

Table 2. Summary of racial genetic data of naturally occurring variations of human drug transporters

Gene	Mutation	Location	Effect	Allelic frequency (n)		
				Japanese (48 – 220)	Caucasians (37 – 461)	African- Americans (23 – 200)
ABCB1 (MDR1)	-129T→C	Exon 1b	Noncoding	0.94/0.06	0.94 – 0.97/ 0.03 – 0.06	
	1236C→T	Exon 12	Synonymous	0.35/0.65	0.54 – 0.66/ 0.34 – 0.46	0.79 – 0.85/ 0.15 – 0.21
	2677G→(T/A)	Exon 21	893Ala→(Ser/Thr)	0.36 – 0.44/ 0.36 – 0.42/ 0.20 – 0.22	0.50 – 0.56/ 0.38 – 0.46/ 0.02 – 0.10	0.85 – 0.89/ 0.10 – 0.15/ 0.00 – 0.01
	3435C→T	Exon 26	Synonymous	0.51 – 0.62/ 0.38 – 0.49	0.43 – 0.54/ 0.46 – 0.57	0.74 – 0.84/ 0.16 – 0.26
ABCC2 (MRP2)	-24C→T	Promoter	Noncoding	0.81/0.19		
	1249G→A	Exon 10	417Val→Ile	0.88/0.13		
	2302C→T	Exon 18	768Arg→Trp	0.99/0.01		
	2366C→T	Exon 18	789Ser→Phe	0.99/0.01		
	4348G→A	Exon 31	1450Ala→Thr	0.99/0.01		
ABCG2 (BCRP)	34G→A	Exon 2	12Val→Met	0.81 – 0.83/ 0.17 – 0.19	0.90 – 0.96/ 0.04 – 0.10	0.94/0.06
	376C→T	Exon 4	126Gln→ (stop codon)	0.98 – 0.99/ 0.01 – 0.02	1.00/0.00	1.00/0.00
	421C>→A	Exon 5	141Gln→Lys	0.67 – 0.73/ 0.27 – 0.33	0.86 – 0.89/ 0.11 – 0.14	0.95 – 0.97/ 0.02 – 0.05
	1515C (deletion)	Exon 13	509Met→ (stop codon)	0.995/0.005		
SLCO1A2 (OATP-A)	38T→C	Exon 1	13Ile→Thr		0.89 – 0.94/ 0.06 – 0.11	0.98/0.02
	382A→T	Exon 4	128Asn→Tyr		1.00/0.00	0.99/0.01
	516A→C	Exon 5	172Glu→Asp		0.95 – 0.98/ 0.02 – 0.05	0.98/0.02
	559G→A	Exon 5	187Ala→Thr		0.99/0.01	1.00/0.00
	2003C→G	Exon 14	668Thr→Ser		0.99/0.01	0.96/0.04
SLCO1B1 (OATP-C)	-11187G→A	Promoter	Noncoding		0.93/0.07	
	388A→G	Exon 4	130Asn→Asp	0.37/0.63	0.53 – 0.69/ 0.31 – 0.47	0.25/0.75
	521T→C	Exon 5	174Val→Ala	0.84 – 0.89/ 0.11 – 0.16	0.82 – 0.88/ 0.12 – 0.18	0.98/0.02
SLCO1B3 (OATP8)	334T→G	Exon 3	112Ser→Ala	0.70/0.30	0.76/0.24	0.49/0.51
	699G→A	Exon 6	233Met→Ile	0.70/0.30	0.76/0.24	0.49/0.51
SLCO2B1 (OATP-B)	9-bp deletion	Exon 2	Frame shift	0.93/0.07		
	1457C→T	Exon 10	486Ser→Phe	0.69/0.31	0.86/0.14	0.63/0.37
SLC22A6 (OAT1)	149G→A	Exon 1	50Arg→His		1.00/0.00	0.97/0.03
	311C→T	Exon 1	104Pro→Leu		1.00/0.00	0.99/0.01

BCRP: Breast cancer-resistance protein; MDR: Multi-drug resistance; MRP: Multi-drug resistance-associated protein; OAT: Organic anion transporter; OATP: Organic anion-transporting polypeptide; OCT: Organic cation transporter.

Table 2. Summary of racial genetic data of naturally occurring variations of human drug transporters (continued)

Gene	Mutation	Location	Effect	Allelic frequency (n)		
				Japanese (48 – 220)	Caucasians (37 – 461)	African- Americans (23 – 200)
	677T→C	Exon 4	226Ile→Thr		0.99/0.01	1.00/0.00
	767C→T	Exon 4	256Ala→Val		1.00/0.00	0.99/0.01
	877C→T	Exon 5	293Arg→Trp		1.00/0.00	0.98/0.02
	1361G→A	Exon 8	454Arg→Gln		1.00/0.01	0.99/0.01
<i>SLC22A8</i> (OAT3)	1166C→T	Exon 8	389Ala→Val	0.99/0.01		
<i>SLC22A1</i> (OCT1)	181C→T	Exon 1	61Arg→Cys	1.00/0.00	0.91 – 0.93/ 0.07 – 0.09	1.00/0.00
	262T→C	Exon 1	88Cys→Arg	1.00/0.00	0.99/0.01	1.00/0.00
	480C→G	Exon 2	160Phe→Leu	0.89/0.11	0.78 – 0.93/ 0.07 – 0.22	0.99/0.01
	1022C→T	Exon 6	341Pro→Leu	0.84/0.16	1.00/0.00	0.92/0.08
	17857G→A	Exon 7	401Gly→Ser	1.00/0.00	0.97 – 0.99/ 0.01 – 0.03	0.99/0.01
	17878A→G	Exon 7	408Met→Val	0.17/0.83	0.40/0.60	0.26/0.74
	17914(ATG) deletion	Exon 7	420Met deletion	1.00/0.00	0.81 – 0.84/ 0.16 – 0.19	0.97/0.03
	32870G→A	Exon 9	465Gly→Arg	1.00/0.00	0.96 – 0.99/ 0.01 – 0.04	1.00/0.00
<i>SLC22A2</i> (OCT2)	495G→A	Exon 2	165Met→Ile	1.00/0.00	1.00/0.00	0.99/0.01
	601C→T	Exon 3	200Thr→Met	0.99/0.01	1.00/0.00	1.00/0.00
	808G→T	Exon 4	270Ala→Ser	0.83 – 0.87/ 0.13 – 0.17	0.84/0.16	0.89/0.11
	1198C→T	Exon 7	400Arg→Cys	1.00/0.00	1.00/0.00	0.98/0.02
	1294A→C	Exon 8	432Lys→Gln	1.00/0.00	1.00/0.00	0.99/0.01
<i>SLC22A3</i> (OCT3)	1270A→T	Exon 7	424Thr→Ser	0.99/0.01		

BCRP: Breast cancer-resistance protein; MDR: Multi-drug resistance; MRP: Multi-drug resistance-associated protein; OAT: Organic anion transporter; OATP: Organic anion-transporting polypeptide; OCT: Organic cation transporter.

(n = 48), Ito *et al.* [79] analysed the entire *ABCC2* gene and found six SNPs. Among them, 1249G→A in exon 10, a non-synonymous mutation (417Val→Ile) was frequently observed with an allelic frequency of 12.5%. Only one heterozygote (allelic frequency is 1%) was observed out of 48 volunteers for 2302C→T (768Arg→Trp in exon 18), 2366C→T (789Ser→Phe in exon 18) and 4348G→A (1450Ala→Thr in exon 31).

The *ABCG2* gene is located at 4q22 and encodes a 72-kDa membrane protein composed of 655 amino acids [22,94]. So far, systematic mutation analysis of the *ABCG2* gene has been performed in various ethnic populations, and > 40 SNPs have been identified [95-98]. The two most frequent non-synonymous mutations identified in humans are 34G→A (12Val→Met in exon 2) and 421C→A (141Gln→Lys in exon 5). When comparing the frequencies of the three major variants (i.e., 34G→A, 376C→T and 421C→A) among three

ethnic populations (Japanese, Caucasian and African-American), Japanese subjects had significantly higher frequencies of 34G→A and 421C→A than the other two ethnic groups. Interestingly, these three variants occurred simultaneously, and the following four haplotypes were identified: G-C-C, G-C-A, A-C-C and G-T-C with their corresponding allelic frequencies of 46, 35, 18 and 1%, respectively [97]. Thus, similar to other transporter genes, the genetic frequency of *ABCG2* appears to be dependent on ethnicity.

Two recent studies have been conducted to identify SNPs in the *SLCO1A2* gene using genomic DNA samples from various ethnic populations [99,100]. Iida *et al.* [99] screened 27-kb wide for *SLCO1A2* in a Japanese population (n = 48). They did not detect SNPs in the exonic regions, but identified several variations in the 5'-flanking region. Among them, three variations (-916G→A, -526T→C and -189A/ins) are of interest because they are located within important transcriptional regulatory

regions (e.g., hepatic nuclear factor 1 α). In contrast, Lee *et al.* [100] screened all 14 exons of *SLCO1A2* and identified 6 nonsynonymous SNPs with an allelic frequency in the range of 1.0 – 11.1%. They also demonstrated that allelic frequencies of six identified SNPs are dependent on ethnicity using ethnically defined DNA (European, African, Chinese and Hispanic-Americans).

So far, at least 15 nonsynonymous *SLCO1B1* SNPs have been identified in various ethnic populations. Among them, two commonly occurring nonsynonymous SNPs, 388A→G (130Asn→Asp in exon 4) and 521T→C (174Val→Ala in exon 5), are of special interest, due to not only their marked consequences in transport capability, but also interethnicity in allelic frequency. In addition to SNP-based analysis, haplotype-oriented assessment has also been well documented [101–104]. At least 17 haplotypes have been recognised so far. Major haplotypes in humans are as follows: *SLCO1B1*1a* (130Asn174Val), *-*1b* (130Asp174Val), *-*5* (130Asn174Ala), *-*15* (130Asp174Ala) and *-*17* (-11187G→A130Asp174Ala). Although the frequency of *SLCO1B1*5* is extremely low in Asian and black populations, the frequency in Caucasians is ~15%. In contrast, *SLCO1B1*15* is more common in Asian populations [69,101,103]. Interestingly, although the allelic frequency of 521T→C is similar between Asians and Caucasians (~15%), their haplotype patterns are different. In Asian populations, the 521T→C polymorphism is combined with the 388A→G variant. In recent Japanese data, the -11187G→A variant is also tightly linked (~100%) to the *SLCO1B1*15* allele [105].

A recent report has described the identification of SNPs in *SLCO1B3* in a population of Japanese individuals [99]. Based on this study and the authors' unpublished data, at least two nonsynonymous SNPs, 334T→G (112Ser→Ala in exon 3) and 699G→A (233Met→Ile in exon 6), exist with an allelic frequency ranging 0.24 – 0.51. Because the frequencies of these two SNPs were identical in all ethnic populations studied, these SNPs may occur simultaneously (being haplotyped).

Interestingly, collective evidence indicates that the frequency of nonsynonymous SNPs in OAT family genes (*SLC22A6*, *SLC22A7* and *SLC22A8*) appears extremely lower (<1%) [99,103,106,107], suggesting these genes are relatively intolerant of nonsynonymous changes. Fujita *et al.* [106] focused on *SLC22A6* (OAT1 gene) and identified 6 nonsynonymous SNPs using 267 DNA samples from an ethnically diverse population. Only two SNPs, 149G→A (50Arg→His in exon 1) and 877C→T (293Arg→Trp in exon 5), were present at $\geq 1\%$ in at least one ethnic population. They also identified 17 distinct haplotypes. Xu *et al.* [107] resequenced the coding regions of four OAT member genes from an ethnically diverse, healthy population ($n = 192$), and identified two nonsynonymous SNPs in *SLC22A6*, three in *SLC22A7*, one in *SLC22A8* and eight in *SLC22A9*, with an allelic frequency in the range of 0.01 – 0.03.

Some groups have screened for genetic variants of *SLC22A1* (encoding OCT1) in various ethnic populations [108–110].

Kerb *et al.* [110] identified 4 nonsynonymous SNPs (61Arg→Cys, 88Cys→Arg, 160Phe→Leu, 401Gly→Ser) and 1 deletion (420Met→del) in 57 Caucasian samples, with respective allelic frequencies of 9.1, 0.6, 22.0, 3.2 and 16.0%. Subsequently, Shu *et al.* [109] also detected numerous variations from five different ethnic groups. Some known SNPs (e.g., 41Phe→Leu and 117Pro→Leu) were observed in at least one ethnic population, suggesting ethnic diversity in *SLC22A1* polymorphism.

Similar to *SLC22A1*, several genetic variants in the coding region of *SLC22A2* (OCT2 gene) have been identified [111,112]. *SLC22A2* polymorphism was recently investigated comprehensively by screening all 11 exons as well as intronic sequence using 247 ethnically diverse DNA samples [112]. Among eight nonsynonymous SNPs, four (165Met→Ile, 270Ala→Ser, 400Arg→Cys and 432Lys→Gln) were polymorphic, with ethnic-specific allelic frequencies $\geq 1\%$. Novel variations, including SNPs and deletion, have also been reported in recent Japanese studies [111,113].

4. Impact of polymorphisms on pharmacotherapy

4.1 Pharmacokinetic consequences

In the pharmacogenomics of the *ABCB1* gene, Hoffmeyer *et al.* [77] first reported that a synonymous SNP, 3435C→T, was associated with significantly reduced intestinal P-gp content in subjects with the T/T genotype in comparison with subjects homozygous for the C allele (C/C), leading to higher steady-state plasma concentrations after the oral administration of digoxin in T/T subjects. After this report, a remarkably large number of clinical studies have been conducted around the world on the association of the *ABCB1* genotype and pharmacokinetic phenotypes. Most studies have focused on SNPs in the following two exons, 21 (2677G→T/A) and 26 (3435C→T); however, as summarised in recent reviews [50,82,114–116] and Table 3, the published observations conflict even when using the same probe drug and even among the same racial group. For example, Sakaeda *et al.* [117] conducted an *ABCB1* genotype–phenotype study using digoxin as a probe and found that the AUC of digoxin in the absorption phase was significantly lower in subjects with 3435T/T genotype than in 3435C/C subjects. These observations are in line with a finding by Kim *et al.* [81], but are in contrast to the findings of Hoffmeyer *et al.* [77] and Kurata *et al.* [118]. In order to overcome these disagreements, some researchers have incorporated haplotype-oriented analysis into the genotype–phenotype study [83–86,119]. Recent studies have demonstrated that haplotype assessment represents more precise prediction of the pharmacokinetics of certain drugs such as digoxin [119] and ciclosporin [83].

Wang *et al.* [120] introduced new approach for the evaluation of the 3435C→T variant. The level of mRNA expression can be regulated in a *cis* or *trans* fashion, and the *cis*-acting polymorphism changes the expression of the gene transcript

Table 3. Impact of *ABCB1* gene variants on PK of drug substrates

Polymorphism	Population	Drug	Functional effect of the variant allele	Ref.
3435C→T	Caucasian HV	Digoxin	Increased AUC after single dose for T/T	[77]
	Caucasian HV	Digoxin	Higher AUC and C _{max} under steady state for T/T*	[119]
	Japanese HV	Digoxin	Higher BA after single dose for T/T*	[118]
	Caucasian and African HV	Digoxin	Higher AUC after single dose for T/T	[191]
	Caucasian HV	Digoxin	No difference in PK data after single dose	[192]
	Japanese HV	Digoxin	Decreased AUC after single dose for T/T	[117]
	Korean HV	Fexofenadine	Higher AUC and C _{max} after single dose for T/T*	[193]
	Caucasian HV	Fexofenadine	No difference in PK data after single dose	[194]
	Caucasian and African HV	Fexofenadine	Decreased AUC after single dose for T/T	[81]
	Asian HT patients	Ciclosporin	Higher AUC under steady state for T/T*	[84]
	Caucasian RT patients	Ciclosporin	No difference in C _{min} under steady state	[195]
	Caucasian and African HV	Ciclosporin	No difference in AUC after single dose	[196]
	Caucasian RT patients	Ciclosporin	Decreased AUC under steady state for C/T and T/T	[197]
	LT patients	Ciclosporin	Higher plasma (or serum) level/dose ratio under steady state for T/T	[198]
	RT patients	Tacrolimus	Higher C _{min} under steady state for T/T	[199]
	HT paediatric patients	Tacrolimus	Higher C _{min} under steady state for C/T and T/T	[200]
	RT patients	Tacrolimus	No difference in C _{min} under steady state	[201]
	Caucasian HV	Talinolol	No difference in AUC*	[202]
	Chinese HV	Talinolol	No difference in AUC after single dose*	[203]
	Caucasian HIV-1 patients	Nelfinavir, efavirenz	Lower C _{min} under steady state for T/T	[143]
	HIV patients	Atazanavir	Lower drug level under steady state for T/T	[204]
	Caucasian HV	Loperamide	No difference in PK data after single dose	[205]
	HV	Dicloxacillin	No difference in C _{max} after single dose	[206]
Turkish HV	Phenytoin	Higher drug level under steady state for T/T	[207]	
Japanese schizophrenic patients	Risperidone	No difference in C _{min} under steady state	[208]	
ALL paediatric patients	Vincristine	No difference in PK data*	[160]	
2677G→(T/A)	Japanese HV	Digoxin	Higher BA after single dose for T/T*	[118]
	Caucasian and African HV	Digoxin	Higher AUC after single dose for T/T	[191]
	Caucasian HV	Digoxin	No difference in PK data after single dose	[192]
	Japanese HV	Digoxin	Lower AUC after single dose for T/T	[209]
	Caucasian and African HV	Fexofenadine	Decreased AUC after single dose for T/T	[81]
	Korean HV	Fexofenadine	Decreased AUC after single dose for A/A*	[193]
	Asian HT patients	Ciclosporin	Higher AUC under steady state for T/T*	[84]
	HT paediatric patients	Tacrolimus	Higher C _{min} under steady state for G/T and T/T	[200]

*Including haplotype assessments.

ALL. Acute lymphoblastic leukaemia; BA: Bioavailability; HT: Heart transplant; HV: Healthy volunteers; LT: Liver transplant; PK: Pharmacokinetics; RT: Renal transplant.

Table 3. Impact of *ABCB1* gene variants on PK of drug substrates (continued)

Polymorphism	Population	Drug	Functional effect of the variant allele	Ref.
	RT patients	Tacrolimus	Higher drug level under steady state for T/T*	[210]
	Caucasian HV	Talinolol	Slightly higher in AUC for T/A and T/T*	[202]
	Chinese HV	Talinolol	No difference in AUC after single dose*	[203]
	ALL paediatric patients	Vincristine	No difference in PK data*	[160]

*Including haplotype assessments.

ALL: Acute lymphoblastic leukaemia; BA: Bioavailability; HT: Heart transplant; HV: Healthy volunteers; LT: Liver transplant; PK: Pharmacokinetics; RT: Renal transplant.

from the allele carrying the polymorphism, leading to the allelic expression imbalance. In order to test for the presence of *cis*-acting polymorphisms in human *ABCB1* that might be responsible for altered mRNA expression of the 3435T allele, they measured differences in allelic mRNA expression between the 3435T and 3435C allele using liver samples from heterozygous individuals carrying the 3435C→T SNP. They indicated that mRNA expression of the 3435C allele was significantly higher than that of the 3435T allele (3435C/3435T ratios in the range of 1.06 – 1.16). Based on the experiments including *in vitro* transfection of mixtures of *ABCB1* variants carrying all possible combinations of 1236C→T, 2677G→T and 3435C→T, they concluded that 3435C→T is the main functional polymorphism affecting mRNA levels, by altering mRNA stability. Interestingly, allelic expression imbalance has been observed in other pharmacokinetic genes such as *ABCG2*, *CYP3A5* and *CYP3A4* [97,121,122].

The question arises as to why the contribution of SNPs to the pharmacokinetics of some probes (e.g., digoxin and fexofenadine) differs among reports. The reasons for this discrepancy remain to be addressed; however, multiple tissue expression of P-gp with various vectorial movements and no suitable specific probe drug for P-gp function may contribute. Recently, Brunner *et al.* [123] measured the brain distribution of a model P-gp substrate, the calcium-channel inhibitor verapamil [124], using positron emission tomography in two groups of healthy volunteers. To these authors' knowledge, this is the first evaluation of P-gp function, as a 'gatekeeper' (i.e., regulating drug uptake to highly sensitive tissue brain), in the BBB directly. They indicate no difference in the brain distribution of [¹¹C]verapamil between the TTT haplotype (1236T, 2677T and 3435T) and the wild-type CGC haplotype (1236C, 2677G and 3435C). Because positron emission tomography has sensitivity in the lower picomolar range for tissue concentrations of drug molecules to be measured, and because P-gp-triggered active efflux may be an unyielding barrier in the brain penetration of substrate drugs, their findings that failed to show an effect of *ABCB1* gene polymorphisms on P-gp functions in the BBB are notable.

Sparreboom *et al.* [125] first studied the effects of naturally occurring, common variant *ABCG2* 421C→A on the pharmacokinetics of diflomotecan, a synthetic derivative of camptothecin, in 22 adult white patients with cancer. They

found that plasma levels of diflomotecan after intravenous administration were significantly higher (~ 300%) in patients with 421C/A genotype than in 421C/C patients. However, despite expectations of significant genotype-dependent regulation in intestinal absorption due to its enriched localisation, the pharmacokinetics of diflomotecan did not differ between the two genotype groups after oral administration. Although further investigation is required to resolve this issue, these observations partially agree with some *in vitro* studies, indicating that the *ABCG2* 421C→A allele is associated with low BCRP expression levels [97,126,127]. These *in vitro* observations suggest that carriers of the 421C→A allele may have decreased clearance (increased plasma levels) and/or increased bioavailability. In a preliminary fashion, Sparreboom *et al.* [128] also reported that the heterozygous 421C/A allele observed in 2 patients was associated with a 1.34-fold increased oral bioavailability of topotecan compared with that in 10 patients with the 421C/C genotype. In contrast, de Jong *et al.* [129] reported no difference in the pharmacokinetic parameters of irinotecan and SN-38 between patients with and without the *ABCG2* 421C→A allele. They noted that other processes involved in irinotecan metabolism and elimination that exhibit great interindividual variation might be overshadowing any effect of this *ABCG2* polymorphism.

As described previously, because MRP2 (*ABCC2* protein) is responsible for the export of conjugated drug metabolites from hepatocytes to bile, and because many *ABCC2* variants are known to be associated with DJS, naturally occurring *ABCC2* variants are expected to be involved in large interindividual differences in pharmacokinetic and pharmacodynamic consequences of substrate drugs; however, no sufficient human data have been reported so far. To the authors' knowledge, at least two studies have been carried out to assess possible associations of genetic variants in *ABCC2* with phenotypes (i.e., cellular exposure of nelfinavir [130] and pharmacokinetics of pravastatin [102]). However, there were no significant associations between phenotype indices and SNPs and/or haplotypes at *ABCC2*.

Facilitative hepatic uptake from the portal circulation by OATP1B1 is thought to contribute to tissue selectivity and therapeutic response to HMG-CoA reductase inhibitors (statins). Nishizato *et al.* [103] screened genetic polymorphism in the *SLCO1B1* using DNA from 120 Japanese healthy

volunteers and conducted a clinical study to examine whether variants alter transport activity with pravastatin as a selective probe drug. Subjects with the *SLCO1B1**15 allele (130Asp174Ala) had reduced total and nonrenal clearance, as compared with those with the *SLCO1B1**1b allele (130Asp174Val), and the difference between *1b/*1b and *1b/*15 subjects was significant. In their study, only one subject harboured the *15/*15 genotype, with nonrenal clearance about a tenth of that in the *1b/*1b genotype. They first demonstrated that commonly occurring SNPs in the *SLCO1B1* gene are likely to be associated with altered pharmacokinetics of substrate drugs in humans. Niemi *et al.* [102] also evaluated the relationship between *SLCO1B1* variants and the pharmacokinetics of pravastatin. In heterozygous carriers of *15B (130Asp174Ala), the mean pravastatin AUC₀₋₁₂ was 93% higher compared with noncarriers and, in heterozygous carriers of *17 (-11187G→A and 130Asp174Ala), it was 130% higher compared with noncarriers. They also reported no significant associations between *SLCO2B1*, *ABCC2* or *ABCB1* polymorphisms and the pharmacokinetics of pravastatin. Based on the healthy volunteers study, Mwinyi *et al.* [104] found that *5 allele (130Asn174Ala) delayed the hepatocellular uptake of pravastatin, whereas *1b allele seemed to accelerate OATP1B1-dependent uptake of the drug. Chung *et al.* [69] characterised the effects of *SLCO1B1* alleles, *1a, *1b and *15 on the pharmacokinetics of pitavastatin. Despite small sample size, the dose-normalised AUC and C_{max} of pitavastatin were 1.4- and 1.8-fold higher, respectively, in subjects heterozygous for the *15 allele versus subjects not varying this allele. Similar to pravastatin, the *15 allele is suggested to be associated with decreased pitavastatin uptake from blood into hepatocytes. Systematic exposure to rosuvastatin had been observed to be ~2-fold higher in Japanese subjects living in Japan compared with white subjects in Western Europe or the US [131-133]. Because OATP1B1 contributes to the hepatic uptake of rosuvastatin [134], in order to determine whether polymorphisms in the *SLCO1B1* gene contribute to any pharmacokinetic differences, Lee *et al.* [135] conducted a pharmacokinetic study including four racial populations. They found that *SLCO1B1* 521T→C did not account for the clear population differences in rosuvastatin exposure among white subjects and Asian groups. Although no 521C/C homozygote in Asian subjects seems to be the most likely reason for failed to show up the differences, they concluded that the pharmacogenetics of other rosuvastatin disposition pathways may better explain the ethnic differences in pharmacokinetics [136].

The H₁-receptor antagonist fexofenadine is a P-gp substrate [65]; however, association between the pharmacokinetics of fexofenadine and polymorphism of the *SLCO1B1* gene has recently been reported [137]. The mean total AUC of fexofenadine in the -11187G/G521C/C subjects was 76% higher in subjects with the 521T/C genotype and 127% higher in subjects with the 521T/T genotype. These results suggest that OATP1B1 is involved in fexofenadine exposure, and may

partly explain the conflicting observations between fexofenadine pharmacokinetics and *ABCB1* polymorphism.

Niemi *et al.* [138] investigated possible associations between the pharmacokinetics of repaglinide, a meglitinide analogue antidiabetic drug, and SNPs in genes encoding for OATP1B1, P-gp, CYP2C8 and CYP3A5 in 56 healthy subjects. Multiple regression analysis indicated that the *SLCO1B1* 521T→C and *CYP2C8**3 allele were independent predictors of the AUC_{0-∞} and C_{max} of repaglinide; the AUC_{0-∞} in the subjects with 521C/C genotype was 107 and 188% higher, respectively, than in subjects with the 521C/T or 521T/T genotype; however, surprisingly, only *SLCO1B1* -11187G→A was significantly associated with an enhanced effect of repaglinide on blood glucose, even though SNPs at positions -11187 and 521 are haplotyped.

Very recently, the authors studied the effects of polymorphism of *SLCO1B1*, particularly the *1b allele, on the pharmacokinetics of three anionic drugs, pravastatin, valsartan and temocapril in a three-way crossover manner in 23 healthy Japanese volunteers [139]. The authors found that AUC of pravastatin in *1b/*1b carriers was 65% of that in *1a/*1a carriers, and AUC of valsartan and temocapril in each subject was significantly correlated with that of pravastatin. These results suggest that: i) *SLCO1B1**1b allele enhances the hepatic uptake activity of pravastatin; and ii) OATP1B1 is one of the determinant factors governing interindividual variability in the pharmacokinetics of these three drugs. Reduction of pravastatin AUC in the *1b subjects was well consistent with the above-mentioned study conducted by Mwinyi *et al.* [104].

Two studies have examined the effects of *SLCO1B1**5 and *15 on the functional properties of OATP1B1 using cDNA transfected cells. Kameyama *et al.* [140] evaluated transport capability by transient expression system of HEK293 and HeLa cells using endogenous conjugates, estradiol-17β-D-glucuronide and estrone-3-sulfate, and statins as substrates. Kinetic analysis of pravastatin and atorvastatin showed that K_m values were not altered, but V_{max} values decreased significantly in cells expressing the variants. Immunocytochemical study showed that the variant-typed proteins were localised not only at the plasma membrane, but also in the intracellular space. In contrast, Iwai *et al.* [141] indicated that all SNP variants expressed in HEK293 cells were predominantly located on the cell surface without changes in K_m values for the transport of 17β-estradiol 17β-D-glucuronide. However, the normalised V_{max} value (by the protein expression level estimated from western blotting) for *SLCO1B1**15 was drastically decreased to < 30% compared with *1a. Although the observation of lower V_{max} values in the *SLCO1B1**15 cells was similar between the studies, the expression manner was clearly controversial. There is no good reason for this discrepancy; however, we need to be careful in expecting *in vivo* cellular localisation from the results of *in vitro* expression system.

Although many SNPs have been identified in the OCT2 gene, no pharmacogenomic human study has yet been

Table 4. Impact of the *ABCB1* (*MDR1*) genetic variant on PD of drug substrates and their consequences on disease states

Population (disease)	Polymorphism	Drug	Outcome marker	Effect	Ref.
Caucasian RT patients	3435C→T	Ciclosporin	Acute rejection	No significant difference	[195]
Caucasian patients (depression)	3435C→T	Nortriptyline	Nortriptyline-induced postural hypotension	Higher in T/T	[211]
Caucasian patients (HIV infection)	3435C→T	Nelfinavir, efavirenz	CD4 recovery with treatment	Higher in T/T	[143]
HIV patients	3435C→T		Virological suppression	Higher in T/T	[212]
Caucasian patients (HIV infection)	3435C→T		CD4 recovery with treatment	No significant difference	[145]
HIV patients	3435C→T	Efavirenz	Drug-induced HDL-cholesterol level	Higher in C/C	[213]
Paediatric HT patients	3435C→T 2677G→T	Corticosteroids	Steroid weaning 1 year after HT	Higher in T/T	[214]
AML patients	1236C→T 2677G→(T/A) 3435C→T	Menu of SHG-AML-96	OS and PR	Higher OS and low PR in T/T	[155]
AML patients	2677G→T		OS and PR	No significant difference	[156]
ALL patients	3435C→T		OS	No significant difference	[157]
Colon cancer patients	3435C→T		Susceptibility to colon cancer	Higher in T/T	[179]
Japanese LT patients	2677G→(T/A)	Tacrolimus	Tacrolimus-induced neurotoxicity	Higher in T/T	[161]
RT patients	3435C→T	Ciclosporin	Ciclosporin-induced tremor	No significant difference	[215]
RT patients	2677G→(T/A) 3435C→T	Ciclosporin	Ciclosporin-induced nephrotoxicity	Higher in T/T (donor side)	[162]
Lung transplant patients	2677G→T 3435C→T	Tacrolimus + predonisolone + azathiopurine	Acute persistent rejection	Higher in patients with the C allele	[216]
RT patients	3435C→T	Ciclosporin	Gingival overgrowth	No significant difference	[163]
Caucasian patients (renal epithelial cell cancer)	3435C→T		Susceptibility to renal tumour	Higher in T/T	[181]
Caucasian patients (ulcerative colitis)	3435C→T		Susceptibility to ulcerative colitis	Higher in T/T	[168]
Caucasian patients (Crohn's disease)	3435C→T		Susceptibility to Crohn's disease	No significant difference	[168]
White Spanish patients	2677G→(T/A) 3435C→T		Susceptibility to Crohn's disease	Higher in 2677T/3435C	[175]
White Scottish patients	2677G→T 3435C→T		Susceptibility to ulcerative colitis	Higher in 3435T/T	[174]
Caucasian patients (Parkinson's disease)	3435C→T		Susceptibility to Parkinson's disease	Early onset in T/T (trend)	[164]

5-HT: 5-Hydroxytryptamine; ALL: Acute lymphoblastic leukaemia; AML: Acute myeloid leukaemia; HDL: High-density lipoprotein; HT: Heart transplant; LT: Liver transplant; OS: Overall survival; PD: Pharmacodynamics; PR: Probability of relapse; RT: Renal transplant.

Table 4. Impact of the *ABCB1* (*MDR1*) genetic variant on PD of drug substrates and their consequences on disease states (continued)

Population (disease)	Polymorphism	Drug	Outcome marker	Effect	Ref.
Parkinson's disease patients	3435C→T		Susceptibility to pesticide-induced Parkinson's disease	Higher in C/T	[165]
Breast cancer patients	3435C→T		Response to preoperative chemotherapy	Decreased resistance in T/T	[217]
Epileptic patients	3435C→T		Response to antiepileptics	Higher in T/T	[152]
Epileptic patients	3435C→T		Response to antiepileptics	No significant difference	[154]
Cancer patients	3435C→T	5-HT ₃ receptor antagonists	Antiemetic response (granisetron)	Higher in T/T (first 24 h)	[218]
Japanese patients (schizophrenia)	2677G→(T/A) 3435C→T	Bromperidol	Response to bromperidol		[219]

5-HT: 5-Hydroxytryptamine; ALL: Acute lymphoblastic leukaemia; AML: Acute myeloid leukaemia; HDL: High-density lipoprotein; HT: Heart transplant; LT: Liver transplant; OS: Overall survival; PD: Pharmacodynamics; PR: Probability of relapse; RT: Renal transplant.

reported. A recent study of monozygotic twin pairs showed that genetic factors contribute substantially to the renal clearance of metformin [142]. Genetic variation in the *OCT2* is expected to explain the large interindividual variability in the pharmacokinetics of metformin.

Fujita *et al.* [106] conducted a small family based clinical study to determine the renal elimination of a model *OAT1* substrate, adefovir, with regard to a nonfunctional variant, *OAT1* 1361G→A. They did not observe significant differences in renal clearance or renal secretory clearance in family members heterozygous for 1361G→A allele, in comparison with family members who did not carry this variant.

In *OAT3* pharmacogenomics, one study reported no remarkable differences in both the mean renal and renal secretory clearances of pravastatin among the genotypic groups evaluated (reference, 723T→A and 1166C→T) [103].

4.2 Pharmacodynamic consequences

An association of *ABCB1* gene polymorphisms with pharmacodynamic consequences was reported for HIV therapy, antiepileptic pharmacotherapy, chemotherapy, adverse effects of P-gp substrates such as immunosuppressants and tricyclic antidepressants, and others (Table 4).

Fellay *et al.* [143] have studied the association between the response to antiretroviral treatment and the *ABCB1* genotype in 123 HIV-1-infected patients treated with efavirenz or nelfinavir. They found that patients with 3435T allele have a better response to the drugs after treatment for 6 months, as determined by an increased CD4⁺ count. Haas *et al.* [144] also found that the 3435T/T genotype was associated with a decreased likelihood of virological failure and decreased emergence of efavirenz-resistant virus, but not with plasma efavirenz exposure after long-term follow up lasting up to 3 years. In contrast, Nasi *et al.* [145] and Winzer *et al.* [146] failed to find an

association between the *ABCB1* genotype and virological and immunological responses to antiretroviral therapy.

P-gp can transport antiepileptic drugs [147], and the association of the multiple-drug resistance phenotype in epilepsy with increased lesional P-gp expression levels in resected brain tissues has been speculated [148-151]. Based on these backgrounds, Siddiqui *et al.* [152] genotyped 3435C→T in 315 patients with epilepsy, and demonstrated that patients with drug-resistant epilepsy were more likely to have the C/C genotype than T/T, when compared with patients with drug-responsive epilepsy. However, similar to antiretroviral therapy, controversial observations have been reported by at least two independent laboratories [153,154].

Multi-drug resistance is one of the most serious problems in the failure of chemotherapy, and some clinical studies with regard to *ABCB1* polymorphism and clinical outcomes have been conducted; however, as shown in Table 4, contribution of the *ABCB1* gene variants on outcome markers (e.g., overall survival and probability of relapse) in acute lymphoblastic leukaemia or acute myeloid leukaemia patients is controversial among the studies [155-160].

Numerous adverse reactions including neurotoxicity, nephrotoxicity and gingival hyperplasia are sometimes observed in patients treated with immunosuppressants. Yamauchi *et al.* [161] studied the correlation of the *ABCB1* polymorphism with tacrolimus-induced neurotoxicity (e.g., convulsion and tremor) in patients after living-related donor liver transplantation, and found that the 2677T allele might be a positive predictor of toxicity. Similarly, although daily dose, trough levels, and the concentration per dose ratio were not different between case and control groups, the donor's *ABCB1* 3435T/T genotype was reported to be a predictor of ciclosporin-induced nephrotoxicity [162]. Drug transporters in salivary glands have not yet been characterised; however,

Table 5. Impact of gene variants of the drug transporters PK and PD of substrates and their effect on disease states.

Gene	Polymorphism	Population	Drug/substrate	PK	PD	Functional effect of the variant allele	Ref.
<i>ABCG2</i> (BCRP)	421C→T (141Gly→Lys)	White cancer patients (n = 22)	Difflomotecan	Yes		Higher AUC for C/T	[125]
	421C→T (141Gly→Lys)	European Caucasian cancer patients (n = 84)	Irinotecan (SN-38 and SN-38G)	Yes		No significant change in PK	[129]
	421C→T (141Gly→Lys)	2 cancer patients	Topotecan	Yes		Higher BA for C/T	[128]
<i>SLCO1B1</i> (OATP1B1)	*15	Japanese HV	Pravastatin	Yes		Higher AUC for *15/*15	[103]
	521T→C	Japanese hyperlipidaemia (n = 66)	HMG-CoA reductase inhibitors		Response to cholesterol-lowering effect	Lower in C/T	[188]
	*15	Japanese patients	Pravastatin, atorvastatin		Susceptibility to statin-induced myopathy	Higher in *15 patients	[220]
	521 T→C -11187G→A	Caucasian HV (n = 20)	Fexofenadine	Yes		Higher AUC in C/C	[137]
	*17	Caucasian HV (n = 41)	Pravastatin	Yes	Response to cholesterol synthesis inhibition	Higher AUC and smaller response in *17 subjects	[189]
	521 T→C -11187G→A	HV (n = 56)	Repaglinide	Yes	Response to glucose-lowering effect	Higher AUC in C/C Increased response in G/A	[138]
	388A→G 521T→C	4 racial populations (HV)	Rosuvastatin	Yes		Higher AUC for C/T and C/C	[135]
	*1a, *1b, *15	Korean HV (n = 24)	Pitavastatin	Yes		Higher AUC for *15 subjects	[69]
	*1a, *1b, *5	Caucasian HV (n = 30)	Pravastatin	Yes		Higher AUC for *5 subjects Lower AUC for *1b subjects	[104]
	*15B, *17	Caucasian HV (n = 41)	Pravastatin	Yes		Higher AUC for *15B and *17 subjects	[102]
<i>SLC22A6</i> (OAT1)	1361G→A (454Arg→Gln)	1 African-American family	Adefovir	Yes		No change in renal clearance	[106]
<i>SLC22A8</i> (OAT3)	1166C→T (389Ala→Val)	1 heterozygote (Japanese HV)	Pravastatin	Yes		No change in renal clearance	[103]

BA: Bioavailability; BCRP: Breast cancer-resistance protein; HMG-CoA: 3-Hydroxy-3-methylgluaryl coenzyme A; HV: Healthy volunteers; OAT: Organic anion transporter; OATP: Organic anion-transporting polypeptide; PD: Pharmacodynamics; PK: Pharmacokinetics.

Drozdik *et al.* [163] reported no association between *ABCBI* polymorphism and gingival overgrowth in kidney transplant patients with ciclosporin treatment.

Although the physiological role of P-gp is not fully elucidated, it is conceivable that P-gp acts as a cellular barrier at numerous levels in the human body. Therefore, genotype-dependent P-gp function may contribute to disease

susceptibility. So far, a number of studies have been reported on the association of *ABCBI* polymorphism with the following diseases: Parkinson's disease [164-167], inflammatory bowel diseases (ulcerative colitis and Crohn's disease) [168-175], cancers (leukaemia [155,176,177], colon cancers [178-180], renal epithelial tumours [181] and glioma [182]), primary biliary cirrhosis [183], rheumatoid arthritis [184] and hypertension [185].

Previous human studies agree that among numerous SNPs in the *SLCO1B1* gene, 521T→C (174Val→Ala) plays an important role in the transport capability, reducing hepatic uptake of pravastatin (Table 5) [103,104]. Because the target tissue of pravastatin is hepatocytes [186,187], subjects with this allele may exhibit reduced cholesterol-lowering effect of pravastatin due to lower pravastatin concentration in the hepatocytes, despite high plasma levels and AUC of pravastatin. At least two studies have been conducted to clarify this hypothesis. Tachibana-Iimori *et al.* [188] conducted a retrospective study on 66 patients who underwent treatment for hyperlipidaemia with HMG-CoA reductase inhibitors. They found that patients with the 521C allele showed an attenuated total-cholesterol-lowering effect compared with those homozygous for the 521T allele. Niemi *et al.* [189] investigated the association between polymorphism in the *SLCO1B1* and plasma concentrations of lathosterol and cholesterol up to 12 h after the intake of a single dose of pravastatin 40 mg in 41 healthy Caucasian subjects, and found that the plasma lathosterol level and lathosterol to cholesterol level ratio, markers of the rate of cholesterol synthesis *in vivo*, were significantly lower among the three heterozygous carriers of the *SLCO1B1**17 haplotype as compared with noncarriers. Both studies suggest that the 521T→C polymorphism modulates the lipid-lowering efficacy of HMG-CoA reductase inhibitors.

5. Conclusion

The polymorphism of genes encoding drug transporters is a useful marker to interpret large interindividual differences in the pharmacokinetics and response (pharmacodynamics) of clinically important drugs, and a great deal of effort is now being directed at assessing genotype–phenotype relationships not only in the clinical setting, but also at all stages of drug development. Numerous drug transporters, except the transporters described here, may also play an important role in the human body. Gene-knockout animals and expression cell systems are now available for the characterisation of basic traits such as substrate specificity, localisation and vectorial movement. Thus, in order to elucidate their *in vivo* functions more precisely, it seems appropriate to integrate the results from *in vitro* experiments/animal studies into the human study. Further refining of this integration will provide more precise and useful observations, allowing for truly genome-based scientific pharmacotherapy.

6. Expert opinion

Genetic polymorphisms have been identified in most known drug transporters. Some of these variants were shown to have an impact on pharmacokinetic and pharmacodynamic consequences in pharmacotherapy, but unfortunately, functional confirmation remains to be elucidated for most of these variants. We are now beginning to elucidate and understand the consequences of these variants in the human body. So far, except for a few cases (e.g., the *SLCO1B1* genotype and statins pharmacokinetics/pharmacodynamics), there are still discrepancies in the results of functional confirmation (i.e., phenotype and genotype relationship), thus necessitating some concerns for further investigations.

Controversial and confused observations relating to the *in vivo* pharmacokinetic relevance of the polymorphisms of some drug transporter genes (e.g., *ABCB1* and *ABCG2*) may have arisen from the nonspecific substrate drugs used in the various studies. For example, in the *ABCB1* polymorphism, although digoxin and fexofenadine have been used as probed drugs for P-gp function, these are also known to be substrates, at least for polymorphic *SLCO1B3* and *SLCO1B1*, respectively.

Despite considerable effort, it is difficult to find specific substrates to corresponding specific transporters because the substrate specificity of most transporters is extremely broad and shows substantial overlap between different members of the superfamily. For this perspective, multiple gene analysis of the network of genes involved in drug metabolism, transport, and response (e.g., receptors), is preferable. For example, previous *in vitro* experiments reported that at least two transporters, but no cytochrome P450s, are involved in the pharmacokinetics of pitavastatin; OATP1B1 for uptake into hepatocytes and BCRP for efflux into the bile and gut lumen [190]. A pharmacogenomic human study of pitavastatin conducted with polymorphisms in *SLCO1B1* and *ABCG2* is of interest. Again, in order to establish a pharmacokinetic gene network, the integration of *in vitro* and animal experiments into the human study is essential.

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Further development of multiplex single nucleotide polymorphism typing method, the DigiTag2 assay

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Abstract

A number of single nucleotide polymorphisms (SNPs) are considered to be candidate susceptibility or resistance genetic factors for multifactorial disease. Genome-wide searches for disease susceptibility regions followed by high-resolution mapping of primary genes require cost-effective and highly reliable technology. To accomplish successful and low-cost typing for candidate SNPs, new technologies must be developed. We previously reported a multiplex SNP typing method, designated the DigiTag assay, that has the potential to analyze nearly any SNP with high accuracy and reproducibility. However, the DigiTag assay requires multiple washing steps in manipulation and uses genotyping probes modified with biotin for each target SNP. Here we describe the next version of the assay, DigiTag2, which works with simple protocols and uses unmodified genotyping probes. We investigated the feasibility of the DigiTag2 assay by genotyping 96 target SNPs spanning a 610-kb region of human chromosome 5. The DigiTag2 assay is suitable for genotyping an intermediate number of SNPs (tens to hundreds of sites) with a high conversion rate (> 90%), high accuracy, and low cost.

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As a consequence of the Human Genome Project and single nucleotide polymorphism (SNP)¹ discovery projects, several million SNPs have been uploaded onto public SNP databases. It is estimated that there are 5 million SNPs with a greater than 10% minor allele frequency and 11 million SNPs with a greater than 1% minor allele frequency in the human genome [1]. Among these SNPs, many are candidate susceptibility or resistance genetic factors for multifactorial diseases and have been identified based on linkage analysis

in families or association analysis with unrelated patients (cases) and healthy controls [2–6]. Large-scale case-control analyses using a dense set of SNP markers across the human genome have revealed associations between various diseases and SNPs with the highest detection power [7–9].

During recent years, genome-wide association studies using SNP markers have attempted to search for susceptibility and/or resistance genes by using emerging genome-wide SNP typing technologies such as Affymetrix GeneChip arrays and Illumina BeadArray genotyping technology [10–13]. These genome-wide SNP typing technologies would detect candidate regions, including susceptibility or resistance genes. However, to identify primary SNPs or genes, it is necessary to perform association analysis using an intermediate number of SNPs (tens to hundreds of sites) located within the candidate regions. Currently, there are a variety of SNP genotyping methods that are suitable for genotyping large numbers of samples for a modest number of SNPs such as 5' exonuclease fluorescence-based assay

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¹ Abbreviations used: SNP, single nucleotide polymorphism; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ED, end digit; D1, first digit; PCR, polymerase chain reaction; dNTP, deoxynucleoside triphosphate; ATP, adenosine triphosphate; DTT, dithiothreitol; NAD, nicotinamide adenosine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Cy3-ED-1, Cy3-labeled ED-1; Cy5-ED-2, Cy5-labeled ED-2; SDS, sodium dodecyl sulfate; DCN, DNA coded number.

(TaqMan) [14], pyrosequencing [15], single-base extension [16], matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [17,18], and SNPlex assay [19]. However, many applications need to select relevant SNPs for their assay by *in silico* assay design, and some candidate SNPs are then excluded from investigation. Moreover, it is difficult or impossible for some assays to perform multiplex SNP genotyping.

To accomplish successful SNP typing for all candidate SNPs at low cost, new technologies must be developed. We previously reported a multiplex SNP typing method, designated the DigiTag assay, that has a high conversion rate (>90%) and reliable accuracy [20]. However, the DigiTag assay requires improvement with regard to simplifying assay protocols and reducing assay cost. In this study, we developed the DigiTag2 assay, which has simplified assay protocols, and performed typing for 96 SNP sites located in a 610-kb region on human chromosome 5 using 48 individual genomic DNA samples.

Materials and methods

DNA samples

Genomic DNA samples from 48 unrelated healthy donors were obtained from the Japan Health Science Foundation (Osaka, Japan). All donors provided written informed consent, and samples were anonymized. For each sample, 1 µg of purified genomic DNA was dissolved in 20 µl of TE buffer (pH 8.0, Wako, Osaka, Japan) for use and was stored at -20 °C.

End digits and first digits

We designed the end digits (EDs) and first digits (D1s) to be 23-mer oligonucleotides and attached the EDs and D1s to 5' query probes and 3' query probes, respectively. We prepared two EDs (ED-1 and ED-2) for two alleles at each SNP. All EDs and D1s are used for the priming site in the labeling step, and D1s are also used as probes that are attached to DNA microarray in the detection step. The EDs and D1s have the following properties: (i) uniform melting temperature (58.8 ± 1.0 °C) and length, (ii) specific hybridization only to complementary EDs and D1s, (iii) minimal interaction with other EDs and D1s, and (iv) no formation of secondary structures [21]. These properties ensure uniform polymerase chain reaction (PCR) efficiency, even if all of the EDs and D1s are used in multiplex PCR. Furthermore, precise hybridization on DNA microarray is possible using a set of D1s with high reproducibility. Sequence information for EDs and D1s is listed in Supplementary Table 1.

Multiplex PCR from sample DNA

We designed multiplex PCR primers for each of the 96 SNP sites to have relatively long length (average length 40-mer) and to give PCR products of between 181 and 798 bp

(average length 527 bp). Sequence information for the multiplex PCR primers is listed in Supplementary Table 2.

We performed multiplex PCR using a two-step protocol (denature and extension steps) with a 6-min extension step using specifically designed primer pairs. Multiplex PCR was performed with 2.5 µl genomic DNA and 250 fmol of each primer for 96 SNP sites in 10 µl of 2× Qiagen Multiplex PCR Master Mix containing HotStarTaq DNA polymerase, multiplex PCR buffer and deoxynucleoside triphosphate (dNTP) mix (Qiagen Multiplex PCR Kit, Qiagen, Valencia, CA, USA). Cycling was performed using a Bio-Rad PTC-200 Peltier thermal cycler (Bio-Rad, Hercules, CA, USA) as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s and 68 °C for 6 min. When necessary, fragment length of the 96 PCR products was confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent, Palo Alto, CA, USA) to evaluate PCR efficiency.

Encoding reaction

We performed multiplex oligonucleotide ligation assay using the multiplex PCR products as targets. For 96-plex oligonucleotide ligation assay, we prepared mismatch-induced 5' query probes for 91 target SNPs and perfect match 5' query probes for 5 target SNPs (SNP 7, SNP 9, SNP 18, SNP 49, and SNP 93). The assignment of D1s to the SNPs analyzed in this study and sequence information for the probes are listed in Supplementary Table 3.

Prior to the encoding reaction, 96 unmodified 3' query probes were simultaneously phosphorylated at the 5' end in 40 µl of 1× protruding end kinase buffer containing 30 mM adenosine triphosphate (ATP), 40 U polynucleotide kinase, and 4 pmol of 3' query probes for 96 SNP sites (Kination Kit, Toyobo, Osaka, Japan). The reaction mixture was incubated for 30 min at 37 °C and for 3 min at 95 °C using a Bio-Rad PTC-200 Peltier thermal cycler. The encoding reaction was prepared by mixing 1 µl of multiplex PCR products in 15 µl of *Taq* DNA ligase buffer containing 20 mM Tris-HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol (DTT), 1 mM nicotinamide adenosine dinucleotide (NAD), and 0.1% Triton X-100 (New England Biolabs, Beverly, MA, USA) with 10 fmol of probes (192 5' query probes and 96 phosphorylated 3' query probes) and 10 U *Taq* DNA ligase. All components of the encoding reaction were mixed on ice. The encoding reaction initially was held at 95 °C for 5 min, followed by 58 °C for 15 min using a Bio-Rad PTC-200 Peltier thermal cycler. The reaction was stopped by holding the temperature at 10 °C.

Labeling reaction

For the labeling reaction, 6 µl of ligation products was directly mixed in 12 µl of *Ex Taq* buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, 0.5% Tween 20, 0.5%