Table IV. Effects of genetic polymorphism of *MDR1* on the expression of P-glycoprotein in human tissues and *in vivo* pharmacokinetics and pharmacodynamics of P-glycoprotein substrates

Study	Polymorphism	Substrate	Subject/material	Pharmacodynamic-pharmacokinetic outcome	*In vitro* efflux	Expression level
Hoffmeyer et al.[9]	C3435T	Digoxin	HV/human duodenum	T/T > C/C (C_{max})		C/C > C/T > T/T (protein concentration)
Kerb et al.[76]	C3435T	Phenytoin	HV	T/T > C/C (plasma concentration)		
Kurata et al.[11]	C3435T, G2677T	Digoxin	HV	M/M > W/M > W/W in both loci (F). W/W > W/M > M/M in both loci (CL_R, CL_{sec})		
Min and Ellingrod[77]	C3435T	Ciclosporin	HV	(T/T + C/T) > C/C (C_{max} and AUC, but not significantly different)		
Johne et al.[78]	C3435T, G2677T	Digoxin	HV	TT > TC > CC (C3435T, AUC and C_{max}). Haplotype 12 > haplotype 11 (AUC, C_{max})		
von Ahsen et al.[60]	C3435T	Ciclosporin	RTR	C/C = C/T = T/T (dose-adjusted C_{min} and rejection incidence)		
Chowbay et al.[57]	C1236T, G2677T, C3435T	Ciclosporin	HTR	TT-TT-TT > CT-GT-CT > CC-GG-CC. T-T-T > C-G-C haplotype. (AUC, C_{max}, C_{min})		
Drescher et al.[79]	C3435T	Fexofenadine, rhodamine 123	HV/CD56+ cell	C/C = T/T (AUC)	C/C > C/T > T/T	

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Table IV. Contd

Study	Polymorphism	Substrate	Subject/material	Pharmacodynamic-pharmacokinetic outcome	*In vitro* efflux	Expression level
Goto et al.[7]	C+139T, C1236T, T–76A, G2677T/A, C3435T	Tacrolimus	LDLTR/human intestine	No significant effect of SNPs on tacrolimus concentration/dose ratio		No effect (mRNA level)
Siegmund et al.[53]	C3435T, G2677T/A	Talinolol	HV/human duodenum	W/W = W/M = M/M in both loci (AUC)		No significant effect (protein and mRNA levels)
Goh et al.[80]	C3435T	Docetaxel	Cancer patients	C/C = C/T = T/T (CL)		
Kim et al.[52]	G2677T *2 allele	Fexofenadine, digoxin	HV/NIH-3T3 GP+E86 cells	*1/*1 > *1/*2 > *2/*2 (AUC)	*MDR1*-Ser893 > *MDR1*-Ala893	
Sakaeda et al.[55]	C3435T	Digoxin	HV	C/C > subjects with the T allele (i.e. C/T and T/T) [AUC_4]		
Roberts et al.[1]	C3435T	Nortriptyline	Depressed patients	T/T > C/T > C/C (frequency of drug-induced postural hypotension)		
Fellay et al.[2]	C3435T	Nelfinavir, efavirenz	HIV-1-infected patients/PBMC	T/T > C/T > C/C (CD4+ cell count and recovery of naive CD4+ cells). C/C > C/T > T/T (C_{min})		C/C > C/T > T/T (protein and mRNA levels)
Zheng et al.[5]	C3435T, G2677T	Corticosteroids	Pediatric heart transplant recipients	W/W > W/M > M/M (duration of corticosteroid therapy)		
Illmer et al.[4]	C1236T, G2677T, C3435T		AML patients/blast samples	W/M > M/M > W/M in all 3 loci (overall survival). W/W > M/M > W/M in all 3 loci (probability of relapse)		C/T > T/T > C/C (1236), G/T > T/T > G/G (2677), C/T > T/T > C/C (3435) [mRNA level]

Continued next page

Table IV. Contd

Study	Polymorphism	Substrate	Subject/material	Pharmacodynamic-pharmacokinetic outcome	*In vitro* efflux	Expression level
Potocnik et al.[81]	T-129C, IVS1-81delG		Tumour samples (colorectal adenocarcinoma)	Association with lymphoid infiltration		W > M (protein level)
Yamauchi et al.[6]	G2677T/A	Tacrolimus	LDLTR	Positive predictor of drug-induced neurotoxicity		
Siegsmund et al.[3]	C3435T		Healthy control and non-CCRCC patients/ non-cancerous renal tissues	T allele as a risk factor		C/C > T/T (protein level)
Schwab et al.[70]	C3435T		Inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients	T > C allele, T/T genotype > other types (allelic frequency in patients with ulcerative colitis)		
Hitzl et al.[82]	C3435T	Rhodamine 123	CD56+ cell		C/C > C/T > T/T	C/C > C/T > T/T (mRNA level)
Calado et al.[83]	T–129C, G2677T, C3435T	Rhodamine 123	CD34+ cells		W/W = W/M = M/M	
Tanabe et al.[10]	T–129C, G2677T/A, C3435T		Human placenta			T/T > T/C (–129), W/W > W/M > M/M (2677), C/C = C/T = T/T (3435) [protein level]
Nakamura et al.[8]	C3435T		Human duodenum			T/T > C/T > C/C (mRNA level)
Meissner et al.[84]	C3435T		Human heart			Reduced in T/T samples (protein and mRNA levels)

AML = acute myeloid leukaemia; **AUC** = area under the plasma concentration-time curve; **CCRCC** = clear cell renal cell carcinoma; **CL** = systemic clearance; $\mathbf{CL_R}$ = renal clearance; $\mathbf{CL_{sec}}$ = renal secretory clearance; $\mathbf{C_{max}}$ = peak plasma concentration; $\mathbf{C_{min}}$ = trough plasma concentration; **F** = bioavailability; **HTR** = heart transplant recipients; **HV** = healthy volunteers; **LDLTR** = living donor liver transplantation recipients; **M** = mutant allele; **mRNA** = messenger RNA; **PBMC** = peripheral blood mononuclear cells; **RTR** = renal transplant recipients; **SNP** = single nucleotide polymorphism; **W** = wild-type allele.

mRNA levels.[53] Thus, the collected evidence indicates that the contribution of the *MDR1* variants to expression (both at protein and mRNA levels) is still controversial.

4. Impact of Polymorphisms on Pharmacotherapy

4.1 Pharmacokinetic Consequences

To date, polymorphisms of the *MDR1* gene that alter *in vivo* transport activity have been focused on: the silent mutation in exon 26 (C3435T) and the non-synonymous mutation in exon 21 (G2677T/A).

Subjects with the T/T genotype at position 3435 had higher steady-state plasma concentrations after oral administration of digoxin in comparison with the C/C subjects.[9,78] Similar results were observed by Kurata et al.,[11] who showed that the mean absolute bioavailability (estimated from oral and intravenous administrations) of digoxin was significantly higher in 2677TT/3435TT subjects (homozygotes for thymine at both positions 2677 and 3435) than 2677GG/3435CC subjects in a gene dose-dependent manner, in that maximum bioavailability was observed in homozygotes for the mutant allele (mean, 87.1%) > heterozygotes (80.9%) > homozygotes for the wild-type allele (67.6%). They also indicated that the renal clearance of digoxin was almost 32% lower in 2677TT/3435TT subjects than in 2677GG/3435CC subjects, with 2677GT/3435CT subjects having an intermediate value. These results suggest that reductions in the intestinal secretion of digoxin into the gut lumen and renal excretion into the urine occur simultaneously in subjects with SNPs.

The histamine H_1 receptor antagonist fexofenadine, which is used for the treatment of seasonal allergic rhinitis and chronic idiopathic urticaria, is also a P-glycoprotein substrate. Kim et al.[52] demonstrated that the *2 allele was associated with differences in fexofenadine concentrations, with the area under the plasma concentration-time curve (AUC) being almost 40% greater in *1/*1 subjects compared with *2/*2 subjects, with *1/*2 heterozygotes having an intermediate value, suggesting enhanced *in vivo* P-glycoprotein activity among subjects with the *MDR1 *2* allele. In their study, the *2 allele was defined as a haplotype in which three SNPs at different polymorphic sites (T1236, T2677 and T3435) occurred simultaneously. However, they reported that fexofenadine is also a good substrate for organic anion transporting polypeptide (OATP).[87,88] In contrast, Drescher et al.[79] did not find any significant differences in fexofenadine disposition between subjects homozygous for the C allele and T allele at position 3435. Siegmund et al.[53] also did not find a significant influence of three *MDR1* variants (C3435T and G2677T/A) on talinolol disposition.

Because of a lack of metabolic biotransformation, digoxin is often used as a probe drug for pharmacogenetic testing (i.e. *in vivo* phenotype-genotype relationship studies) of the *MDR1* gene polymorphism. Sakaeda et al.[55] studied the relationship between the *MDR1* genotype and the pharmacokinetics of digoxin after a single oral administration in healthy subjects. They found that the AUC_4 of digoxin was significantly lower in subjects with the T/T3435 genotype than in C/C3435 subjects. Their observations are in line with a finding by Kim et al.,[52] but in contrast to the findings of Hoffmeyer et al.[9] and Kurata et al.[11]

One study has investigated the relationship between *MDR1* polymorphisms and the pharmacokinetics of oral ciclosporin in healthy subjects. Although the peak concentration and AUC of ciclosporin in the C/T3435 and T/T3435 subjects were 15% and 22% larger than those in C/C3435 subjects, differences in these values did not reach statistical significance.[77]

Functional consequences of *MDR1* polymorphism have also been investigated in two *in vitro* studies. In *in vitro* experiments conducted by Kim et al.[52] with cultured cells expressing MDR1-Ala893 (G2677) and MDR1-Ser893 (T2677) revealed that the Ser893 variant transporter resulted in a 47% lower intracellular digoxin concentration than did the Ala893 variant. Based on these results, they concluded that Ser893 variant-containing cells exhibit enhanced efflux characteristics compared with

those cells in which Ala893 was expressed. Kimchi-Sarfaty et al.[89] also investigated functional consequences of *MDR1* polymorphisms (Asn21Asp, Phe103Leu, Ser400Asn, Ala893Ser, and Ala998Thr) using a vaccinia virus-based transient expression system by two approaches; cell surface localisation and transport function. In contrast to the findings by Kim et al.,[52] they found that cell surface expression and transport capabilities were not substantially affected by any of the polymorphisms tested.

Taking all these findings into consideration, published observations, even when made using the same probe drug and even among the same racial group, are conflicting. The question arises as to why the contribution of C3435T and/or G2677T/A mutations to the pharmacokinetics of digoxin and fexofenadine differs among reports. Discussing possible reasons for this discrepancy will be useful for future studies of the involvement of polymorphisms of *MDR1*, as well as other drug transporters, in *in vivo* transport activity.

Both digoxin and fexofenadine are transported across cells by the OATPs, which are also expressed in various human tissues such as liver, intestine, and kidney. Although the intestinal transport mechanisms responsible for fexofenadine uptake have not yet been defined, fexofenadine has been shown to be a substrate of human OATP-A.[87,88] Similarly, digoxin was reported to be a substrate of liver-specific OATP8, another member of the OATP family.[90] In addition to the *MDR1* gene, the *OATP-C* and *OATP8* genes exhibit genetic variability.[91,92] Although the effects of *OATP8* variants have not yet been elucidated, certain mutations in the *OATP-C* gene could alter the *in vivo* pharmacokinetics of a clinically used drug.[93] Thus, it is possible that other transport mechanisms apart from those involving P-glycoprotein contribute to the variations in digoxin and fexofenadine pharmacokinetics in humans.

Grapefruit is known to inhibit the intestinal metabolism of numerous drugs, including terfenadine, saquinavir, ciclosporin, triazolam and nisoldipine, by inhibiting CYP3A enzymes, resulting in elevated drug bioavailability and then serum concentrations.[94] Recently, a new mechanism for the drug-grapefruit juice interaction has been reported; the bioavailability and serum concentrations of fexofenadine were reduced when grapefruit juice was taken.[95] In the intestine, P-glycoprotein and OATPs are located on the luminal membrane of the enterocyte, but they have opposite vectors for efflux back into the bowel and for uptake into the portal circulation, respectively.[95] Although the specific OATP member(s) responsible for the fexofenadine-grapefruit juice interaction has not been elucidated, OATP-B was recently identified as an OATP member localised at the apical membrane of intestinal epithelial cells in humans.[96] In addition to OATP-B, OATP-D and OATP-E are reported to be expressed in the human small intestine.[97] Since grapefruit juice is a more potent *in vitro* inhibitor of OATP than of P-glycoprotein activities,[95] the entry of fexofenadine from the intestinal lumen to blood may be inhibited by grapefruit juice, resulting in a reduced bioavailability. As grapefruit is able to inhibit P-glycoprotein-mediated drug efflux when present in sufficient concentrations,[95,98,99] the net bioavailability of fexofenadine will depend on the relative contribution of both efflux and uptake mechanisms. Indeed, in an interaction study with grapefruit juice, non-significant but moderate changes in digoxin pharmacokinetics were observed.[99] These findings clearly indicate that the intestinal transport of fexofenadine is determined by at least two drug transporters (P-glycoprotein and OATPs). Thus, multi-transporter-mediated drug transport with genetic variability needs to be considered when evaluating transport activities in the human body. It is clear that the identification of specific probe substrates and inhibitors for P-glycoprotein is required to elucidate the *in vivo* effect of *MDR1* polymorphisms on pharmacotherapy.

The possibility of the existence of functional unobserved SNPs cannot be excluded. As described in section 2, three SNPs, C1236T, G2677T/A and C3435T, have been haplotyped.[10,13,52,54] Recently, Tang et al.[54] demonstrated linkage disequilibrium between the different pairs of these SNPs and speculated on unobserved causal SNP(s) near position

3435, which might provide a plausible explanation for the conflicting findings among reports. However, based on the collective evidence from previous systematic analyses of the entire *MDR1* gene,[9,10,50,51] it is anticipated that such functional unobserved mutation(s) would not be localised to the coding region. Nevertheless, haplotype-based approaches, which take into consideration the combination of SNPs present in one allele, are expected to offer greater ability to predict changes in phenotype than SNP-based approaches.[100,101] Johne et al.[78] recently reported that the analysis of *MDR1* haplotypes is superior to an unphased SNP analysis for predicting *MDR1* phenotype. They speculated that haplotype 12 (i.e. 2677GT/3435TT) is a key genotype to describe interindividual differences in the pharmacokinetics of substrate drugs and account for divergent results among reports.

There are some reports about specific mechanisms of upregulation of *MDR1* transcription. Nakayama et al.[102] reported hypomethylation of the *MDR1* promoter as a predictive factor for *MDR1* upregulation in patients with AML, and Mickley et al.[103] showed gene rearrangements as causative events for *MDR1* expression. DNA methylation is one plausible regulator of gene expression. Mammalian DNA is heavily methylated at cytosine residues within CpG dinucleotides, with 60–80% of such residues being methylated.[104,105] Histone acetylation is associated with an increase in the accessibility of DNA to transcriptional machinery. The presence or absence of methylation at CpG sites (i.e. epigenetic mechanisms) is closely associated with transcriptional activation of the *MDR1* gene in various cultured cell lines and human tumour samples.[102,106-109] However, unfortunately, there are currently no data about differential *MDR1* gene regulation in normal tissues.

4.2 Pharmacodynamic Consequences

Besides the direct effect of genetic polymorphism on the pharmacokinetic profiles of substrate drugs, which may be responsible for the intended therapeutic effect and/or toxicity, the association between genetic variations and clinical outcomes remains largely unexplored.

As described previously, Fellay et al.[2] have studied the association between response to antiretroviral treatment and allelic variants of *MDR1* in 123 HIV-1-infected patients who were treated with efavirenz or nelfinavir. They found an association of T3435 with lower P-glycoprotein expression levels in peripheral blood mononuclear cells and a better response to anti-HIV-1 drugs as determined by an increased CD4+ cell count. CD4+ T-lymphocytes are the major cellular target of HIV-1 protease inhibitors.[110] A more pronounced P-glycoprotein activity in subjects with the C/C genotype compared with T/T subjects could limit intracellular concentrations of these drugs, thereby limiting their therapeutic efficacy.[2,82] They also reported the surprising finding that the T allele was associated with lower concentrations of nelfinavir and efavirenz in plasma, even with a low expression of P-glycoprotein in peripheral blood mononuclear cells. In order to address this paradox, they explored the following two hypotheses for a reduction of plasma drug concentrations: overexpression of other transporters with affinity for antiretroviral drugs and/or induction of CYP3A as compensatory adaptations to low concentrations of P-glycoprotein, as have been observed in *mdr1*-knockout mice.[111,112] However, they could not identify such compensatory mechanisms by analysis of transcription levels of *ABCC1* (MRP1) and *ABCC2* (MRP2), which encode for multidrug-resistant protein with affinity for antiretroviral agents,[113,114] or by assessment of CYP3A activity using midazolam as a probe drug.[115] The results about *MDR1* polymorphisms in their study raise another issue, in that the plasma concentrations of efavirenz, which is not a known substrate of P-glycoprotein, had a similar distribution pattern (i.e. lower concentrations in patients with the T allele) to those of nelfinavir, which is a well defined substrate of P-glycoprotein.[113] Interestingly, in addition to genetic variation in *MDR1*, *CYP2D6* genotypic status was a weak predictor for the interindividual variations in plasma concentrations of the two study drugs. Indeed, patients having the *CYP2D6* allele

associated with a poor metaboliser phenotype had higher concentrations in plasma of both drugs than did patients with a *CYP2D6* extensive metaboliser genotype when the patients' *MDR1* genotype was matched. Although CYP2D6 has not been reported to be a predominant enzyme for the metabolism of these drugs, the *CYP2D6* genotype may be a partial explanation for the paradoxical results.

Roberts et al.[1] evaluated the association between drug-induced adverse effects and *MDR1* gene polymorphism. Postural hypotension is a problematic adverse effect of tricyclic antidepressant medication that occurs in 10–50% of patients at therapeutic dosages, and is characterised by dizziness, palpitations and headache. They found that patients homozygous for the T allele at exon 26 (C3435T) had an increased risk of nortriptyline-induced postural hypotension, although neither the nortriptyline dose nor blood concentrations of drug differed significantly by genotype group.

Neurotoxicity is one of the most important and serious adverse effects of tacrolimus. Yamauchi et al.[6] have recently evaluated the correlation of *MDR1* gene polymorphism with tacrolimus-induced neurotoxicity (e.g. convulsions, tremor and leukoencephalopathy) in patients after living-related donor liver transplantation, and found that a high tacrolimus concentration, liver dysfunction and a mutation at position 2677 in exon 21 (i.e. the T2677 allele) were positive predictors of toxicity by a stepwise discriminant function analysis. Since P-glycoprotein regulates the distribution of substrate drugs (e.g. nortriptyline and tacrolimus) through the blood-brain barrier into the brain, a reduction in P-glycoprotein function and expression could lead to an abnormal accumulation of prescribed drugs in the brain. In living-donor liver transplantation, intestinal *MDR1* expression is also found to predict both tacrolimus pharmacokinetics and patient survival.[116] In this study, G2677T/A was a positive predictor for the development of tacrolimus neurotoxicity, whereas C3435T negatively contributed to toxicity, suggesting functional differences between the two SNPs.

Although these two SNPs are in tight linkage disequilibrium, their functional linkage to other SNPs is not identical. One systematic analysis of entire placental cDNA has indicated that heterozygous samples for the T–129C allele also had a mutant 2677T/A allele; however, an association between T–129C and C3435T was not observed.[10] Although the T–129C polymorphism is not located on known regulatory elements, it was shown to be associated with a lower P-glycoprotein expression in placenta.[10] Thus, whether the major three polymorphisms (i.e. C1236T, G2677T/A and C3435T) are functionally linked to polymorphic positions at regulatory sites of the *MDR1* promoter is of interest.

Corticosteroids are frequently prescribed with tacrolimus for the purpose of immunosuppression in transplant recipients. Zheng et al.[5] recently demonstrated an association between polymorphisms of the *MDR1* gene and corticosteroid weaning in 65 paediatric heart transplant patients, and indicated that homozygotes for the C3435 allele or G2677 allele required longer (at 1 year after transplantation) prednisone therapy than did patients having SNPs. Patients with the C/C3435 genotype and the G/G2677 genotype may require more aggressive alternative therapy if corticosteroids are going to be deleted from the immunosuppressive regimen.[5]

MDR1 gene polymorphisms are also reported to affect the outcome of therapy in patients with AML. Illmer et al.[4] compared the clinical course of AML treatment among patients with various *MDR1* genotypes, and demonstrated that patients homozygous for the wild-type allele at any locus investigated (exons 12, 21 and 26) exhibited a significantly decreased overall survival with a higher probability of relapse. Theoretically, a reduced intracellular concentration of anticancer drugs attributable to the action of P-glycoprotein (i.e. efflux transport of drugs to the outside of cells) in AML blasts may be related to resistant disease and failure of AML therapy. The patients were uniformly treated with anticancer drugs[4,117] that are P-glycoprotein substrates, including etoposide,[28] mitoxantrone[118] and daunorubicin.[119] Although they did not determine

whether the association between *MDR1* polymorphism and survival of AML patients was attributable to altered P-glycoprotein-mediated drug pharmacokinetics, increased clearance of these drugs in patients homozygous for the wild-type allele at the three loci might explain these findings. However, they showed a clear correlation between homozygosity for the wild-type allele(s) and lower *MDR1* expression in blast samples. Taken together, these findings, as well as those of Fellay et al.,[2] raise the possibility of differential gene regulation in different tissues, especially between normal tissues and leukaemic blasts. Nevertheless, a number of studies have indicated that overexpression of P-glycoprotein caused by *MDR1* gene amplification can be applied as a prognostic marker in certain diseases, such as leukaemia or ovarian cancer; high *MDR1* levels being indicative of a poor prognosis.[120-123]

Since P-glycoprotein is expressed in lymphocytes,[124,125] it has been hypothesised that overexpression of P-glycoprotein may be one of the reasons for acute and chronic rejection episodes despite adequate ciclosporin concentrations in blood.[126,127] However, von Ahsen et al.[60] reported no remarkable differences between stable renal transplant recipients with and without the C3435T mutation regarding renal function and the incidence of acute rejection as determined by biopsy during ciclosporin immunosuppression therapy. They also observed no differences in dose-adjusted ciclosporin trough concentrations between the two genotype groups. By contrast, Chowbay et al.[57] recently investigated the influence of *MDR1* polymorphisms on the pharmacokinetics of oral ciclosporin in 14 stable heart transplant patients. They focused on the haplotype of C1236T, G2677T/A and C3435T polymorphisms. Of the four major haplotypes identified in Asian populations (C-A-C, C-G-C, T-G-C and T-T-T), the T-T-T haplotype was frequently observed in all three racial populations (50% for Indians, 41% for Chinese and 37% for Malays). They also indicated that patients with the CC-GG-CC genotypes (C-G-C haplotype) had lower ciclosporin exposure, as determined from AUC_4, AUC_{12} and peak concentration, compared with patients with the TT-TT-TT genotypes (T-T-T haplotype), and suggested that Indians, in whom the T-T-T haplotype frequency was highest, may require a ciclosporin dosage regimen different from that in Chinese and Malays.

4.3 Interaction Profiles

In contrast to the extensive analysis of the pharmacological and physiological role of P-glycoprotein, surprisingly little has been reported regarding *MDR1* polymorphisms and drug-drug interaction profiles. Hoffmeyer et al.[9] first provided an interesting finding that the mean of the rifampicin (rifampin)-induced digoxin concentration of the C3435 population was lower than that of the T3435 population. Several studies have reported a digoxin-clarithromycin interaction, which is characterised by a significant elevation of the steady-state plasma concentration of digoxin.[128-131] Recently, Kurata et al.[11] have provided evidence that the oral bioavailability of digoxin during administration of clarithromycin was significantly increased in 2677GG/3435CC subjects, whereas no such significant change was observed in subjects with the *MDR1* gene SNPs. Although the molecular mechanism behind the inhibition of P-glycoprotein by clarithromycin is not precisely known, the most plausible reason for the remarkable changes in oral bioavailability in 2677GG/3435CC subjects would be that they have relatively greater amounts of intestinal P-glycoprotein to be inhibited by clarithromycin. Similar genotype-specific drug interactions have been reported for a known polymorphic metabolising protein, CYP2C19, including moclobemide-omeprazole,[132] diazepam-omeprazole[133] and proguanil-omeprazole[134] interactions.

It is highly likely that certain clinically relevant interactions between P-glycoprotein substrates and inhibitors and/or inducers depend on the route of administration. A number of clinically important drug interactions with rifampicin have been reported that are caused by the potent induction of intestinal CYP3A4.[135,136] However, recent findings indicate that coadministration of rifampicin 600 mg/day for 10 days was associated with substantially reduced

digoxin plasma concentrations after oral administration, but to a lesser extent after intravenous administration.[137] When duodenal biopsies were analysed before and after administration of rifampicin, the treatment was found to increase intestinal P-glycoprotein content 3.5-fold, which correlated with the extent of reduction of AUC after oral but not after intravenous administration of digoxin.[137] These results suggest that intestinal P-glycoprotein plays a key role in the systemic availability of digoxin. Likewise, in the study conducted by Kurata et al.,[11] coadministration of clarithromycin increased the digoxin AUC substantially after oral administration but to a lesser extent after intravenous administration. Interestingly, the AUC values of digoxin after intravenous administration were comparable among the three genotypic groups.

5. Conclusion

Although many factors, such as diet, race and disease state, may influence interindividual variability in the pharmacokinetic and pharmacodynamic outcomes of treatment with P-glycoprotein substrate drugs, the premise that genetic variations in the *MDR1* gene are one of the prime determinants of this variability is supported by a number of human studies. The clinical usefulness of genotyping would be expected to increase if it allowed a more accurate prediction of transport activity in humans. In order to achieve this, at least four points of research will be of importance.

Firstly, although the effects of *MDR1* gene variations on phenotypic indices (pharmacokinetics and pharmacodynamics) are controversial, most studies agree that P-glycoprotein expression correlates inversely with phenotype indices, e.g. less protein on the apical surface of intestinal enterocytes to pump substrates back into the intestinal lumen, resulting in increased bioavailability, and vice versa. Thus, additional studies of expression mechanisms (e.g. translation efficacy) with regard to *MDR1* gene variations are needed; expression levels can be influenced by structural differences in the genome, such as chromatin alterations and methylation.

Secondly, the identification of new functionally important mutations and/or haplotypes is needed to more accurately explain the variability in transport activity. The majority of *in vivo* data on the importance of *MDR1* polymorphisms in humans are from single-dose pharmacokinetic studies focused on a single polymorphism (e.g. G2677T/A or C3435T). Like many genes, the *MDR1* gene has multiple polymorphisms, some of which are in linkage disequilibrium. Thus, haplotypes or mutation patterns should be considered when clinical studies are conducted.

Thirdly, a 'candidate gene' pharmacogenomic approach,[138] where polymorphisms in multiple genes known or suspected to contribute to drug responses and kinetics are considered, is also useful. Combined genotyping of the *MDR1* and *CYP2C9* genes, allowing a more accurate prediction of phenytoin (a substrate for both proteins) plasma concentrations, is one example.[76]

Finally, rapid progress in the study of drug transporters in recent years has allowed us to identify the specific transporters involved in the disposition and distribution of certain drugs. For example, by use of recent technologies (e.g. site-directed mutagenesis and gene knockout in mice), digoxin was found to be a dual substrate for both P-glycoprotein and OATP-8, meaning that the contribution of both transporters with regard to genetic variation needs to be considered in order to describe more accurately the pharmacokinetics, and thus the clinical outcome, of digoxin treatment. As can be seen from this review, digoxin and fexofenadine may not be suitable substrates for *in vivo* pharmacogenetic testing. It is clear that the identification of specific probe drugs for P-glycoprotein is required.

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Note in Proof

Additional important works regarding *MDR1* polymorphisms have been published since acceptance of this review.[139-141]

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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	aggaGatca	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

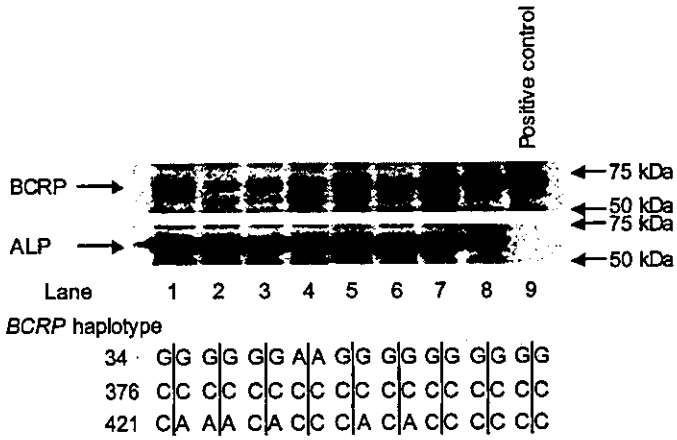

FIG. 1. Western blot analysis of BCRP expression in human placentas. Lane 1, sample 100 (control for calculations); lanes 2 through 8, placental samples; lane 9, positive control. The position of the molecular mass marker is indicated on the right. The *BCRP* haplotype was also shown for each placental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

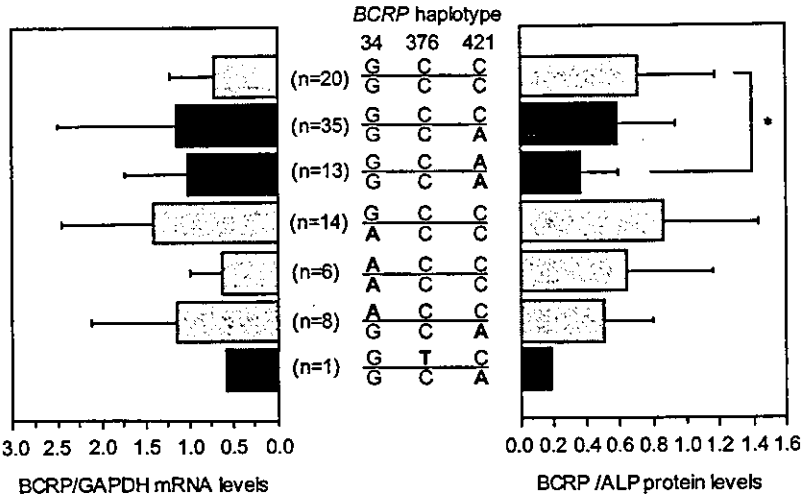

FIG. 2. Placental BCRP mRNA (left) and protein (right) expression levels in various *BCRP* haplotypes. Data are shown as the mean ± S.D. *, significantly different from values in wild-type subjects as determined by one-way analysis of variance with the Tukey-Kramer multiple comparisons test ($p < 0.05$).

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

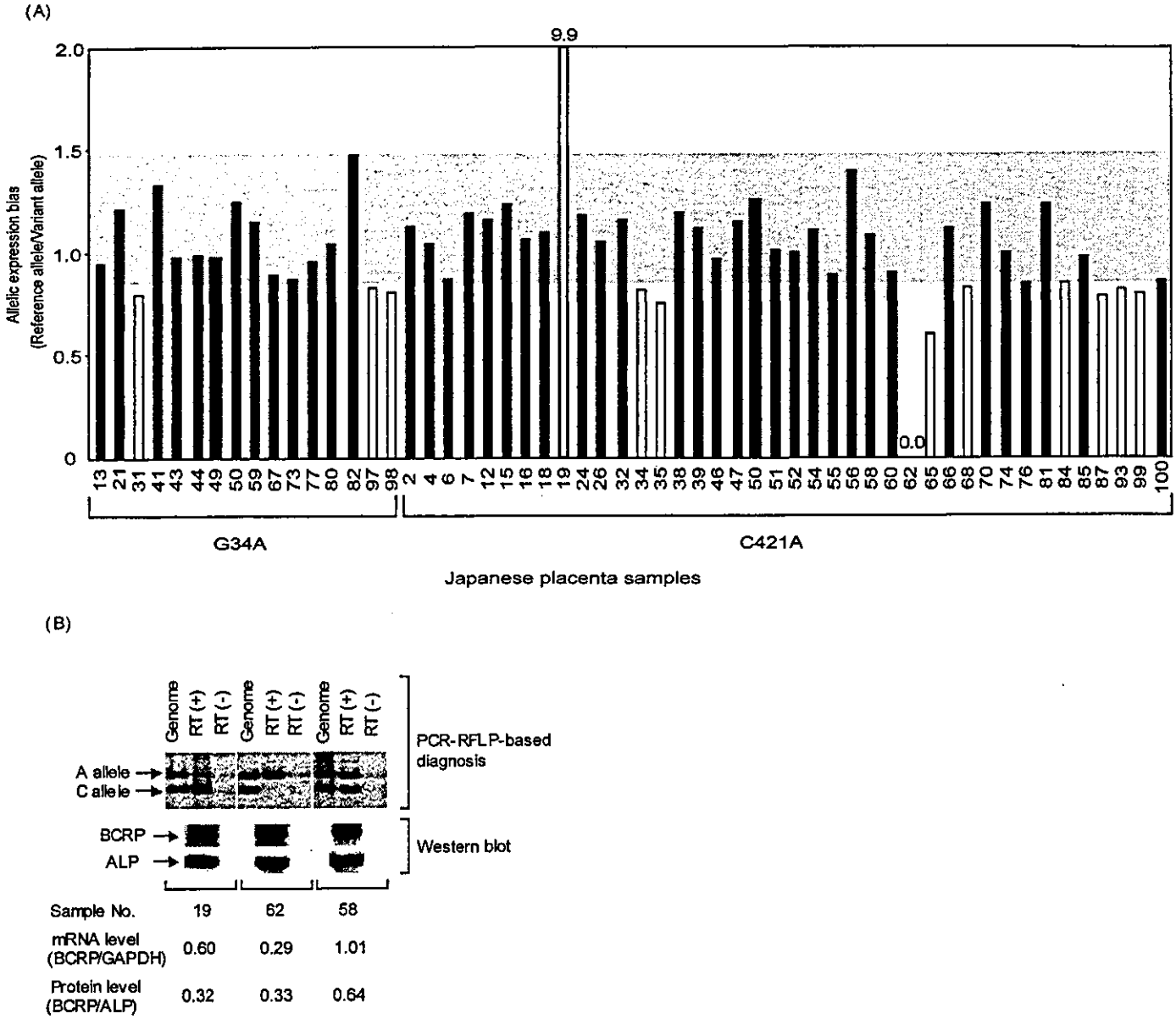

FIG. 3. A, allelic expression bias of the *BCRP* gene in Japanese placentas. The expression bias was estimated based on the reference/variant ratios (replicated data points for each sample) at either the G34A or C421A polymorphism, and corrected using the genomic ratio. The shaded box represents the approximated 95% confidence interval and the open (white) bars indicate individuals displaying significant variations. B, the PCR-RFLP-based diagnosis for allelic imbalance. Monoallelic samples (19 and 62) and biallelic sample (58) are presented with their BCRP mRNA and protein levels.

charged amino acid substitution. These characteristics suggest that the C421A variant alters the tertiary structure of the BCRP protein, leading to greater susceptibility to degradation (Imai et al., 2002). In addition, Mizuarai et al. (2004) reported that ATPase activity in the membrane of SF9 cells infected with the C421A variant showed a decrease of 1.3 below that of wild-type cells. They also indicated that the C421A variant exhibited reduced drug resistance in polarized LLC-PK1 cells along with increased intracellular drug accumulation.

In an in vivo human study, Sparreboom et al. (2004) recently evaluated the effects of the C421A variant on the pharmacokinetics of diflomotecan, a synthetic derivative of camptothecin, in 22 cancer patients, and provided the first evidence linking variant *BCRP* alleles to altered drug exposure. Patients with this variant as heterozygosity indicated about 3-fold higher plasma levels than did patients with wild-type 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