparent effect of these two variants and the *UGT1A1*28* on direct bilirubin levels, the *OATP-C* variants were associated with differences in unconjugated bilirubin levels. Subjects with the *OATP-C*15* allele had higher bilirubin levels; unconjugated bilirubin levels in *1b/*1b ($n = 3$), *1b/*15 ($n = 7$), and *15/*15 ($n = 1$) subjects were 0.40 ± 0.10, 0.77 ± 0.35, and 0.70 (mg/dL), respectively. In addition, the correlation between unconjugated bilirubin levels and pharmacokinetic parameters of pravastatin revealed that the subjects with higher bilirubin levels had lower non-renal clearance values, and then higher serum concentrations of pravastatin. Large clinical studies are needed to confirm a role of OATP-C in the carrier-mediated uptake of bilirubin in the human liver.

Keywords: Organic anion transporter; Polymorphism; Hepatobiliary transport; Pravastatin

1. Introduction

After rapidly and selectively being taken up from blood into the liver [1], bilirubin is conjugated with glucuronate by uridine diphosphate glucuronosyltransferase (UGT) isoform 1A1 (UGT1A1) [2,3], and then secreted into bile across the canalicular membrane of hepatocytes by multidrug resistance-associated protein 2 (MRP2, symbol ABCC2) [4,5]. Although the mechanisms responsible for the uptake of bilirubin by hepatocytes have not yet been well defined in humans, passive diffusion and/or carrier-mediated transport by specific membrane proteins have been proposed [6–8]. Among known transporters expressed in the basolateral membrane of human hepatocytes [9,10], a recent in vitro study indicated that organic anion transporting polypeptide C (OATP-C, also known as OATP2 and LST-1; symbol

Abbreviations: UGT1A1, uridine diphosphate glucuronosyltransferase 1A1; MRP2, multidrug resistance-associated protein 2; OATP-C, organic anion transporting polypeptide C; DJS, Dubin–Johnson syndrome; AUC, area under the serum concentration–time curve; CLnr, non-renal clearance; SSCP, single-strand conformational polymorphism analysis

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SLC21A6) may play a key role in the hepatocellular transport of unconjugated bilirubin [11]. We have recently studied in vivo the functional significance of genetic polymorphisms of the human *OATP-C* gene to the disposition kinetics of pravastatin (a substrate for OATP-C) and indicated that certain variants, such as Val174Ala (*OATP-C*15*), are likely to be associated with a reduction in hepatocellular uptake of pravastatin, resulting in less non-renal clearance and higher serum concentrations [12]. Thus, it is hypothesized that polymorphisms of the *OATP-C* gene could modulate the hepatic transport of unconjugated bilirubin. In contrast to the uptake process-mediated predominantly by OATPs, MRP2 is known to be involved in the hepatobiliary excretion of various organic anions. A deficiency of MRP2 is known to cause Dubin–Johnson syndrome (DJS), a rare autosomal recessive liver disorder characterized by chronic conjugated hyperbilirubinemia [13–15]. Although various causative mutations for DJS have been identified in the *MRP2* gene [13–16], no study has examined the contribution of naturally occurring and relatively frequently observed variants to bilirubin levels. In contrast to transporter-mediated DJS, the molecular defect in Gilbert's syndrome, a benign unconjugated hyperbilirubinemia, is reported to be the presence of an additional TA repeat [$(TA)_6TAA \rightarrow (TA)_7TAA$] in the TATA sequence of the *UGT1A1* gene [17], designated as *UGT1A1*28* [18]. Thus, both transport and metabolism processes should be taken into consideration, when examining the hepatobiliary kinetics of bilirubin.

It is useful to accumulate findings with regard to the factors responsible for large inter-individual differences in hepatic uptake and efflux processes of endogenous and xenobiotic compounds not only for individualized drug therapy but also for understanding the pathogenesis of liver disease. In the present study, in order to gain further insight into the relationship between *OATP-C* polymorphism and hepatic bilirubin transport, we have re-evaluated our previously published study [12], but have focused on the bilirubin transport capability. In addition, we examined the effects of certain mutations of the *MRP2* gene on direct bilirubin levels in serum. This is the first in vivo preliminary report that unconjugated bilirubin is likely to be taken up into the liver by OATP-C.

2. Subjects and methods

2.1. Subjects and study design

After approval by the ethics review board of Kyushu Pharmacology Research Clinic and Tottori University Hospital, 23 healthy male volunteers (age, 21–40 years) gave written informed consent to participate in the study. They were nonsmokers who had not taken any medication or alcohol in the 7 days before the study. Their health status was judged to be normal on the basis of a physical examination with screening of blood chemistries, a complete blood count and urinalysis before the study. Hemolysis was excluded on the basis of normal hemoglobin and haptoglobin values and reticulocyte counts. The pharmacokinetic variables of pravastatin [area under the serum concentration–time curve (AUC) and non-renal clearance (CLnr)] were obtained from our previous study [12]. In that study, the volunteers received a single oral dose of 10 mg of pravastatin (Mevalotin; Sankyo Co. Ltd., Tokyo, Japan), after an overnight fast. Serial blood samples were collected from time 0 to 24 h, after pravastatin administration. In addition, urine samples were collected for 24 h. Serum concentrations of pravastatin and its chemical degradation product, RMS-416, and urinary concentrations of pravastatin were quantified by a highly specific and sensitive HPLC-triple-quadrupole mass spectrometry [12].

2.2. Serum bilirubin concentrations

To examine whether certain mutations in the three genes of interest alter transport activity, direct and unconjugated bilirubin values were used as phenotypic indexes; the unconjugated bilirubin value for each subject was calculated by subtracting the direct bilirubin value in serum from the total value. The samples were stored at 4 °C in the dark, and serum bilirubin concentrations were measured using the vanadate oxidation method within 12 h (Hitachi 7070; Hitachi, Tokyo, Japan).

2.3. Genotyping of OATP-C, MRP2 and UGT1A1*28

DNA was isolated from blood samples using the Toyobo blood kit on a Toyobo HMX-2000 robot (Toyobo, Osaka, Japan). The genotype of *MRP2* was identified by single-strand conformational polymorphism (SSCP) analysis [19]. Details of the genotyping and haplotype analysis of the *OATP-C* gene were described previously [12]. Among known functional variants, a promoter variant of the *UGT1A1* gene, *UGT1A1*28*, is the most common in Asian populations [20]. The genotype of *UGT1A1*28* was determined by previously described methods [21,22]. In the present study, alleles with the GenBank™/EMBL accession numbers AB026257 and AJ132573, and U63970 were used as a reference for *OATP-C* and *MRP2*, respectively.

2.4. Statistical analysis

The statistical analysis to evaluate differences between groups was carried out using Mann–Whitney *U*-test. For examining the correlation between unconjugated bilirubin and certain pharmacokinetic variables, Spearman's rank correlation test was used. $P < 0.05$ was considered as significant.

3. Results

In the *MRP2* gene, two non-synonymous mutations, 1249G > A (Val417Ile) in exon 10 and 2366C > T (Ser789Phe) in exon 18, were observed; three were heterozygotes for isoleucine at position 417, one was homozygote

Table 1
Bilirubin values in healthy subjects with various *MRP2*, *UGT1A1*, and *OATP-C* genotypes

Gene	Genotype pattern		n	T.Bil (mg/dL)	D.Bil (mg/dL)	(T-D). Bil (mg/dL)
MRP2						
	No mutation		18	0.75 ± 0.34	0.19 ± 0.08	0.56 ± 0.27
	Val417 > Ile	Heterozygotes	3	0.83 ± 0.35	0.17 ± 0.06	0.67 ± 0.31
		Homozygote	1	0.50	0.10	0.40
	Ser789 > Phe		1	0.90	0.20	0.70
UGT1A1						
	$(TA)_6/(TA)_6$		19	0.75 ± 0.30	0.18 ± 0.06	0.56 ± 0.26
	$(TA)_6/(TA)_7$		3	0.57 ± 0.12	0.13 ± 0.06	0.43 ± 0.06
	$(TA)_7/(TA)_7$		1*	1.50	0.40	1.10
OATP-C						
	*OATP-C*1a/*1b*		2	0.80	0.20	0.60
	*OATP-C*1b/*1b*		3	0.57 ± 0.15	0.17 ± 0.06	0.40 ± 0.10
	*OATP-C*1b/*15*		7	0.99 ± 0.40	0.21 ± 0.07	0.77 ± 0.35
	*OATP-C*15/*15*		1	0.90	0.20	0.70
	*OATP-C*1b/*16*		2	0.60	0.20	0.40

Values are the mean ± S.D. *His *OATP-C* genotype was *OATP-C*1b/*15*, T.Bil, total bilirubin; D.Bil, direct bilirubin; (T-D).Bil, unconjugated bilirubin.

for isoleucine, and one was heterozygote for phenylalanine at position 789. There were no remarkable differences in the mean direct bilirubin levels among the genotypic groups, and values for all volunteers were within the normal range (i.e., 0.1–0.3 mg/dL) (Table 1).

Among 23 healthy volunteers, three were heterozygous and one was homozygous for the *UGT1A1*28* allele. Although the mean (±S.D.) total (0.80 ± 0.48 versus 0.75 ± 0.30) and unconjugated (0.60 ± 0.34 versus 0.56 ± 0.26) bilirubin levels were not significantly different between subjects with (n = 4) and without the **28* allele (n = 19), one homozygote for the **28* allele also having the *OATP-C*15* allele as heterozygosity, had the highest bilirubin values among the study subjects.

Serum bilirubin levels in various *OATP-C* genotypic groups are also shown in Table 1. In this comparison, we excluded subjects having either *MRP2* or *UGT1A1* polymorphisms in order to evaluate net in vivo effect of the *OATP-C* gene polymorphism. The mean (±S.D.) unconjugated bilirubin levels in *OATP-C*1a/*1b*, *1b/*1b, *1b/*15, and *15/*15 subjects were 0.6, 0.40 ± 0.10, 0.77 ± 0.35 and 0.7, respectively; although the difference did not reach the significant level, the mean value tended to be larger in heterozygotes for the **15* allele (**1b/*15*) than homozygotes for the **1b* allele (**1b/*1b*).

To determine whether and to what extent OATP-C contributes to the uptake of bilirubin, correlations between unconjugated bilirubin levels and pharmacokinetic parameters of pravastatin were evaluated. These results are shown in Fig. 1. There was a positive correlation of the AUC of pravastatin with unconjugated bilirubin levels (r_s = 0.26; P < 0.05). In contrast, for the association between unconjugated bilirubin and non-renal clearance of pravastatin, there was a significant negative trend (r_s = 0.36; P < 0.05).

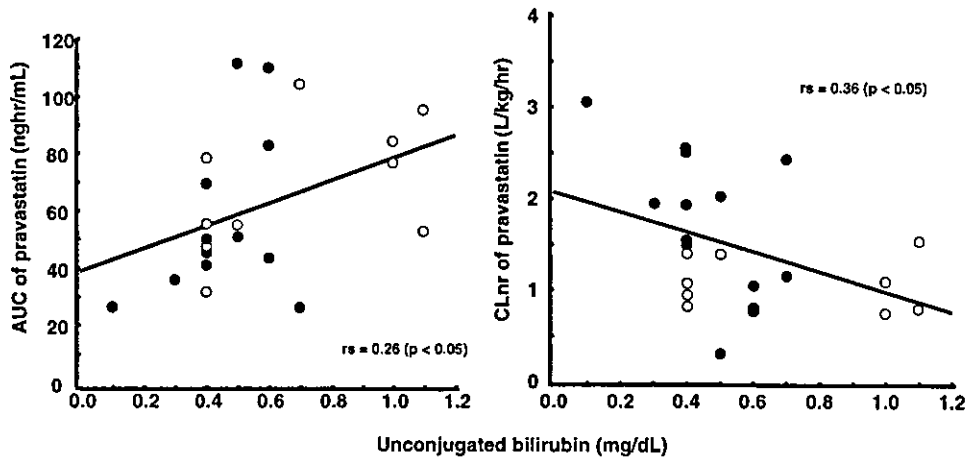

Fig. 1. Pharmacokinetic parameters of pravastatin and unconjugated bilirubin level. Spearman rank correlation (r_s) between the unconjugated bilirubin level and area under the serum concentration–time curve (AUC) (left panel), and non-renal clearance (CLnr) of pravastatin (right panel) after administration of a single 10-mg dose in 23 healthy subjects. Open and closed circles indicate the subjects with and without the *OATP-C*15* allele, respectively.

4. Discussion

In the present study, two non-synonymous variants in the *MRP2* gene [i.e., 1249G > A (Val417Ile) and 2366C > T (Ser789Phe)] were unlikely to be associated with changes in the hepatic excretion of bilirubin. A deficiency of MRP2 is known to cause DJS, and several causative molecular alterations for DJS have now been identified [13–16]. Among them, the Ser789Phe variant is reported to be an alteration for DJS [14]. Previous studies have indicated that homozygous carriers for these causative alterations suffer from the syndrome, whereas heterozygous carriers have a moderately elevated urinary coproporphyrin I fraction (~40%) with normal total and direct bilirubin values [13,15]. Thus, the lack of an apparent effect of the two variants in the *MRP2* gene can be explained by the fact that all subjects having mutations in the *MRP2* gene were heterozygotes except one. Although the net in vivo effect of these polymorphisms on the hepatic excretion of bilirubin remains to be investigated, it is noteworthy that total and direct bilirubin values in one homozygote for the Ile417 variant were 0.5 mg/dL and 0.1 mg/dL, respectively, and much lower than those reported in patients with DJS (1.3–6.9 mg/dL for total bilirubin) and within normal ranges [15]. The Val417Ile variant is frequently observed in some racial populations (allele frequency among Japanese subjects is 0.125) [19,23]; however, the present findings suggest that this non-synonymous mutation is not associated with changes in total or direct bilirubin levels in serum.

We have recently evaluated the functional significance of polymorphisms in the *OATP-C* gene to the pharmacokinetics of pravastatin, and indicated that subjects with the *OATP-C*15* allele had significantly lower non-renal clearances, which is probably due to a reduction in the hepatocellular uptake of pravastatin [12]. Bilirubin is taken up from blood into hepatocytes by basolateral (sinusoidal) membrane transporters and then excreted into bile mainly as bilirubin glucuronides. Although various mechanisms of bilirubin uptake into hepatocytes have been proposed to date [7,8,24,25], there has been a long-standing debate on the mechanism of transport of unconjugated bilirubin in the basolateral membrane. If OATP-C plays a role in the sinusoidal uptake of unconjugated bilirubin, subjects with the *OATP-C*15* allele, a functionally impaired allele, may have higher unconjugated and total bilirubin levels. As would be expected, subjects with the *OATP-C*15* allele had higher unconjugated bilirubin levels than subjects without the allele. In addition, significant correlations were observed between unconjugated bilirubin levels and pharmacokinetic parameters of pravastatin, suggesting a functional consequence of the **15* allele in the hepatic transport of bilirubin.

It is reasonable to assume that impaired function of OATP-C due to **15* allele would reduce the hepatic uptake of pravastatin and thus result in increased systemic exposure to pravastatin (i.e., increased serum concentration) and in unconjugated hyperbilirubinemia. In such circumstances, because pravastatin reduces cholesterol levels primarily by inhibiting the hepatic HMG-CoA reductase, patients with an impaired OATP-C might show a reduced cholesterol-lowering efficacy of pravastatin. Clinical studies are required to address the question whether *OATP-C* polymorphism affects the efficacy of pravastatin.

Although no disease state that is associated with mutations in the *OATP-C* gene has been identified so far, impaired OATP-C-mediated bile transport could be one possible pathogenesis of Gilbert's syndrome, a form of unconjugated hyperbilirubinemia [26,27]. We found that one homozygous carrier for the *UGT1A1*28* allele had the highest bilirubin levels among our study subjects. This finding was in keeping with reports that the **28* allele is less efficient in binding regulatory proteins which control transcription of the *UGT1A1* gene [17,28]. However, this subject had the *OATP-C*15* allele as heterozygosity at the same time. Thus, the net in vivo effect of the homozygous status of the **28* allele on bilirubin levels remains unclear. Although, the *OATP-C*15* allele does not seem to be a causative molecular alteration, the genetic characterization of *OATP-C* and *UGT1A1* in patients with Gilbert's syndrome is of interest. Indeed, some investigators indicated that a mild reduction in the enzyme activity due to the **28* allele is not always sufficient for the full manifestation of the phenotypic bilirubin levels [17,29,30].

The results of the present study appear to be supportive of previous in vitro findings indicating the involvement of OATP-C in hepatic bilirubin transport [11,31]. However, as shown in Fig. 1, the observed correlations were significant, but weak. In addition, other authors have failed to observe unconjugated bilirubin uptake by OATP-C using HeLa cells and HEK293 cells as expression systems [32]. These results suggest the involvement of additional carriers in this process. For example, OATP-8, another basolateral hepatic transporter, has been found to be able to transport unconjugated and monoconjugated bilirubin, when expressed in *Xenopus laevis* oocytes and HEK293 cells [11,31]. The substrate specificity of most OATPs (eg, OATP-A, OATP-C and OATP-8) is extremely broad and shows substantial overlap [14].

In conclusion, our results suggest that OATP-C plays a role in the carrier-mediated uptake of bilirubin in the human liver. Large clinical studies with regard to polymorphisms of the *OATP-C* gene are needed to confirm these observations.

5. Disclosure

All of the authors have no commercial associations (e.g., consultancies, stock ownership, equity interests) that might pose a conflict of interest in connection with the submitted article.

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FUNCTIONAL ASSESSMENT OF *ABCG2* (*BCRP*) GENE POLYMORPHISMS TO PROTEIN EXPRESSION IN HUMAN PLACENTA

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ABSTRACT:

The aim of the present study was to assess the contribution of polymorphisms in the breast cancer resistance protein/ATP-binding cassette transporter G2 (*BCRP/ABCG2*) gene to the placental expression from a new perspective, allelic imbalance. Polymorphisms were screened by polymerase chain reaction (PCR)-single-strand conformation polymorphism analysis followed by sequencing with DNA extracted from 100 placentas. To examine whether polymorphisms of the *BCRP* gene correlate with the placental BCRP expression, we determined mRNA and protein levels by quantitative real-time PCR and Western blotting, respectively. In placentas, G34A (Val^{12}Met) and C421A (Gln^{141}Lys) were frequently observed (18–36%), but C376T, which creates a stop codon (Gln^{126} stop codon), was found with an allelic frequency of 1%. The mean of the BCRP protein level was significantly lower ($p < 0.05$) in homozygotes for the A421 allele than in those for the C421 allele, and heterozygotes had an intermediate value. To evaluate whether the C421A polymorphism acts as a *cis*-element in BCRP transcription, allelic imbalance was determined using informative lymphoblasts and 56 samples of placental cDNA. In most of the placental samples we tested, the difference in expression levels between the two alleles was small, and only two samples indicated a monoallelic expression (i.e., preferential expression of one allele). These results suggest that 1) the predominant allelic expression pattern of *BCRP* in placental samples is biallelic, and 2) the mutation C421A is not a genetic variant acting in *cis*, but is considered to influence the translation efficiency.

Breast cancer resistance protein (BCRP), also called mitoxantrone-resistant protein, is the second member of the G family of ATP-binding cassette transporters (ABCG2) (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999; Doyle and Ross, 2003). The *BCRP* gene is located at 4q22 and encodes a 72-kDa membrane protein composed of 655 amino acids (Allikmets et al., 1998; Doyle et al., 1998; Allen et al., 1999; Bailey-Dell et al., 2001). In contrast to many other ABC transporters, BCRP has only one ATP-binding region and one transmembrane domain. Therefore, BCRP is referred to as a half-transporter, and its homodimerization may be necessary to transport substrates (Kage et al., 2002).

In normal human tissues, BCRP is highly expressed in the placenta, colon, small intestine, and liver (Maliepaard et al., 2001). On the basis of its tissue distribution and findings in knockout mice, BCRP is speculated to have a major influence on the pharmacokinetic and pharmacodynamic profiles of certain xenobiotics and endogenous substrates. For example, inhibition of mouse Bcrp 1 by GF120918, a dual inhibitor for BCRP and P-glycoprotein, has been demonstrated to increase the bioavailability of topotecan when GF120918 was administered orally to *mdr1a/1b*(−/−) mice (Jonker et al., 2000). In a clinical study, coadministration of GF120918 was also associated with a marked increase in the bioavailability of and systemic exposure to topotecan (Kruijtzer et al., 2002).

Recent clinical studies indicate that the large interindividual variability in drug response occurs as a result of molecular alterations to various proteins such as drug-metabolizing enzymes, drug targets and receptors, and drug transporters. Most studies on molecular alterations have focused on the impact of single-nucleotide polymorphisms (SNPs) on the expression and function of these proteins (Evans and Relling, 1999; Evans and Johnson, 2001). Several groups have reported naturally occurring SNPs in the *BCRP* gene. G34A and C421A occur at relatively high frequency in most ethnic populations (Imai et al., 2002; Bäckström et al., 2003; Zamber et al., 2003; Mizuarai et al.,

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ABBREVIATIONS: BCRP, breast cancer resistance protein; ABC, ATP-binding cassette transporter; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; RT, reverse transcriptase; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; bp, pase pair(s); SNP, single-nucleotide polymorphism; PCR-RFLP, PCR-restriction fragment length polymorphism; ALP, alkaline phosphatase; TPBS, 1× phosphate-buffered saline, 0.1% Tween 20; EB, Epstein-Barr.

2004). Although effects of these SNPs on the drug resistance mechanism have not been well documented, the mutation C421A has been reported to reduce BCRP protein levels in PA317 cells (Imai et al., 2002). In contrast, no significant difference was observed in human intestinal samples (Zamber et al., 2003). Since BCRP may function as a maternal-fetal barrier, effects of these SNPs on expression levels in the placenta are of interest.

Recently, it has been suggested that various gene expression mechanisms, such as genomic imprinting (Constância et al., 1998; Brannan and Bartolomei, 1999) and expression imbalance (Yan et al., 2002; Bray et al., 2003), contribute to human diversity. Genomic imprinting is an epigenetic modification leading to a functional inequality of paternal and maternal genomes in somatic cells (McGrath and Solter, 1984; Surani et al., 1984). In addition to epigenetic modifications, *cis*-acting SNP(s) has been reported to be a causative factor for mRNA expression imbalance. In that case, the *cis*-acting SNP(s) alters the expression of the gene transcript from the chromosome carrying it, resulting in an imbalance of expression between the two alleles. For example, Wojnowski and Brockmöller (2004) have recently indicated a hepatic transcriptional imbalance of the *CYP3A5* gene in *CYP3A5*1A/*3* heterozygous samples. In the *CYP3A5* gene, *cis*-acting *1A variant, which increases the expression of the *CYP3A5* gene transcript from the allele carrying the variant, is a possible mechanism for the imbalance. Thus, allelic expression imbalance is useful for assessing the functional characterization of SNPs.

The initial aim of this study was to determine polymorphisms of the *BCRP* gene using 100 Japanese placentas and 420 genomic DNA samples obtained from Japanese, Caucasian, and African-American populations and then to compare the allelic frequency among these three populations. The second and major aim of this study was to evaluate the substantial variability in placental BCRP expression from two perspectives: genetic polymorphisms and expression imbalance.

Materials and Methods

Placentas and Lymphoblast Samples. Human full-term placentas (highly enriched placental trophoblast populations) were obtained from 100 Japanese patients at Tottori University Hospital. These tissues were immediately frozen in liquid nitrogen and stored at −80°C for the preparation of DNA and RNA (Tanabe et al., 2001). We also obtained 18 lymphoblast samples for which the parental origin of the *BCRP* alleles was determined (Mitsuya et al., 1997). EB virus-transformed lymphoblast cultures were obtained using standard procedures. This study was approved by the Ethical Board of the Faculty of Medicine, Tottori University, and informed consent was obtained from all individuals.

RNA Extraction and cDNA Synthesis. Total RNA extraction and RT-PCR procedures for placental samples were previously described (Tanabe et al., 2001). For EB virus-transformed lymphoblasts, the RNeasy Kit (QIAGEN GmbH, Hilden, Germany) was used to extract total RNA. Prior to RT, total RNA samples were first treated with RNase-free DNase I and digested with BsrI (New England Biolabs, Beverly, MA). BsrI digests the potential DNA template, which would lead to the amplification of both alleles and thus mask allelic imbalance. RT from total RNA was performed in a 20-μl reaction mixture containing 5 μg of total RNA in 1× First-strand Buffer, 25 mM DTT, 0.5 μg of the random primers (Promega, Madison, WI), a 2 mM concentration of each deoxynucleoside-5′-triphosphate, and SuperScript II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA). Samples were incubated at 42°C for 1 h. As a negative control, template RNA was processed without reverse transcriptase.

Screening of Variants in the *BCRP* Gene (PCR-SSCP). Genomic DNA was isolated from placental samples using the QIAamp DNA Mini Kit (QIAGEN). The primer design was based on published sequences (GenBank accession number AC084732) of *BCRP* to avoid the amplification of sequences from homologous genes. PCR was carried out in a total volume of 25 μl in the presence of 50 ng of DNA, a 0.25 μM concentration of each of the forward and reverse primers (totally, 25 sets of primers were designed and presented in the Appendix), 1× PCR buffer II, 1.5 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside-5′-triphosphate, and 0.625 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). After an initial denaturation at 94°C for 5 min, 40 to 45 cycles of 0.5 to 1 min at 94°C, 0.5 to 1 min at 50–68°C, and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were carried out. PCR products were analyzed on 3% agarose gels to check both the size and specificity of the products. To screen variants of the *BCRP* gene, SSCP analysis was performed. The details of the procedures were described elsewhere (Tanabe et al., 2001).

DNA Sequence. All PCR products were sequenced either directly or after subcloning on an ABI 3100 automatic sequencer (Applied Biosystems) using a Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). If the direct sequencing was incomplete, each amplified PCR product was subcloned into the vector pGEM T-easy (Promega) and transformed into competent JM109 cells (Promega). Before the sequencing, reaction mixtures were purified with Sephadex G-50 (Amersham Biosciences AB, Uppsala, Sweden). The sequencing primers were those used in the PCR amplifications. The sequences of both strands were analyzed for products from at least two independent PCR amplifications to ensure that the identified mutations were not PCR-based artifacts.

Genotyping for *BCRP* in Three Ethnic Populations. We examined the allelic frequencies of the G34A, C376T, and C421A mutations using genomic DNA samples from unrelated Japanese (n = 120), Caucasian (n = 150), and African-American (n = 150) volunteers. Genomic DNA was isolated from blood samples with use of the Toyobo blood kit on a Toyobo HMX-2000 robot (Toyobo, Osaka, Japan). The three polymorphisms were genotyped by the PCR-RFLP method. The PCR conditions were the same as for PCR-SSCP, but mismatch primers were designed for the genotyping of G34A and C421A. Primer sequences were as follows: BseMI RFLP for G34A, forward, 5′-CAGTAATGTCGAAGTTTTTATCGCA-3′ and reverse, 5′-AAATGTTCATAGCCAGTTTCTTGGA-3′; AfaI RFLP for C376T, forward, 5′-ATAGCATGTGTTGGAGGGAAAAA-3′ and reverse, 5′-ATTGGTATCACTGTCCTTACAAG-3′; TaaI RFLP for C421A, forward, 5′-GTTGTGATGGGCACTCTGATGGT-3′ and reverse, 5′-CAAGCCACTTTTCTCATTGTT-3′. After digestion with an appropriate restriction enzyme, all RFLP products were analyzed on a 3% agarose gel.

Haplotype Analysis. In addition to the unphased SNP analysis, we performed a haplotype analysis for the three major polymorphisms, G34A, C376T, and C421A, using the following two approaches. 1) Haplotypes in individuals who were homozygous at all SNP sites or heterozygous at no more than one of the variable sites were assigned directly from the result of the unphased SNP analysis; 2) haplotypes in the remaining multiheterozygous subjects were determined by a combination of allele-specific PCR and RFLP methods. For the allele-specific PCR, the BseMI RFLP forward primer and the allele-specific reverse primers for the C421 allele or A421 allele were used: the reverse primer for the C421 allele was 5′-GAAGAGCTGCTGAGAACTG-3′ and for the A421 allele was 5′-GAAGAGCTGCTGAGAACTT-3′. All allele-specific PCR products were digested with both *Bse*MI (G34A) and *Afa*I (C376T).

Quantitative Real-Time PCR Analysis. The mRNA levels were measured by TaqMan quantitative real-time PCR with an ABI PRISM 7000 sequence detection system (Applied Biosystems). The following primers and TaqMan probe were used for determining the BCRP mRNA: forward primer, 5′-TTCTGCCCAGGACTCAATGC-3′; reverse, 5′-GCCACGTGATTCTTCCACAA-3′; TaqMan probe, 5′-FAM-CCAAATATTCTTCGCCAGTACATGTTGC-TAMRA-3′. The endogenous reference gene was determined using the commercially available human GAPDH TaqMan PreDevelopped Assay Reagent (Applied Biosystems). The quantitative PCR was carried out in a total volume of 25 μl in the presence of 1 μl of cDNA, 200 nM each of the forward and reverse primers, 100 nM of probe and 1×TaqMan universal PCR master mix (Applied Biosystems).

Assessment of Expression Imbalance. To assess the allelic imbalance of *BCRP*, the *Bse*MI RFLP (G34A, for lymphoblast and placental samples) and *Taa*I RFLP (C421A, for placental samples) were used. Primer sequences for lymphoblast samples were as follows: forward and reverse primers for genomic DNA were 5′-CAGTAATGTCGAAGTTTTTATCGCA-3′ and 3′-AAATGTTCATAGCCAGTTTCTTGGA-3′, respectively; whereas those for cDNA were 5′-CAGTAATGTCGAAGTTTTTATCGCA-3′ and 5′-TAACGAAGATTTGCCTCCACCTGTG-3′, respectively. A 291-bp and a 259-bp

TABLE 1

Genetic polymorphism in the BCRP gene in Japanese placentas (n = 100)

Location	Position[a]	Reference Allele[b]	Variant Allele	Amino Acid Substitution	Genotype			Frequency of Variant Allele
					R/R	R/V	V/V	
5′-Flanking region	−20445	gtctCctcc	gtctTctcc		98	2	0	0.010
	−20296	agctAttaa	agctGttaa		80	18	2	0.110
	−19781	aaaaAttat	aaaaGttat		99	1	0	
	−19572_−19569	ctcaCTCAcaaa	ctca--caaa		60	33	7	0.235
Exon 2	34	cccaGtgtc	cccaAtgtc	Val12Met	70	24	6	0.180
Intron 2	203 + 16	tttaAttta	tttaGttta		70	24	6	0.180
Intron 3	263 + 10	tataAgaga	tataGgaga		85	14	1	0.080
	263 + 72	ttttGtgtg	ttttTGtgtg		99	1	0	0.005
Exon 4	376	ggtaCaagt	ggtaTaagt	Gln126stop	98	2	0	0.010
Exon 5	421	cttaCagtt	cttaAagtt	Gln141Lys	42	45	13	0.355
Intron 5	532–16	ttatAatat	ttatGatat		99	1	0	0.005
Exon 9	1098	aggaAatca	aggaAatca	Synonymous	98	2	0	0.010
Intron 10	1277 + 95	atagTgtaa	atagAgtaa		97	3	0	0.015
Exon 11	1322	agcaGtgtt	agcaAtgtt	Ser441Asn	99	1	0	0.005
Intron 11	1367 + 20	ttctAggaa	ttctGggaa		71	25	4	0.165
Exon 12	1465	tataTttac	tataCttac	Phe489Leu	99	1	0	0.005
Intron 12	1492 + 49	ctatGggtg	ctatCggtg		44	45	11	0.335
Exon 13	1515	atgcCttct	atgc-ttct	Phe506Ser Phe507Leu Val508Leu Met509stop	99	1	0	0.005
Intron 13	1648–42	tgaaAttac	tgaaTttac		99	1	0	0.005
	1648–21	gactCttag	gactTttag		71	25	4	0.165
Intron 14	1738–46	tcttAaaat	tcttGaaat		24	52	24	0.500
3′-UTR	2332	cttcAgtct	cttcTAgtct		86	14	0	0.070
	2364	tgccAttat	tgccCttat		99	1	0	0.005
	2512	agaaCttac	agaaTttac		99	1	0	0.005

R, reference allele; V, variant allele.

[a] Position is in respect to the translation start site of the *BCRP* gene; the A in ATG is + 1 and the base immediately 5′ is −1.

[b] Reference allele: GenBank/EMBL accession no. AC084732.

PCR product were obtained from genomic DNA and cDNA samples, respectively. After digestion of the PCR products from genomic DNA samples with BseMI, homozygotes of the G allele yielded a 291-bp fragment, whereas heterozygotes of the A allele yielded 291-, 261-, and 30-bp fragments. In contrast to the genomic DNA, homozygotes of the G allele yielded a 259-bp fragment, and heterozygotes of the A allele yielded 259-, 229-, and 30-bp fragments in the PCR products from cDNA samples.

In placental samples, the primer sets for genomic DNA and cDNA were the same: BseMI RFLP, forward, 5′-CAGTAATGTCGAAGTTTTTATCGCA-3′ and reverse, 5′-TTCGACAAGGTAGAAAGCCACTCTT-3′; and TaaI RFLP, forward, 5′-GTTGTGATGGGCACTCTGATGGT-3′ and reverse, 5′-CCTAACTCTTGAATGACCCTGTT-3′. PCR was carried out under the same conditions for the PCR-SSCP analysis, but only for 24 to 31 cycles. RFLP products were electrophoresed on a 3% agarose gel, then stained with SYBR Green I (Takara, Kyoto, Japan). Allelic expression was quantified on a fluorescence image analyzer (Hitachi, Tokyo, Japan) using Analysis Version 7.5 software. As a control, genomic DNA PCR-RFLP products were included and ratios of the allele-specific band intensities were taken as a 1:1 allelic representation. To eliminate sampling or measurement error, we conducted the experiment for each sample with three replicates.

Western Blotting. Human placental trophoblast samples were homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% SDS, 1 mM dithiothreitol, and 1×Complete Protease Inhibitor Cocktail (Roche Diagnostics, Germany). The lysate was centrifuged at 15,000*g* for 30 min at 4°C, and the supernatant was separated. Protein concentrations of the supernatants were determined by the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) using bovine serum globulin as a standard. The supernatants (80 μg of protein) were loaded onto SDS 4 to 20% (w/v) gradient polyacrylamide gels (Tefco, Tokyo, Japan) and transferred to Sequi-Blot polyvinylidene difluoride membranes (Bio-Rad) at 180 mA for 1 h. Thereafter, the membranes were blocked with 5% skim milk in TPBS (1× phosphate-buffered saline, 0.1% Tween 20) for 2 h at room temperature, then incubated overnight at 4°C with anti-BCRP, clone BXP-21 (1:50; Kamiya Biomedical, Thousand Oaks, CA) in skim milk in TPBS. The membrane was washed five times with TPBS and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000; DakoCytomation Denmark A/S, Glostrup, Denmark). Polyvinylidene difluoride membranes were rinsed five times for 10 min with TPBS and then evenly coated using the ECL Western blotting detection system (Amersham Biosciences Inc.) for 1 min. The membrane was immediately exposed to Kodak X-OMAT AR film (Kodak, Tokyo, Japan) at room temperature. To assure the quantitative expression of BCRP, an additional marker protein expressed in placenta, alkaline phosphatase (ALP), was measured according to the same protocol except that different primary (polyclonal rabbit anti-human placental ALP; Biomeda, Foster City, CA) and secondary (horseradish peroxidase-conjugated goat anti-rabbit IgG; MP Biomedicals, Irvine, CA) antibodies were used. The immunoblots were quantitated using a public domain NIH Image program.

Statistical Analysis. Data are shown as the mean ± S.D. The statistical differences between various groups were determined with either the Mann-Whitney *U* test or the one-way analysis of variance (with the Tukey-Kramer multiple comparisons test), as appropriate. A χ^2 test was used to compare the allele frequency of each variant with that expected for a population in Hardy-Weinberg equilibrium. $p < 0.05$ was taken to be the minimum level of statistical significance.

Results

Identification of Variants in the *BCRP* Gene. For the identification of polymorphisms in all 16 exons and the 5′-flanking region of the *BCRP* gene, PCR-SSCP analysis was performed with genomic DNA obtained from 100 placental samples. Twenty polymorphisms were detected by SSCP analysis and identified by subsequent sequencing (Table 1). Of these, five SNPs resulted in the following amino acid substitutions: G34A (Val12Met), C376T (Gln126stop), C421A (Gln141Lys), G1322A (Ser441Asn), and T1465C (Phe489Leu). G34A (18.0%) and C421A (35.5%) variants were found at a relatively high incidence in this study. C376T, which is associated with an amino acid substitution from Gln to a stop codon at codon 126 (Gln126stop), was detected in only two placental samples (1.0%) as

TABLE 2

Frequencies of BCRP alleles in different ethnic populations

Values in parentheses indicate 95% confidence intervals.

SNP	Amino Acid Change	Population	Genotypes			Frequency of Variant Allele
			R/R	R/V	V/V	
G34A	Val12Met	Japanese (n = 120)	81	37	2	0.17 (0.12–0.22)
		Caucasian (n = 150)	139	11	0	0.04 (0.02–0.06)
		African American (n = 150)	132	17	1	0.06 (0.04–0.09)
C376T	Gln126stop	Japanese (n = 120)	118	2	0	0.01 (0.00–0.02)
		Caucasian (n = 150)	150	0	0	0.00
		African American (n = 150)	150	0	0	0.00
C421A	Gln141Lys	Japanese (n = 120)	61	45	14	0.30 (0.25–0.36)
		Caucasian (n = 150)	121	25	4	0.11 (0.08–0.15)
		African American (n = 150)	144	5	1	0.02 (0.01–0.04)

R, reference allele; V, variant allele.

heterozygosity. Another polymorphism, a C1515 deletion, which results in a frame shift (Phe506Ser, Phe507Ser, Val508Leu, and Met509stop), was extremely rare (0.5%) in our samples. In the 5′-flanking and 3′ untranslated regions, four and three polymorphisms were identified, respectively: C-20445T, A-20296G, A-19781G, and a CTCA deletion at −19572 to −19569 in the 5′-flanking region; and A2332TA, A2364C, and C2512T in the 3′ untranslated region.

Haplotype Assessment. On the basis of the haplotype analysis for the three major variants (i.e., G34A, C376T, and C421A), four haplotypes were identified: G-C-C, G-C-A, A-C-C, and G-T-C. Their corresponding allelic frequencies were 46.0, 35.0, 18.0, and 1.0%, respectively.

Frequencies of G34A, C376T, and C421A in Different Ethnic Populations. We compared frequencies of the polymorphisms G34A, C376T, and C421A among three ethnic populations (Table 2). The frequency distributions of these three variants in all populations were in Hardy-Weinberg equilibrium. Japanese subjects had significantly higher frequencies of G34A and C421A than were found in the other two ethnic populations ($p < 0.05$). In addition, C376T was only detected in Japanese, but its frequency was extremely low (1.0%), as has been observed in placental samples. These results indicate that genetic frequencies of *BCRP* variants appeared to be dependent on ethnicity.

Polymorphisms and Placental BCRP Expression. The expression levels of the BCRP protein in 99 human placentas were determined by Western blotting using an anti-human BCRP monoclonal antibody, BXP-21 (Fig. 1). The membrane vesicles isolated from wild-type human BCRP-transfected HEK293 cells were used as the positive control (Kondo et al., 2004). The BCRP protein expression level was normalized to the placental ALP level, and the normalized value was compared with the control sample. The BCRP protein levels in various genotypic groups are shown in Fig. 2. The mean BCRP protein level was significantly lower in homozygotes for the A421 allele than in those for the C421 allele (0.37 ± 0.21 versus 0.75 ± 0.50, $p < 0.05$), and heterozygous samples displayed an intermediate value. In contrast to the C421A polymorphism, the mean of the BCRP level for the G/G, G/A and A/A genotypes at nucleotide position 34 was 0.58 ± 0.37, 0.70 ± 0.50, and 0.65 ± 0.51, respectively, and did not differ among these genotypes.

The relationship between various genotypic patterns and mRNA levels is also shown in Fig. 2. In contrast to the findings at the protein level, significant genotype-dependent changes in the mRNA expression were not observed.

Allelic Imbalance of the *BCRP* Gene in Human Placentas. We estimated the allelic expression bias, a measure of the expression of the reference allele divided by the variant allele, using placental

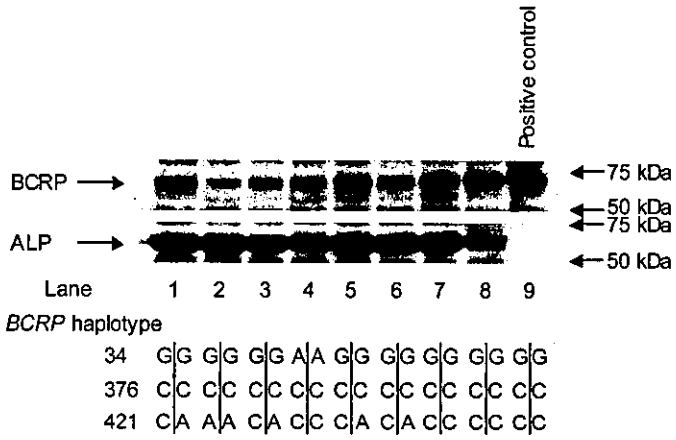


FIG. 1. Western blot analysis of BCRP expression in human placentas. Lane 1, sample 100 (control for calculations); lanes 2 through 8, placental samples; lane 9, positive control. The position of the molecular mass marker is indicated on the right. The *BCRP* haplotype was also shown for each placental sample.

cDNA samples to confirm the interindividual variation in the allelic imbalance of the *BCRP* gene and to characterize the contribution of the SNPs to the allele-specific mRNA expression (Bray et al., 2003; Yan et al., 2002).

After the screening of the genomic DNA from all 100 placental samples, it was possible to identify 56 individuals who were heterozygous for either the BseMI (G34A) or TaaI (C421A) site. These two SNPs were used as markers in the present study. Some of the 56 individuals had fractional allelic expression values outside the 95% confidence interval for the mean (1.16 ± 1.21; 95% confidence interval, 0.85~1.48) (Fig. 3A). Notably, the values in subjects 19 (9.94) and 62 (0.00) were extremely high and low, respectively, being well outside the intervals, indicating monoallelic expression (i.e., preferential expression of one of the two alleles) (Fig. 3B). Although the difference in expression between the two alleles varied among samples, the expression bias in most samples (43 of 56) was within the approximated 95% confidence interval. The allelic expression bias ranged from 0.60 (65) to 1.47 (82), excluding these two outside samples, suggesting that neither variant is associated with large changes in transcription from the allele carrying each variant.

Allelic Expression Pattern in Informative Lymphoblasts. Among the 56 placental samples, 2 indicated a preferential monoallelic expression. To determine whether the two alleles of the *BCRP* gene were differentially expressed according to parental origin, we used RT-PCR of total RNA extracted from EB virus-transformed lymphoblasts, followed by PCR-RFLP. The parental origin of alleles expressed in children was identified by RFLP analysis. Lymphoblasts

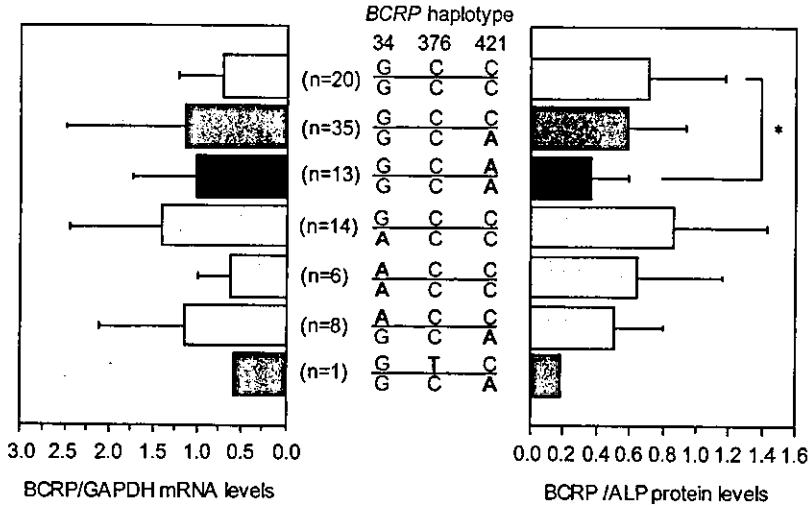


FIG. 2. Placental BCRP mRNA (left) and protein (right) expression levels in various *BCRP* haplotypes. Data are shown as the mean ± S.D. *, significantly different from values in wild-type subjects as determined by one-way analysis of variance with the Tukey-Kramer multiple comparisons test ($p < 0.05$).

were obtained from a panel of 18 healthy Japanese individuals who were members of five distinct families. These samples allowed the precise determination of the parental origin of alleles in the heterozygous children. Of all the subjects, two siblings were heterozygous for a polymorphism at the BseMI site (G34A). All RT reactions in the present study included a negative control to ensure that genomic DNA did not contaminate the subsequent PCR. We first determined the parents' genotypes using genomic DNA samples. Whereas the paternal genotype was homozygous for the G34 allele, the maternal genotype was heterozygous for the A34 allele (Fig. 4). Since the two siblings were heterozygous carriers, their G and A alleles were of paternal and maternal origin, respectively. In contrast to the genomic DNA-based genotypes, both of the siblings showed a monoallelic paternal expression of the RT-PCR products (i.e., cDNA samples) (Fig. 4). These results suggested that the expression pattern of the *BCRP* gene in lymphoblasts is regulated by parental imprinting.

It is interesting to know whether a monoallelic parental expression of the *BCRP* gene is inherited. To address this issue, we further analyzed expression pattern using maternal RT-PCT product, because the maternal genotype was heterozygous for the A34 allele. As shown in Fig. 4, the maternal inactive A34 allele was inherited by both siblings. These results suggest that a monoallelic parental expression is inherited, at least in EB virus-transformed lymphoblasts.

Discussion

Before the functional characterization of the variants, we analyzed genetic polymorphisms in Japanese placental samples and compared allelic frequencies among different ethnic groups. Similar to other ABC-transporters (Ieiri et al., 2004; Marzolini et al., 2004), various synonymous and nonsynonymous polymorphisms were observed (Table 1). Among the nonsynonymous polymorphisms, G34A (Val12Met) and C421A (Gln141Lys) appeared commonly in Japanese subjects, and allelic frequencies of these polymorphisms were in keeping with those of a previous report (Imai et al., 2002). As shown in Table 2, the C421A variant is widespread not only in Japanese subjects but also in Caucasian subjects, with a frequency of between 10% and 30%. However, this variant is rare in African Americans, suggesting an ethnic difference in the frequency of polymorphisms in the *BCRP* gene. These trends were well consistent with the findings reported by de Jong et al. (2004), who indicated that the frequency of C421A variant was the most common (i.e., 34%) in Han Chinese among various ethnic populations they evaluated. It is difficult to assess a plausible explanation for the difference; the C421A allele appears to be very common in Asian populations.

Another polymorphism, C376T in exon 4, which substitutes a stop codon for Gln126, was detected in only four of our study samples as heterozygosity. Although the frequency of the C376T allele was extremely low, C376T may have an impact on protein expression because of the premature stop codon.

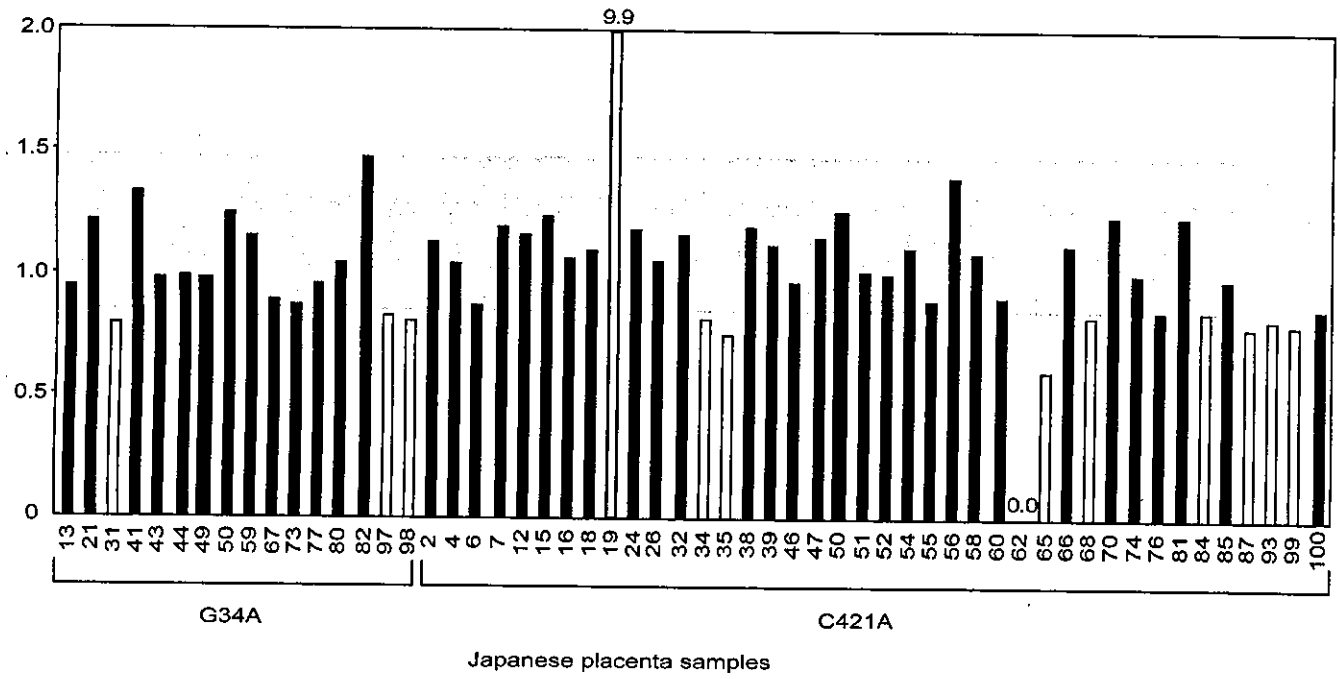
Recent studies indicate that haplotype-based approaches, which take into consideration the combination of SNPs present in one allele, offer greater ability to predict changes in phenotype than do SNP-based approaches (Judson et al., 2000; Stephens et al., 2001). Thus, we determined haplotypes for the three major SNPs (i.e., G34A, C376T, and C421A) in Japanese subjects. In the present study, four allelic patterns were identified. Interestingly, the C421A variant existed only as a G-C-A allele, suggesting that it does not coexist with the other two variants.

In addition to the cording haplotypes, we determined haplotypes for four SNPs in the 5′-flanking (C-20445T, A-20296G, A-19781G, and CTCA deletion at −19572 to −19569) and for three SNPs in the 3′ untranslated (A2332TA, A2364C, and C2512T) regions. Five major haplotypes were identified: C-A-A-CTCA, C-A-A-(CTCA)deletion, and C-G-A-(CTCA)deletion in the 5′-flanking region; and A-A-C and TA-A-C in the 3′ untranslated region. Although we evaluated the relationship between various haplotypes and both mRNA and protein levels, significant haplotype-dependent changes in both expression levels were not observed (data not shown).

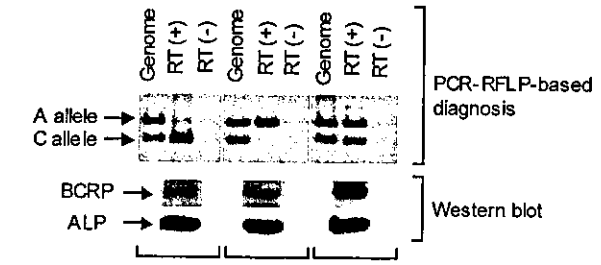
Our findings indicate that the C421A variant may affect the placental expression of BCRP protein; samples with an A421 allele had a reduced protein level, as compared with those without the variant. These findings are consistent with the report that C421A *BCRP*-transfected PA317 cells and KB-3-1 human epidermoid carcinoma cells showed a markedly decreased protein expression and low-level drug resistance (Imai et al., 2002). Similar findings were made in a different cell line, HEK293 (Kondo et al., 2004). Although one study demonstrated no significant correlation between the C421A variant and expression of intestinal BCRP mRNA or protein (Zamber et al., 2003), the present study and collective evidence suggest that C421A is potentially of functional significance.

C421A is located in the functionally important ATP-binding region between Walker A and B motifs and is associated with a differentially

A)



B)



Sample No.	19	62	58
mRNA level (BCRP/GAPDH)	0.60	0.29	1.01
Protein level (BCRP/ALP)	0.32	0.33	0.64

A, allelic expression bias of the *BCRP* gene in Japanese placentas. The expression bias was estimated based on the reference/variant ratios (replicated data points h sample) at either the G34A or C421A polymorphism, and corrected using the genomic ratio. The shaded box represents the approximated 95% confidence interval open (white) bars indicate individuals displaying significant variations. B, the PCR-RFLP-based diagnosis for allelic imbalance. Monoallelic samples (19 and 62) llelic sample (58) are presented with their BCRP mRNA and protein levels.

ed amino acid substitution. These characteristics suggest that the A variant alters the tertiary structure of the BCRP protein, g to greater susceptibility to degradation (Imai et al., 2002). In on, Mizuarai et al. (2004) reported that ATPase activity in the rane of SF9 cells infected with the C421A variant showed a ase of 1.3 below that of wild-type cells. They also indicated that 421A variant exhibited reduced drug resistance in polarized PK1 cells along with increased intracellular drug accumulation. an in vivo human study, Sparreboom et al. (2004) recently ted the effects of the C421A variant on the pharmacokinetics of otecan, a synthetic derivative of camptothecin, in 22 cancer s, and provided the first evidence linking variant *BCRP* alleles red drug exposure. Patients with this variant as heterozygosity ed about 3-fold higher plasma levels than did patients with pe alleles. These results suggest that interindividual variability in substrate drug effects might be influenced, in part, by *BCRP* genotype.

Other nonsynonymous variants, Arg482Gly and Arg482Thr, have been reported to have a crucial role in protein function and in altering the multidrug resistance phenotype by changing substrate specificity (Honjo et al., 2001; Allen et al., 2002). However, these variants ware not detected in the present study, indicating that mutations at position 482 may occur due to drug selection.

In the present study, we assessed the molecular mechanisms responsible for the low protein levels in A421-placental samples from two perspectives, polymorphism and allelic imbalance. Allelic imbalance is a differential expression which can be determined by the measurement of the relative expression level of two alleles of one gene (Yan et al., 2002; Bray et al., 2003; Knight, 2004). One example of this phenomenon is provided by a recent study by Wojnowski and

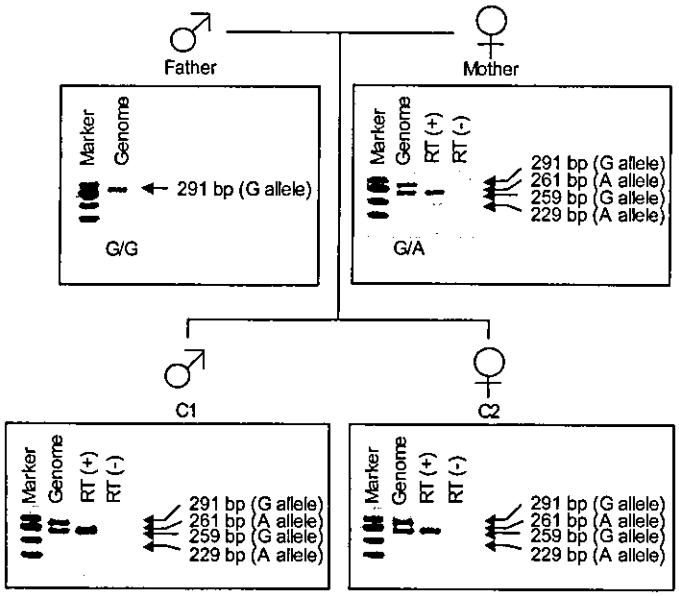


FIG. 4. Monoallelic paternal expression of the *BCRP* gene in informative lymphoblasts. Maternal inactive A34 allele was inherited by both siblings.

Brockmöller (2004), which demonstrated a hepatic transcriptional imbalance of the *CYP3A5* gene in heterozygous *CYP3A5*1A/*3* samples. In that case, *CYP3A5*1A* may act as a *cis*-acting SNP, which increases the expression of the *CYP3A5* transcript from the allele carrying it, resulting in an imbalance of mRNA expression. Thus, allelic imbalance can be used for the functional evaluation of SNPs that affect mRNA expression. If the C421A variant is a functional *cis*-acting polymorphism differentially affecting the expression level of each copy of a gene, a strong over- or under-shift in allelic expression bias will be expected. However, as shown in Fig. 3, although only two samples (19 and 62) exhibited significant allelic imbalance (i.e., preferential expression of one of the two alleles), the allelic expression bias in most samples was within the approximated 95% confidence interval (0.85~1.48). These results suggest that the predominant expression pattern of *BCRP* in placental samples is biallelic, and C421A is not a *cis*-acting SNP. The C421A variant was associated with a reduction in placental protein levels but not in mRNA levels (Fig. 2). These observations are in line with a finding by Imai et al. (2002), who demonstrated that murine PA317 cells expressing C421A had increased intracellular drug accumulation, coupled with reduced protein levels and a similar mRNA expression. Therefore, taking these observations into consideration, the reduction in protein expression levels in the A421-placental samples is considered to be a result of the translation efficiency of BCRP. Nevertheless, we cannot exclude the possibility that the *cis*-acting regulatory polymorphisms, which may form haplotypes with the C421A variant, responsible for the change in BCRP expression reside far up- and down-stream of the gene of the affected allele.

Since protein expression is known to be regulated not only by mRNA and/or DNA sequences but also by epigenetic modifications such as genomic imprinting, chromatin alteration, methylation, and acetylation, we introduced an epigenetic concept in this study. As shown in Fig. 4, we have demonstrated that human lymphoblasts showed a paternal expression of *BCRP*, in contrast with the findings in placental samples, in which a monoallelic expression was observed in only a minority of samples, suggesting a unique allele-specific expression profile of *BCRP*. Among known imprinted genes, the Wilms' tumor suppressor gene (*WT1*) has been reported to exhibit a unique expression profile (Mitsuya et al., 1997); cultured human fibroblasts and lymphocytes showed a paternal or biallelic expression of *WT1* in some cases, whereas a maternal or biallelic expression was observed in human placental villi and fetal brain tissue (Jinno et al., 1994; Nishiwaki et al., 1997). These results suggest that the allele-specific expression profile of certain genes depends on the tissue source. Thus, expression patterns in other BCRP-enriched human tissues such as liver and small intestine are of interest.

The data presented here suggest that the C421A variant in the *BCRP* gene, a common SNP in both Japanese and Caucasian populations, alters protein levels in the human placenta. In addition, a tissue-dependent allele-specific expression profile of *BCRP* is sus-

TABLE 3

Primer sequences used for the PCR-SSCP analysis of the human BCRP gene

Primer Pair	Forward Primer	Reverse Primer
Promoter-1	5'-GTACTAGGATTACAGGCGTGAGC-3'	5'-AATGACCCGAGATCCCACCACTG-3'
Promoter-2	5'-CGCAGATTTTTCAGTTTTTGC-3'	5'-TATTAATGGTTCACCCAATGC-3'
Promoter-3	5'-AGGGTCTTGAAACTGACAGAAAT-3'	5'-AAGGGGAGAAACTTACTGAATGA-3'
Promoter-4	5'-TTTCTAGCCTTTCCACACCATCG-3'	5'-ACGCAGGGACAAGCCAAACACTC-3'
Promoter-5	5'-GCCAGTGACGGCGACCAAACC-3'	5'-GCGCTGACACGAACTTCCTAAGC-3'
Exon 1-1	5'-GGCTCAGCGCGGCAGGACA-3'	5'-GACCCGGACATCCAGGGGACGAG-3'
Exon 1-2	5'-AGCGCGGCTTAGGAAGTTCGTGT-3'	5'-CGCGTCTCTCAATCTCAGTGG-3'
Exon 1-3	5'-CGTGCTGTGCCCACTCAAAAG-3'	5'-ATACACAACGCCCACCAACCT-3'
Exon 2	5'-ATTGTCACCTAGTGTTTGC-3'	5'-AAAAATGTTCATAGCCAGTTTCT-3'
Exon 3	5'-AAAATGGAATAGTAAATCAGTCT-3'	5'-CTGCTACATGTCAATCACCTTAT-3'
Exon 4	5'-ATAGCATGTGTTGGAGGGAAAAA-3'	5'-ATTGGTATCACTGTCCTTACAAG-3'
Exon 5	5'-AAACAGTCATGGTCTTAGAAAAG-3'	5'-TCTCATTGTTATGGAAAGCAACC-3'
Exon 6	5'-CAAATGATAATGACTGGTTGTTA-3'	5'-TTGTTTTTCTTGATAATGCTTTT-3'
Exon 7	5'-AGCAAACAATCTAAAGGCAAGAA-3'	5'-CCAAAGACCAAACAGCACTCCTG-3'
Exon 8	5'-AAGTGAGTTCTCTTTGTTTTCCA-3'	5'-GTTGACTGGTATCAGAAGACTGC-3'
Exon 9	5'-TGTTTGTGTTTCCTTTTTATCCA-3'	5'-CATTGTTCCCATTTGAGTATTTC-3'
Exon 10	5'-TTATCTCTAATTGAAACTCTTCC-3'	5'-AAATAAACTGACTCATCCTACCC-3'
Exon 11	5'-ACGCTTCCCTGTTCCAACCAGAA-3'	5'-TGTAATCAGTCTAACCAATAGCC-3'
Exon 12	5'-GACAAGTCTAGCCTGCCCTGTGG-3'	5'-GTTTGGTTTATAGTTTTGAGAAC-3'
Exon 13	5'-AATAAGCAATCCCAAACATACGG-3'	5'-TTATCAGAGCAAACACAGTTCAG-3'
Exon 14	5'-AGAGGAGAAGAGTTTAGTGAGTG-3'	5'-ACAGTGACAGACAAGGAAGACAT-3'
Exon 15	5'-ACATTAGTTGGTTTGGTGAGACA-3'	5'-ATTCAGTGCCCCTGGAAGGACTC-3'
Exon 16-1	5'-CTGAGTAACATTTGACGGATGCT-3'	5'-GTGATGGCAAGGGAACAGAAAAC-3'
Exon 16-2	5'-TTCAGTATGATTTATCCTCACAT-3'	5'-GCTACTAACCTACCTATTCATTT-3'
Exon 16-3	5'-GTCTCTGGCATTTGTTTCCTCAT-3'	5'-CTCTACTCTACCCACAGTTCCAA-3'

pected. In vivo human studies with regard to these findings are required for the establishment of individualized anticancer therapy.

Appendix

Primer sequences used for the PCR-SSCP analysis of the human *BCRP* gene are shown in Table 3.

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Haplotype-Oriented Genetic Analysis and Functional Assessment of Promoter Variants in the *MDR1* (*ABCB1*) Gene

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ABSTRACT

Recently, a number of nucleotide variants have been described in the multidrug resistance 1 (*MDR1/ABCB1*) gene; however, most studies have focused on the coding region. In the present study, we identified promoter variants of the *MDR1* gene and evaluated their phenotypic consequences using a reporter gene assay and the real-time polymerase chain reaction method. Ten allelic variants were detected in the promoter region (approximately 2 kilobases), seven of which were newly identified. Certain mutations occurred simultaneously, and a total of 10 haplotypes were observed. These promoter polymorphisms were found more frequently in Japanese than Caucasians. Some haplotypes were associated with changes in luciferase activity and placental and hepatic mRNA levels. We also determined DNA methylation status in the proximal promoter region of the *MDR1* gene. The promoter region around potential binding sites for transcription factors was found to be hypomethylated and thus likely to be independent of the gene expression. Nucleotide and/or haplotype variants not only in the coding region but also in the promoter region of the *MDR1* gene may be important for interindividual differences of P-glycoprotein expression.

Polymorphisms in the genes encoding membrane transporters have recently been reported to be associated with variations in the pharmacokinetic and pharmacological effects of clinically used drugs (Fromm, 2002; Kim and Tirona, 2002; Takane et al., 2003). Among various drug transporters, P-glycoprotein, the multidrug resistance 1 (*MDR1/ABCB1*) gene product, is one of the best studied. P-glycoprotein is expressed in various human tissues such as the intestine, liver, and kidney, and functions as a cellular efflux pump for foreign xenobiotics and endogenous substrates.

Although a number of nucleotide variants have been described in the *MDR1* gene, most studies in this area have focused on the association of single nucleotide polymorphisms (SNPs) in the coding region with the altered expression of P-glycoprotein or pharmacokinetics of clinically used drugs. Hoffmeyer et al. (1999) demonstrated that the synonymous C3435T polymorphism (Ile1145Ile) in exon 26 was associated with a low level of expression of P-glycoprotein in the duodenum, resulting in an increase in plasma concentrations after oral administration of digoxin, used as a probe for P-glycoprotein. In contrast, higher level of duodenum P-glycoprotein expression and lower level of serum digoxin after oral administration were observed in the subjects with this variant (Sakaeda et al., 2001; Nakamura et al., 2002). The association of the C3435T polymorphism with P-glycoprotein protein expression and function is controversial. Up to now, various investigators have reported that the variant is associated with decreased or increased expression, or it has no clearly discernible effect (Sparreboom et al., 2003; Ishikawa et al., 2004). In a recent report, an approach using gene-based haplotypes, which are specific combinations of SNPs located throughout the genome, proved superior to the use of individual SNPs for predicting the association between phenotypes and genomic variation (Judson et al., 2000). For example, Drysdale et al. (2000) reported that the bronchodilator response to β agonist is significantly related to haplotype pairs of the β_2-adrenergic receptor gene but not to individual SNPs. With regard to the *MDR1* gene, Johne et al.

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ABBREVIATIONS: SNP, single nucleotide polymorphism; CpG, cytosine-guanosine pair; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; bp, base pair; MDR, multidrug resistance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay.

(2002) indicated that it was important to consider the variability in haplotype structure rather than in SNPs when characterizing the *MDR1* phenotype. However, the association of variants in the promoter region of the *MDR1* gene with the expression of P-glycoprotein has been not well investigated.

DNA methylation, referred to as the methylation of cytosine in a cytosine-guanosine pair (CpG), is the most common eukaryotic DNA modification and one of many epigenetic (an alteration in gene expression without a change in nucleotide sequence) phenomena (Singal and Ginder, 1999). Normally, both the core promoter and transcriptional start site are included within the CpG-rich region, and DNA methylation regulates gene expression by interfering with the binding of specific transcription factors to their recognition sites (Singal and Ginder, 1999; Jones and Takai, 2001). Interestingly, the human *MDR1* gene has a CpG-rich promoter region. Hypomethylation of *MDR1* during chemotherapy resulted in a high level of gene expression in recurrent tumors, and it had important consequences for clinical outcome in acute myeloid leukemias (Nakayama et al., 1998) and bladder cancer (Toda et al., 2000). However, there is currently no data available on the role of DNA methylation in transcriptional regulation in normal tissues.

The aim of this study was to describe variants in the promoter region of *MDR1* in Japanese and Caucasian populations and to evaluate their functional significance with regard to transcriptional activity and mRNA expression in placentas and livers obtained from Japanese subjects. Furthermore, we determined the methylation status of the promoter region of *MDR1* and its association with the interindividual variability in gene expression in normal tissue.

Materials and Methods

Isolation of Genomic DNA and RNA. Human full-term placentas (highly enriched placental trophoblast populations) and livers were obtained from 96 and 19 Japanese patients, respectively. These tissues were immediately frozen in liquid nitrogen and stored at −80°C for the preparation of DNA and RNA. Blood samples were obtained from 96 healthy Caucasian volunteers. Genomic DNA from the samples was prepared using the Toyobo blood kit on a Toyobo HMX-2000 robot (Toyobo, Osaka, Japan). The isolation of genomic DNA from tissues was performed using a DNA preparation kit (QIAamp DNA mini kit; QIAGEN GmbH, Hilden, Germany). Total RNA was extracted using ISOGEN (Nippongene, Tokyo, Japan), and reverse transcription was performed with random hexamers (Promega, Madison, WI) and reverse transcriptase (Invitrogen, Carlsbad, CA). This study was approved by the Tottori University Ethics Committee, and informed consent was obtained from all individuals.

Identification of Variants in the *MDR1* Promoter Region. The genotypes of *MDR1* such as A−41aG, C−145G, T−129C, and C3435T were identified by PCR-restriction fragment length polymorphism analysis as described previously (Tanabe et al., 2001). To identify unknown mutations in the *MDR1* promoter region, SSCP analysis was performed using the GenePhor system (Amersham Biosciences AB, Uppsala, Sweden) as described previously (Ieiri et al., 2000). PCR was performed in a total volume of 25 μl consisting of 50 ng of genomic DNA, 10× PCR buffer II, 1.5 mM $MgCl_2$, 1.25 U of Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 0.25 μM of each primer. The primer sets were designed to divide the promoter region (−1700a to Ex1/+88) of the *MDR1* gene (GenBank accession no. AC002457) into five fragments (~500 bp). After an initial denaturation at 94°C for 5 min, 45 cycles of 40 s at 94°C, 45 s at 50 to 59°C, and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed.

Haplotype Analysis. A 2112-bp fragment, including the promoter region of *MDR1* (−1700a to Ex1/+88), was amplified by using gene-specific primers (5′-GGAGCAAAGAAATGGAATACAATA-3′ and 5′-TTCTCCCGTGAAGACCAAGTTC-3′). The PCR mixture was essentially the same as for the identification of mutations except for the *Taq* polymerase (LA *Taq*; Takara, Shiga, Japan). After an initial denaturation at 94°C for 5 min, 45 cycles of 40 s at 94°C, 15 s at 58.3°C, and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed. The PCR fragments were subcloned into pGEM-T easy vector (Promega) and sequenced.

DNA Sequence. All PCR products were sequenced directly on an ABI 377 automatic sequencer (Applied Biosystems) using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Before the sequencing, reaction mixtures were purified with a DyeEx Spin kit (QIAGEN GmbH). The sequencing primers were those used in the PCR amplifications. The sequencing of both strands was performed for products from at least two independent PCR amplifications to ensure that the identified mutations identified were not PCR-induced artifacts.

Real-Time Quantitative PCR (TaqMan) Analysis. PCR was performed using a master mix based on the TaqMan universal PCR master mix (Applied Biosystems) and run on the ABI PRISM 7000 sequence detection system (Applied Biosystems). The following primers and TaqMan probe were used for determining the MDR1 mRNA: forward primer, 5′-TATCAGCAGCCCACATCATCA T-3′; reverse primer, 5′-CCAAATGTGACATTTCCTTCCA-3′); and probe, 5′-TACAGCACGGAAGGCCTAATGCCGA-3′. The endogenous reference gene was determined using the commercially available human GAPDH TaqMan Predeveloped Assay Reagent (Applied Biosystems). Each primer set and TaqMan probe were used at final concentrations of 200 and 100 nM, respectively. The reactions were run in duplicate. For all experimental samples, the amount of mRNA was determined from a standard curve (serial diluted samples from placental tissue expressed at higher levels of MDR1 and GAPDH mRNA). The mRNA level of MDR1 was expressed as a ratio to that of GAPDH.

Plasmid Construction. To obtain the first plasmid, a 2056-bp fragment (−1704a to Ex1/+28) of *MDR1*, including the promoter region and exon 1, was initially amplified from genomic DNA with gene-specific primers incorporating 5′-KpnI and 3′-NheI, for the 5′ end of inserts 5′-CGGGGTACCGGAGCAAAGAAATGGAATACA-3′ and for the 3′ end of inserts 5′-CTAGCTAGCAGTAGCTCCCAGCTTTGCGTG-3′. The PCR fragment was subcloned into the pGEM-T easy vector and then introduced into competent JM109 cells (Promega). The plasmids obtained were sequenced and digested with KpnI and NheI. The digested fragment was ligated into the KpnI/NheI site of the vector pGL3-enhancer (Promega). Manipulated DNA portions were sequenced again in their entirety.

Cell Culture and Transfection. HepG2 human hepatoma cells were incubated at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. One day before transfection, cells (5.5×10^5) were seed into culture plates (60 mm). The cells were washed two times with serum-free medium. The luciferase reporter gene constructs (5 μg) and the control reporter gene plasmid pRL-TK vector (0.5 μg) (Promega) were mixed with the Tfx-20 reagent (15 μl) (Promega), transferred to serum-free medium, and then incubated at room temperature for 15 min. The mixtures were added to the washed cells and incubated for 1 h at 37°C under 5% CO_2. After the incubation, the cells were cultured in growth medium and harvested after 48 h.

Assay of Luciferase Activity. Luciferase reporter gene activity was evaluated with the Dual luciferase reporter assay system (Promega). HepG2 cells were washed once with a phosphate-buffered saline solution and lysed in passive lysis buffer (400 μl). After incubation at 37°C for 15 min, lysates were mixed in a vortex blender for 15 s and centrifuged at 4°C for 30 s. Supernatants (20 μl) were mixed with the luciferase reagent (100 μl), and the luciferase activity was

measured with a luminometer (Turner Designs, Sunnyvale, CA). After background correction (activity in untreated cells), results were expressed as the level of pGL3 activity divided by pRL activity. The total cellular protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts from HepG2 cells were prepared as reported previously (Takeuchi et al., 2000). Oligonucleotides for the *MDR1* gene, including −1517a T (ACTGTTTAGGGAGGGTTTAAGGCCATTCAAA), −1517a C (ACTGTTTAGGGAGGGCTTAAGGCCATTCAAA), −1459a G (ATAAATGAAAGGTGAGATAAAGCAACAAAGC), −1459a A (ATAAATGAAAGGTGAAATAAAGCAACAAAGC), −1017a T (GAGGCAGGAGAATGGTGTGAACCCGGGAGGC), −1017a C (GAGGCAGGAGAATGGCGTGAACCCGGGAGGC), −145 C/−129 C (CTTTGCCACAGGAAGCCTGA GCTCATTCGAGTAGCGGCTCTTCCAAG), −145 G/−129 T (CTTTGCCACAGGAAGGCTGAGCTCATTCGAGTAGCGGCTCTTCCAAG) and −145 C/−129 C (CTTTGCCACAGGAAGCCTGAGCTCATTCGAGCAGCGGCTCTTCCAAG) were synthesized with both sense and antisense strands, the corresponding pairs of which were annealed and end-labeled with T4 polynucleotide kinase (Takara) and [γ-^{32}P]dATP (Amersham Biosciences AB) according to standard methodology. The γ-^{32}P-labeled probe (1×10^4 cpm) was incubated for 30 min at 0°C with nuclear protein (5 μg) in binding buffer (10 μl) containing 25 mM Hepes (pH 7.9), 40 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 7.5% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet-40, 5 μM ZnSO4, 0.2 μg of poly(dI-dC), and 1 μg of bovine albumin. Competitor oligonucleotides were added at 100-fold molar excesses. Reaction mixtures were electrophoresed on 5% polyacrylamide and 2.5% glycerol gel in a Tris-glycine-EDTA buffer (250 mM Tris, 1.9 M glycine, and 10 mM EDTA) at 4°C and visualized by a BAS-2500 Imaging Analyzer (Fuji Film, Tokyo, Japan).

Determination of Methylation. The methylation status of CpG sites within the proximal promoter region of *MDR1* was confirmed using the bisulfite sequencing method (Frommer et al., 1992). DNA was treated with sodium bisulfite using a CpGenome DNA modification kit (Intergen, Purchase, NY) according to the manufacturer's instructions. PCR (578 bp, −214a to Ex1/+40) was performed in a total volume of 25 μl consisting of 50 ng of bisulfite-modified genomic DNA, 0.625 to 2.5 U of Amplitaq Gold DNA polymerase, and 0.25 μM of each primer, 5′-AAGGTGTTAGGAAGTAGAAAGGT-3′ and 5′-AAACTATCCCATAATAACTCCCAA-3′. After an initial denaturation at 95°C for 5 min, 35 cycles of 45 s at 95°C, 45 s at 55°C, and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed. The PCR product was cloned into the pGEM-T easy vector according to the manufacturer's instructions. The CpG methylation status of individual DNA strands was determined based on a comparison with the sequence obtained from the genomic DNA without the addition of bisulfite modifications. The number of methylated CpGs at a specific site was divided by the number of clones analyzed ($N > 15$) to yield percentage of methylation for each site.

Statistical Analysis. The 95% confidence interval was calculated to compare the differences in genotype or haplotype frequencies between Japanese and Caucasians. Results of MDR1 mRNA expression and mutation (C3435T) were analyzed with a Kruskal-Wallis test. Comparisons between two groups were performed using a Mann-Whitney *U* test. A 5% level of probability was considered to be significant.

Results

Identification of Variants in the Promoter Region of the *MDR1* Gene. Ten variants were detected in the promoter region of the human *MDR1* gene by PCR-SSCP analysis using DNAs obtained from unrelated Japanese and Caucasian subjects (Fig. 1; Table 1). Seven variants, at positions −1517a, −1459a, −1423a, −1132a, −1017a, −824a, and −755a, were newly identified in this study. The most common mutation in Japanese was G–1459aA (allelic frequency 0.250), whereas A–41aG (0.106), T–1517aC (0.080), T–1017aC (0.080), T–129C (0.080) were found at low frequency. The 5-base deletion at position −1132a to −1128a, C–145G and T–824aC were detected at extremely low frequency (0.037, 0.032, and 0.005, respectively). The frequencies of T–1017aC and T–129C were significantly lower in Caucasians than Japanese ($P < 0.05$). The two-base deletion at position −1423a to −1422a and A–755aG were identified only in Caucasian subjects, but at frequencies below 0.010. In contrast, T–1517aC, G–1459aA, a five-base deletion at position −1132a to −1128a, T–824aC, A–41aG, and C–145C were not detected in Caucasian subjects. These results indicate that genotypic frequencies of variants in the promoter region of the *MDR1* gene seemed to be dependent on race.

Identification of *MDR1* Promoter Haplotypes. On the basis of a haplotype analysis using subcloning and direct sequencing, 10 haplotypes derived from all identified promoter variants were found to be present in both populations (Table 2). In Japanese, seven haplotypes were identified with a frequency ranging from 0.005 to 0.665. Unlike in Caucasians, three variants at −1517a, −1017a, and −129 occurred simultaneously in Japanese. In total, 13 different haplotype pairs were found in the subjects examined (Table 3). In Caucasians, the most common haplotype pair was 1/1 (0.923). In contrast, hetero- or homogenous combinations of haplotypes with one or more variant sites were found at a relatively high frequency in Japanese compared with Caucasians (0.553 versus 0.077).

Association of *MDR1* Promoter Haplotypes with mRNA Expression in Placenta and Liver. Before investigating the influence of *MDR1* promoter haplotype combinations on mRNA expression in placental and hepatic tissues, we determined whether the C3435T variant influences MDR1 mRNA expression. As shown in Fig. 2, the synonymous C3435T polymorphism in exon 26 was associated with a low level of placental MDR1 expression ($P < 0.05$; Kruskal-Wallis test). Next, we compared *MDR1* promoter haplotype pairs (haplotypes 1/1, 1/2, 1/3, 1/4, and 4/4) with corresponding placental and hepatic MDR1 levels in 29 and 11 samples with the 3435 C/C and C/T genotype, respectively (Fig. 2). The MDR1 expression in placental tissue with haplotype 1/2 or 1/3 tended to increase compared with that in 1/1 samples ($P = 0.091$; Mann-Whitney *U* test; Fig. 2). However, mean mRNA levels in hetero- and homogygous samples for haplotype 4 was comparable with those in 1/1 samples. Also, the MDR1 expression in hepatic tissue of haplotype 1/2 or 1/3 tended to increase compared with that in 1/1 samples ($P = 0.07$; Mann-Whitney *U* test; Fig. 2).

Luciferase Reporter Gene Assay. To investigate the influence of promoter haplotypes on the potential for transcriptional regulation, 10 reporter plasmids containing *MDR1* promoter sequences were transiently transfected in HepG2 cells, and then luciferase activities were measured. As shown in Fig. 3, haplotypes 2, 3, and 9 increased the luciferase activity by 41, 32, and 30%, respectively, compared with that by haplotype 1. In contrast, the haplotype 6 construct resulted in a 28% reduction in activity. Other haplotypes did not seem to influence the activity.

Binding of Nuclear Proteins to the Promoter Variant Sites. To determine whether the variants in the promoter

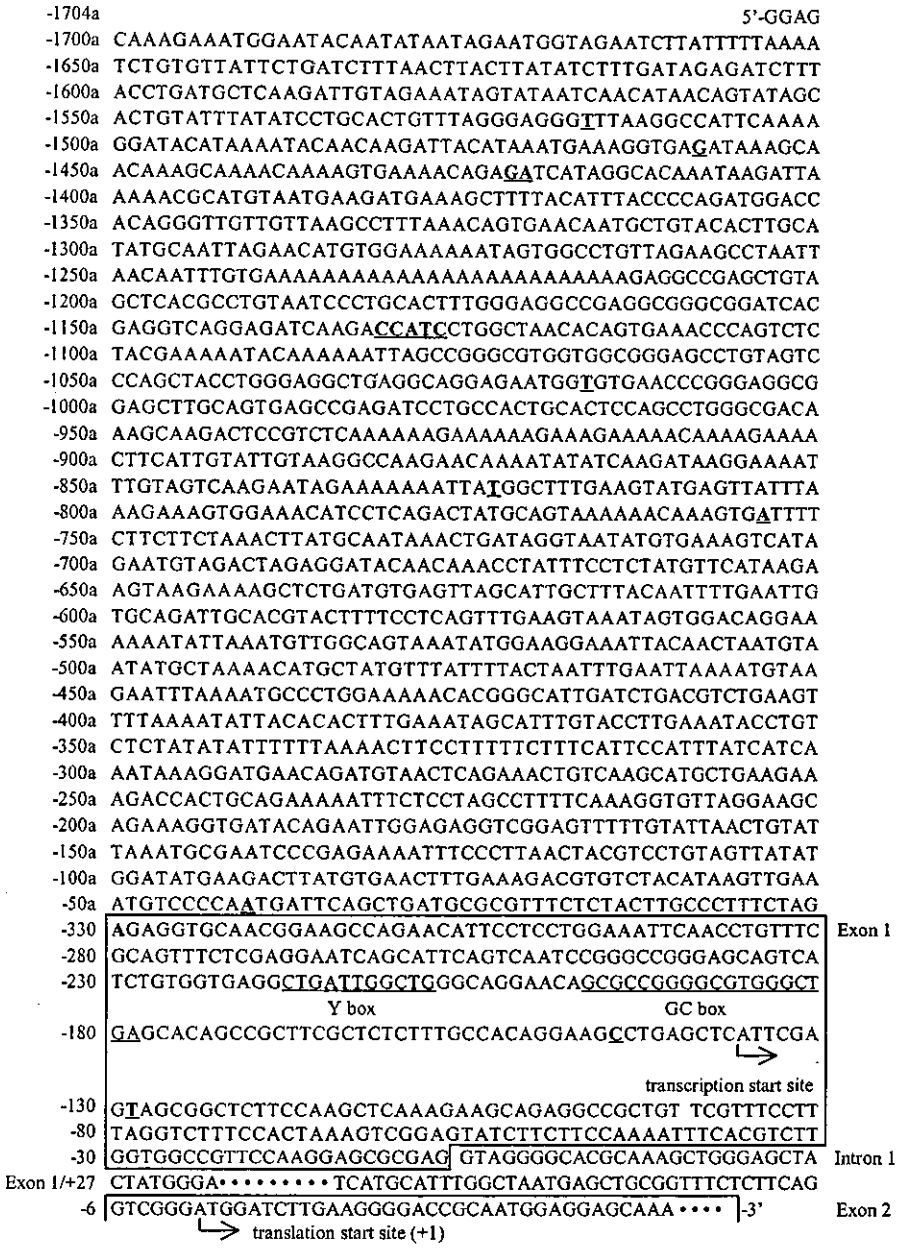

Fig. 1. Nucleotide sequence of the human *MDR1* gene promoter. Positions of nucleotide variants are in bold and are underlined. The exons are boxed. The location of variants in the coding region is relative to the initiation site for translation, which is defined as +1 based on the cDNA nucleotide sequence. The position in the promoter region is relative to the nucleotide sequence immediately preceding exon 1, which is defined as −1a.

region altered binding for transcription factors, we performed EMSA using nuclear extracts prepared from HepG2 cells. By competition assays using an excess of unlabeled probe, allele-specific binding of nuclear proteins was observed when the nuclear extracts were incubated with probes, including −1517a C (complex I) and −1459a G (complex II) (Fig. 4, A and B). Also, strong nuclear protein-DNA binding (complex III) was observed with the probe containing the −1017a T allele when compared with the −1017a C allele (Fig. 4C). The higher binding completely disappeared under an excess of unlabeled −1017a T probe, and weaker inhibition of the binding was observed with the −1017a C probe. With the −145/−129 C/T and G/T probes, nuclear protein-DNA binding (complex VI) was detected, but with the C/C probe it was not detected or was much weaker (Fig. 4D). The protein-DNA binding was completely inhibited by an excess of unlabeled C/T and G/T probes, and slightly competed with by an excess of unlabeled C/C probe. No nuclear protein-DNA binding was observed when the probes containing the −41a A/G and −145 G/C alleles were incubated with nuclear extracts.

Correlation between Placental MDR1 mRNA Expression and Methylation Status at CpG Sites in the *MDR1* Promoter Region. The proximal region of the human *MDR1* is rich in CpG (Fig. 5A). This region including the Y box and GC box elements is required for activation of the *MDR1* promoter. We focused on the CpG-rich proximal promoter region in the *MDR1* gene and determined the relationship between methylation status at each CpG site and MDR1 mRNA expression using placenta with promoter haplotype 1/1 and 3435 C/C genotypes. Results of a bisulfite sequencing analysis of 26 CpG sites in seven subjects, whose MDR1 levels varied considerably, are shown in Fig. 5B. In all samples, methylated CpG sites were found upstream of the promoter region. Moreover, an interindividual difference in methylation status was observed; however, no clear associa-

TABLE 1
Variants in promoter region of the *MDR1* gene in Japanese (n = 94) and Caucasian (n = 96) subjects

Location	Position	Allele	Nucleotide Sequence	Allele Frequency		Genotype	Frequency	
				Japanese	Caucasian		Japanese	Caucasian
Promoter	−1517a[a]	T[b]	agggTttaa	0.920 (0.881–0.959)[c]	1.000	T/T	0.840	1.000
						T/C	0.160	0.000
		C	agggCttaa	0.080 (0.041–0.119)	0.000	C/C	0.000	0.000
	−1459a[a]	G[b]	gtgaGataa	0.750 (0.688–0.812)	1.000	G/G	0.553	1.000
						G/A	0.394	0.000
		A	gtgaAataa	0.250 (0.188–0.312)	0.000	A/A	0.053	0.000
	−1423a[a]	GA[b]	cagaGAtcat	1.000 (0.985–1.005)	0.995	GA/GA	1.000	0.990
						GA/−	0.000	0.010
		Deletion	caga_tcat	0.000	0.005 (−0.027–0.015)	−/−	0.000	0.000
	−1132a[a]	CCATC[b]	aagaCCATCctgg	0.963 (0.936–0.990)	1.000	CCATC/CCATC	0.926	1.000
						CCATC/−	0.074	0.000
		Deletion	aaga_ctgg	0.037 (0.01–0.064)	0.000	−/−	0.000	0.000
	−1017a[a]	T[b]	atggTgtga	0.920 (0.881–0.959)	0.984 (0.966–1.002)	T/T	0.840	0.969
						T/C	0.160	0.031
		C	atggCgtga	0.08 (0.041–0.119)	0.016 (−0.002–0.034)	C/C	0.000	0.000
	−824a[a]	T[b]	attaTggct	0.995	1.000 (0.985–1.005)	T/T	0.989	1.000
						T/C	0.011	0.000
		C	attaCggct	0.005 (−0.027–0.015)	0.000	C/C	0.000	0.000
	−755a[a]	A[b]	agtgAtttt	1.000 (0.985–1.005)	0.995	A/A	1.000	0.990
						A/G	0.000	0.010
		G	agtgGtttt	0.000	0.005 (−0.027–0.015)	G/G	0.000	0.000
	−41a[a]	A[b]	cccaAtgat	0.894 (0.850–0.938)	1.000	A/A	0.798	1.000
						A/G	0.191	0.000
		G	cccaGtgat	0.106 (0.062–0.150)	0.000	G/G	0.011	0.000
	−145[d]	C[b]	gaagCctga	0.968 (0.943–0.993)	1.000	C/C	0.936	1.000
						C/G	0.064	0.000
		G	gaagGctga	0.032 (0.007–0.057)	0.000	G/G	0.000	0.000
Exon 1	−129[d]	T[b]	cgagTagcg	0.920 (0.881–0.959)	0.984 (0.966–1.002)	T/T	0.840	0.969
						T/C	0.160	0.031
		C	cgagCagcg	0.080 (0.041–0.119)	0.016 (−0.002–0.034)	C/C	0.000	0.000

[a] Position is relative to the initiation site of exon 1a, which is defined as +1a.
[b] Reference sequence GenBank accession no. AC002457.
[c] The 95% confidence intervals are given in parentheses.
[d] Position is relative to the initiation site of translation, which is defined as +1.

tion was observed. In addition, methylation was not observed around either the Y box or GC box element in most samples.

Discussion

Despite evidence supporting an association of coding SNPs with pharmacokinetics and pharmacodynamics, little is known about the presence or functional relevance of allelic variants in the promoter region of *MDR1*. We described 10 polymorphic variants in the *MDR1* promoter in Japanese and Caucasian populations. A–41aG, C–145G, and T–129C have been detected in the proximal promoter region of the *MDR1* gene as low frequency variants (Horinouchi et al., 2002; Tang et al., 2002; Kroetz et al., 2003). Here, we identified another seven variants. Their presence and frequency varied according to race. For example, C–145G was identified in Asian-Americans but not Caucasians (Kroetz et al., 2003). In the present study, this variant was observed only in Japanese. In addition, the new G–1459aA variant was the most frequent variant in Japanese (25.0%), but was not found in Caucasians.

The promoter region of the *MDR1* gene has been isolated and sequenced (Ueda et al., 1987; Madden et al., 1993). The promoter has an initiator sequence at the transcriptional start site, without a TATA box (van Groenigen et al., 1993). Numerous studies have shown that the Y box (inverted CCAAT box) and GC box, recognized by the transcription factors NF-Y and Sp1, respectively, are required for efficient transcriptional regulation of the *MDR1* promoter (Goldsmith et al., 1993; Sundseth et al., 1997). Several studies suggest that a region upstream of the Y box negatively regulates the *MDR1* promoter activity (Ogura et al., 1992; Cornwell and Smith, 1993), although the exact positions of the negative element differ. However, our variants are not located within those *cis*-elements.

We identified 10 different *MDR1* promoter haplotypes using subcloning and direct sequencing methods. A comparison of *MDR1* haplotype pairs with placental and hepatic expression showed that haplotypes 1/2 and 1/3 were associated with increased mRNA expression, independent of the C3435T mutation in the coding region. Interestingly, haplotypes 2 and 3, in which T–1517a C, T–1017aC, and T–129C mutations occurred simultaneously in both populations, were associated

TABLE 2
Localization of variants and identification of the *MDR1* promoter haplotypes in Japanese and Caucasian subjects

Nucleotide	−1517a	−1459a	−1423a	−1132a	−1017a	−824a	−755a	−41a	−145	−129	Frequency	
	T/C	G/A	GA/delete	CCATC/delete	T/C	T/C	A/G	A/G	C/G	T/C	Japanese	Caucasians
											n = 94	n = 96
Haplotype 1	T	G	GA	CCATC	T	T	A	A	C	T	0.665 (0.598–0.732)*	0.964 (0.937–0.991)
2	C	G	GA	CCATC	C	T	A	G	C	C	0.043 (0.014–0.072)	0.000
3	C	G	GA	delete	C	T	A	G	C	C	0.037 (0.010–0.064)	0.000
4	T	A	GA	CCATC	T	T	A	A	C	T	0.191 (0.135–0.247)	0.000
5	T	A	GA	CCATC	T	T	A	G	C	T	0.027 (0.004–0.050)	0.000
6	T	A	GA	CCATC	T	T	A	A	G	T	0.032 (0.007–0.057)	0.000
7	T	G	GA	CCATC	T	C	A	A	C	T	0.005 (−0.005–0.015)	0.000
8	T	G	delete	CCATC	T	T	A	A	C	T	0.000	0.010
9	T	G	GA	CCATC	C	T	A	A	C	C	0.000	0.016
10	T	G	GA	CCATC	T	T	G	A	C	T	0.000	0.010

* The 95% confidence intervals are given in parentheses.

TABLE 3
Haplotype configurations in promoter region of the *MDR1* gene in Japanese and Caucasian subjects

Promoter Haplotype Pair	Frequency	
	Japanese (n = 94)	Caucasian (n = 96)
1/1	0.447 (0.376–0.518)*	0.923 (0.885–0.961)
1/2	0.043 (0.014–0.072)	0.000
1/3	0.053 (0.021–0.085)	0.000
1/4	0.234 (0.173–0.295)	0.000
1/5	0.043 (0.014–0.072)	0.000
1/6	0.064 (0.029–0.099)	0.000
1/8	0.000	0.038 (0.011–0.065)
1/9	0.000	0.038 (0.011–0.065)
2/4	0.021 (0.001–0.0041)	0.000
2/5	0.011 (−0.004–0.026)	0.000
2/7	0.011 (−0.004–0.026)	0.000
3/4	0.021 (0.001–0.0041)	0.000
4/4	0.053 (0.021–0.085)	0.000

* The 95% confidence intervals are given in parentheses.

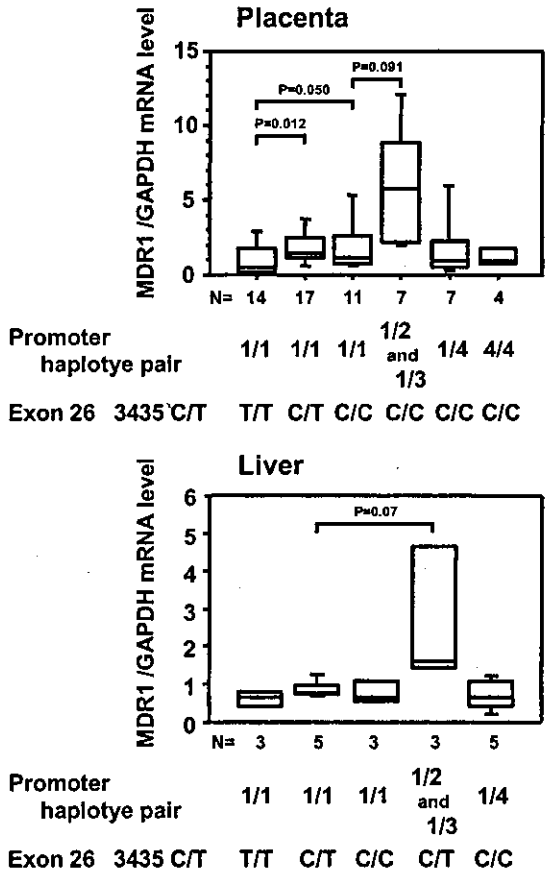

Fig. 2. Influence of *MDR1* promoter haplotypes and SNPs in the coding region on placental and hepatic MDR1 mRNA levels in Japanese. Statistical significance between the two genotypes was analyzed with the Mann-Whitney U test.

with an increase in transcriptional activity in human hepatoma cell line. Moreover, we showed that the T–1517aC, T–1017aC, and T–129C variants affected putative transcriptional protein-DNA binding. Heterozygosity for the –129C allele is associated with a high level of transport activity of P-glycoprotein in hematopoietic stem cells (Calado et al., 2002). The tacrolimus oral dose requirement is higher in renal transplant recipients with the T/C allele than T/T allele at position –129 (Anglicheau et al., 2003). Although these findings are not significant because the T–129C variant was