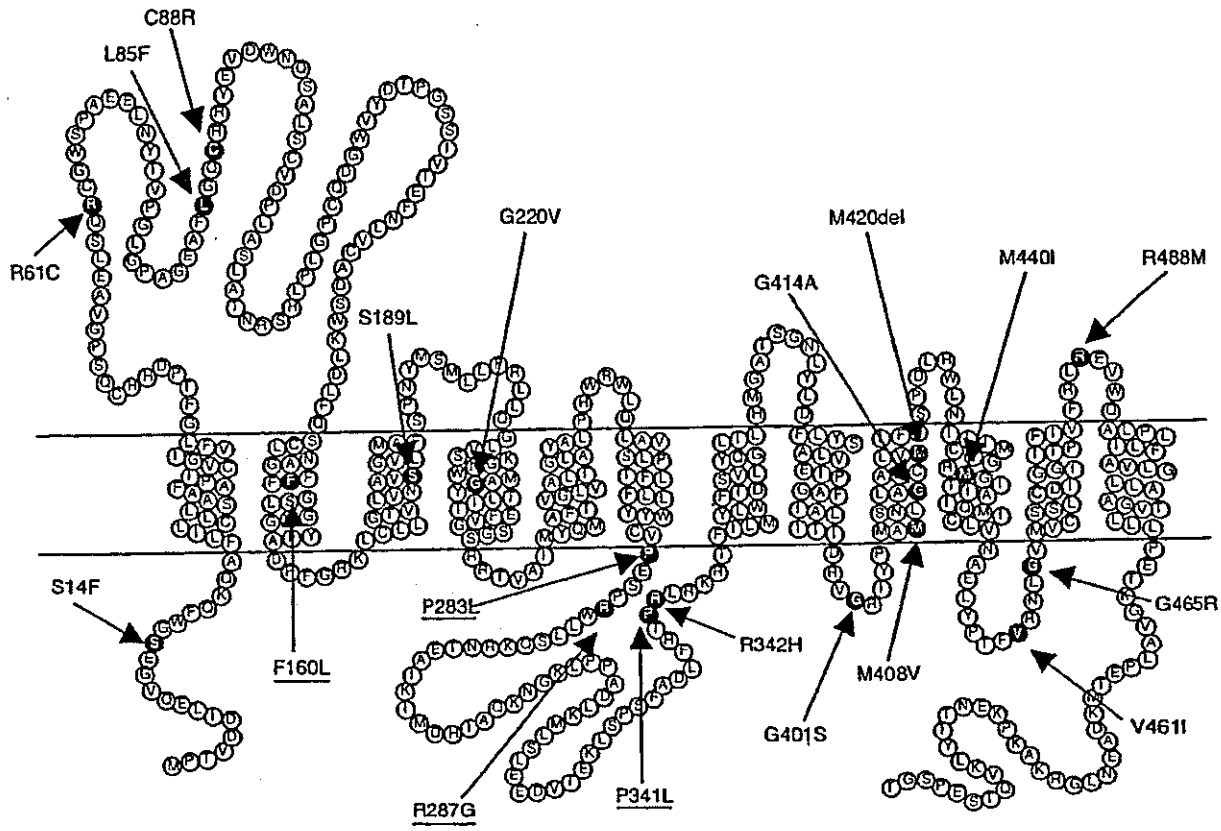
Figure 3. Schematic representation of hOCT1 and SNPs.



Non-synonymous nucleotide substitutions are indicated by arrows. SNPs identified in the Japanese healthy subjects are underlined.
hOCT: Human organic cation transporter; SNP: Single nucleotide polymorphism.

OCTN1 will be published elsewhere (Sai *et al.*, manuscripts in preparation).

Organic cation transporters subfamily

Organic cation transporters (OCTs) mediate electrogenic transport of small organic cations with different molecular structures, independently of sodium gradient [6]. These organic cations include clinically used drugs (e.g., metformin) and endogenous compounds (e.g., dopamin), as well as other cationic substances such as TEA and N^1-methylnicotinamide, and 1-methyl-4-phenylpyridinium (MPP^+). Human organic cation transporter 1 (hOCT1 [*SLC22A1*]) is a member of the organic ion transporter family isolated from human liver [35,36]. While rat ortholog rOCT1 shows relatively broad tissue distribution (e.g., kidney, small intestine and liver), hOCT1 is expressed predominantly in the liver [35-37], where it plays a fundamental role in the cellular uptake and elimination of various cationic substrates including therapeutically important agents. A dramatic difference was reported between the *Oct1*-knockout mice and the wild-type mice with respect to the hepatic distribution of an anticancer drug, metaiodobenzylguanidine (MIBG), and an antidiabetic drug, metformin [38,39].

Polymorphisms in hOCT1

To date, several genetic variants of hOCT1 have been studied. The experimental data on hOCT1 polymorphisms were first presented by Kerb *et al.* [40]. They have identified eight different non-synonymous substitutions (R61C, C88R, F160L, G401S, M408V, G414A, M420del, G465R) in 57 Caucasians. As shown in Figure 3, these mutants are located in a large extracellular loop (R61C, C88R); the second transmembrane domain (TMD; F160L); the highly conserved short intracellular loop between TMD8 and -9 (G401S) [6,35]; TMD9 (M408V, G414V, M420del); or the fifth intracellular loop (G465R).

Table 4. Non-synonymous polymorphisms in the hOCT1 gene in Japanese.

JSNP ID	Sequence	Protein residue	Scoring systems	
			Grantham	BLOSUM62
ssj0008476	cgggc ttctt C/G tttgg ctctc	F160L	22	0
ssj0005319	aggtg tgtgc C/T ggagt cccct	P283L	98	-3
ssj0005320	ggagt cccct C/G ggtgg ctgtt	R287G	125	-2
ssj0008480	ttccg cacgc C/T gcgcc tgagg	P341L	98	-2

hOCT: Human organic cation transporter; JSNP: Japanese Single Nucleotide Polymorphisms database.

Shu *et al.* more recently reported a total of 15 protein-altering variants of hOCT1 from 247 ethnically diverse DNA samples (S14F, R61C, L85F, F160L, S189L, G220V, P341L, R342H, G401S, M408V, M420del, M440I, V461I, G465R, and R488M) [41]. These mutants are located in the intracellular N-terminal (S14F) large extracellular loop (L85F); TMD3 (S189L); TMD4 (G220V); the long intracellular loop between TMD6 and -7 (P341L, R342H); TMD10 (M440I); the fifth intracellular loop (V461I); or the sixth extracellular loop (R488M).

Furthermore, SNPs of the *hOCT1* gene were identified in the PSC project [42,203]. Sakata *et al.* [43] recently reported four SNPs of human OCT1 in Japanese populations: F160L (Japanese SNP database [JSNP] ID: ssj0008476), P283L (ssj0005319), R287G (ssj0005320), and P341L (ssj0008480) (Table 4). While F160L and P341L have been reported in previous studies [40,41], the SNPs of P283L and R287G had hitherto only been found in Japanese healthy subjects. P283 and R287 are located at the 5'-end of the long intracellular loop following the TMD6 (Figure 3). F160 and P341 are characteristic for OCTs; F160 is conserved in both OCT1 and OCT2, whereas P341 is conserved among OCT1, OCT2, and OCT3. Interestingly, however, both P283 and R287 are conserved not only in OCTs (OCT1, OCT2, and OCT3) but also in OCTNs (OCTN1 and OCTN2). These two amino acid residues (P283 and R287) are located in the well-conserved motif (P-E-S-P-R-X-L) of the major facilitator superfamily (MFS), as indicated by Burckhardt and Wolff in their review [44]. In particular, R287 is conserved not only in the OCT family, but also in the OAT family. These findings strongly suggest that these two amino acid residues are critically involved in the transport activity.

Functional analysis of hOCT1 variants in Xenopus oocytes

Kerb *et al.* characterized the above-mentioned five different variants (R61C, C88R, F160L, G401S, and M420del) of hOCT1 by expressing the corresponding cDNA in *Xenopus* oocytes [40]. Among them, the uptake of [^{3}H]MPP$^+$ by R61C, C88R, and G401S were remarkably reduced to 30, 1.4 and 0.9% of the wild-type's level, respectively.

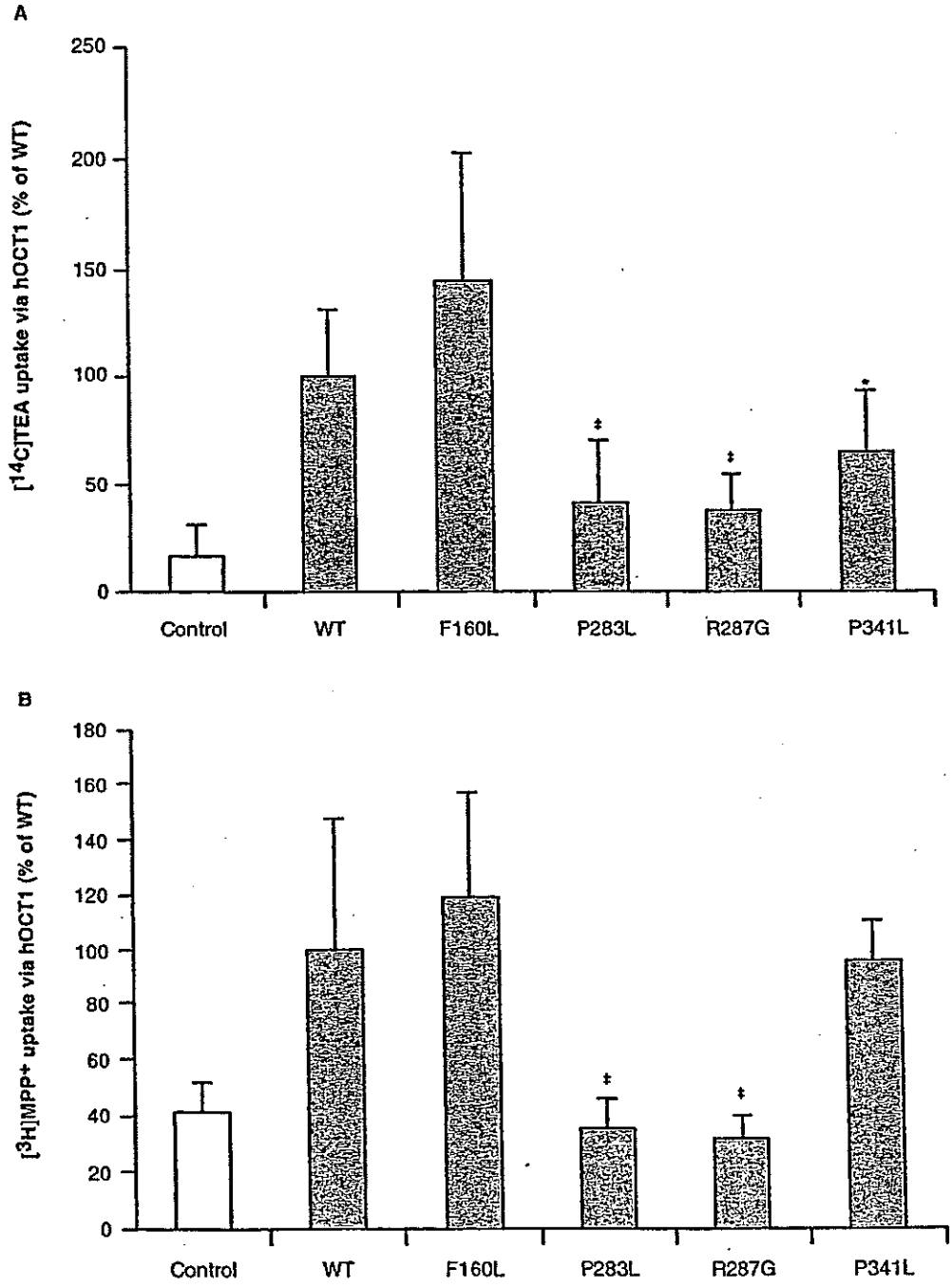
Shu *et al.*, on the other hand, reported that the [^{3}H]MPP$^+$ uptake activities of R61C, P341L, G220V, G401S, and G465R were much lower than that of the wild type [41]. In particular, three variants (G220V, G401S, and G465R) lacked the transport activity. Thus, it is suggested that the glycine residues at those positions (220, 401, and 465) in hOCT1 are critically important for the transport function. In contrast, S14F exhibited increased [^{3}H]MPP$^+$ uptake. The phenylalanine residue at position 14 is conserved among OCT1 orthologs from different mammalian species (except for human), including rat, rabbit, mouse, and chimpanzee. Actually, hOCT1 has a lower transport activity than rat, rabbit, and mouse orthologs [45]. On the other hand, phenylalanine is conserved at the corresponding position in hOCT2 and hOCT3.

As mentioned above, four non-synonymous SNPs (F160L, P283L, R287G, and P341L) were found in Japanese healthy subjects. Figure 4 and Table 5 summarize the uptake of both [^{14}C]TEA and [^{3}H]MPP$^+$ by *Xenopus* oocytes expressing those variants. It is important to note that P283L and R287G almost lacked transport activity [41].

Expression and functional analysis of hOCT1 variants in HEK293 cells

To compare with the data obtained with the *Xenopus* oocyte system, the variants P283L,

Figure 4. Functional characterization of hOCT1 variants.



A. The uptake of [^{14}C]TEA (10 µM) at 60 min in non-injected, and wild-type and mutant hOCT1 cRNA-injected oocytes was measured on day 2 after injection. **B.** The uptake of [^{3}H]MPP^{+} (10 µM) at 60 min in non-injected, and wild-type and mutant hOCT1 cRNA-injected oocytes was measured on day 2 after injection. The data represent mean ± standard deviation for 7–9 oocytes.
*$p < 0.05$, ‡$p < 0.001$ relative to wild-type hOCT using Student's *t*-test.
hOCT: Human organic cation transporter; MPP^{+}: 1-Methyl-4-phenylpyridinium; TEA: Tetraethylammonium; WT: Wild type.

Table 5. Uptake of TEA and MPP+ by hOCT1 variants.

Variant	[^{14}C]TEA uptake (pmol/h/oocyte)	[^{3}H]MPP+ uptake (pmol/h/oocyte)
Control	0.12±0.10	1.19±0.29
Wild type	0.68±0.20	2.85±1.34
F160L	0.97±0.17	3.39±1.09
P283L	0.28±0.19*	1±0.31*
R287G	0.26±0.11*	0.93±0.22*
P341L	0.44±0.19‡	2.73±0.41

The uptake of [^{14}C]TEA (10 μM) at 60 min in non-injected, and wild-type and mutant hOCT1 cRNA-injected oocytes was measured on day 2 after injection. The data represent mean ± standard deviation for 7–9 oocytes.
**p < 0.001, ‡p < 0.05, relative to wild-type hOCT1 using Student's t-test.*
hOCT: Human organic cation transporter; MPP+: 1-Methyl-4-phenylpyridinium; TEA: Tetraethylammonium.

R287G, and P341L, as well as the wild type of hOCT1, were expressed in HEK293 cells and their transport activities were assessed [46]. As shown in Figure 5, HEK293 cells expressing P341L exhibited a decreased but relatively preserved transport activity of TEA (Figure 5). On the other hand, the uptake of TEA by the HEK293 cells expressing P283L and R287G were background levels, similar to that of the mock-transfected cells. These results are consistent with the data obtained with the *Xenopus* oocytes system (Figure 4). In the case of the P341L variant, both the apparent K_m and V_{max} were estimated. The kinetic parameters thus obtained were different from those estimated for the wild type of hOCT1; K_m values were 1.27±0.09 mM for wild type and 0.87±0.05 mM for P341L ($p < 0.05$); V_{max} values were 4.09±0.29 nmol/mg of protein/min for wild type and 1.79±0.15 nmol/mg of protein/min for P341L ($p < 0.01$).

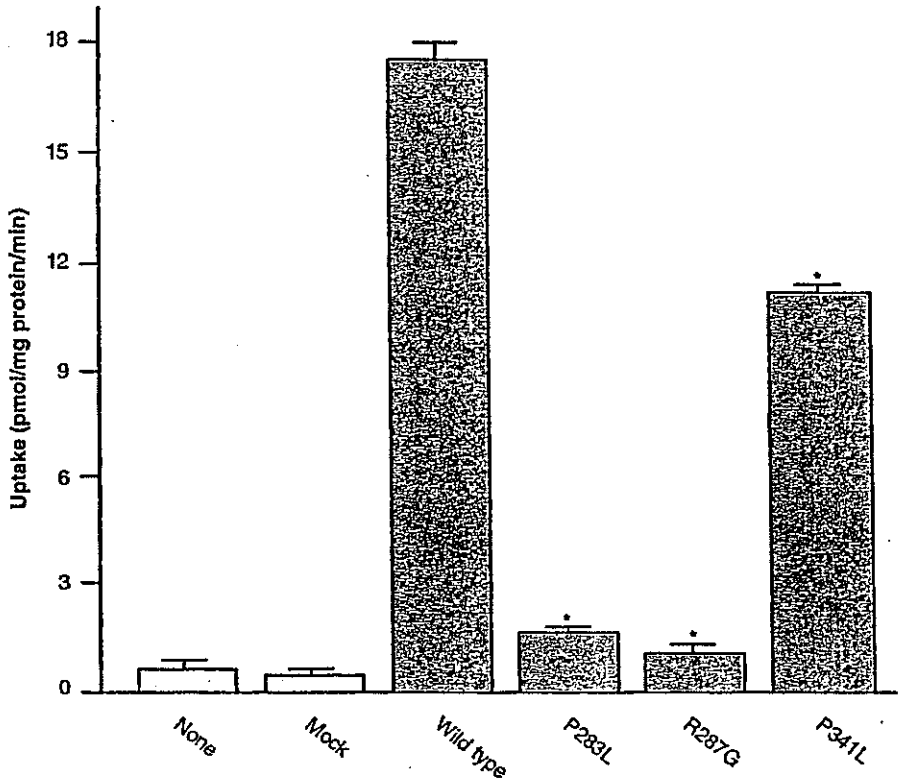
The expression levels of P283L, R287G, P341L, and the wild type of hOCT1 in HEK293 cells were examined by western blot analysis. The immunoreactive protein was clearly detected in those cells expressing the variants and the wild type (Figure 6A). In addition, localization of hOCT1 and its variants in the transfectants was determined by immunofluorescence microscopy (Figures 6B–E). Localization of immunoreactive protein at the plasma membranes was confirmed in HEK293 cells expressing wild-type hOCT1 (Figure 6B). In addition, there were no differences in the localization between wild-type hOCT1 and non-functional variants P283L and R287G, as well as P341L, suggesting that these OCT1 mutants targeted to the plasma membrane correctly (Figures 6C–E). In spite of the completely diminished TEA uptake by HEK293 cells expressing P283L and R287G variants (Figure 5), protein expression and localization of these variants were retained (Figure 6). Therefore, the reduced transport function of those variants is not attributed to alterations in protein expression or membrane localization. Considering that the region between amino acid position P283 and L289, located in the large intracellular loop between TMD6 and -7, is highly conserved among the OCT family in various species (human, rat and mouse OCT1, OCT2, and OCT3), this region may have a substantial role in substrate recognition and/or transport function.

The frequency of the allele causing the P283L variant was ~ 1% in the Japanese population, whereas individuals carrying the homozygous alleles were not found. Another variant, P341L, exhibited a reduced but retained transport activity, and existed at relatively high frequency (16.4%, data not shown). Similar findings were reported previously stating that three non-functional variants of hOCT1, G220V, G401S and G465R, were present at overall allele frequencies of < 2% [41]. It is suggested that non-functional hOCT1 variants are likely to be rare in comparison with P341L.

Prediction of functional alterations in hOCT1 variants

To date, many large-scale screenings of SNPs have been carried out to identify genetic variants that affect disease susceptibility and drug response. To determine which of the variants affect function and contribute to altered phenotype is an important issue. Shu *et al.* evaluated their 15 protein-altering variants of hOCT1 by using amino acid scoring systems (chemical changes and evolutionary conservation) [41]. One

Figure 5. Uptake of [^{14}C]TEA by HEK293 cells transiently transfected with vector alone, wild type hOCT1 and its variants.



HEK293 cells and cells transfected with vector alone (pCMV6-XL4), wild-type hOCT1 and hOCT1 variant (P283L, R287G and P341L) cDNAs were incubated with 5 μM of [^{14}C]TEA for 1 min at 37°C. Each column represents data of the mean ± standard error for three monolayers.
*$p < 0.01$, significant differences from the wild type.
hOCT: Human organic cation transporter; TEA: Tetraethylammonium.

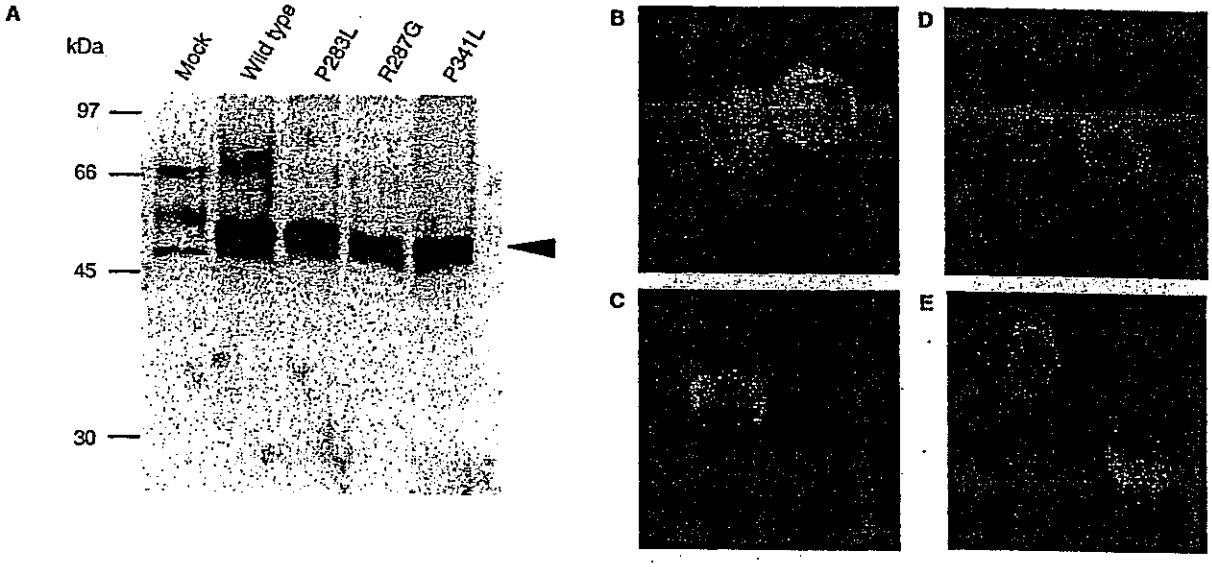
is Grantham value and another is BLOSUM62 value. Grantham value is the criterion used to assess chemical relatedness [47] and is expected to be useful for predicting deleterious function. BLOSUM62, an amino acid substitution matrix, is derived from amino acid changes in an unselected protein set [48] and has been used to infer protein function. It was reported that the variants with decreased function had larger chemical changes (greater Grantham values) than variants that did not reduce function, and it was also observed that the BLOSUM62 values for the decreased-function variants were significantly more negative (evolutionary unfavorable) than values for the variants that retained function [41].

Following the report of Shu *et al.*, Sakata *et al.* attempted to evaluate the changes in hOCT1 variants by chemical changes (Grantham value) and by amino acid substitution scoring matrices (BLOSUM62) [43]. As shown in Table 2, two completely non-functional variants had Grantham values of 98 (P283L) and 125 (R287G), indicating that radical changes are responsible for their reduced function. These two variants had negative BLOSUM62 scores; -3 (F283L) and -2 (R287G), demonstrating that this score was also useful for predicting protein function.

Polymorphisms of hOCT1 and their functional importance

There is no evident phenotype related to the hOCT1 polymorphisms, but our finding, as well as those of two recent reports [40,41], may indicate that these mutations associated with impaired hOCT1 transport function *in vitro* also influence *in vivo* disposition of OCT1 substrates. Studies using *Oct1*-knockout mice exhibited decreased liver accumulation of the anticancer drug MIBG

Figure 6. Western blot analysis and localization of hOCT1 protein in HEK293 cells.



A. Western blot analysis of crude membrane fractions obtained from HEK293 cells transiently transfected with vector alone, wild-type hOCT1 and its variant cDNAs. Crude membrane fractions (1 µg) obtained from HEK293 cells transiently transfected with vector alone (pCMV6-XL4), wild-type hOCT1 and hOCT1 variant (P283L, R287G, and P341L) cDNAs were separated by SDS–PAGE (10%), blotted onto PVDF membranes and immunoblotted by anti-hOCT1 antibody. The arrowhead indicates the position of hOCT1. **B–E.** Localization of hOCT1 proteins in HEK293 cells transiently transfected with wild-type hOCT1 (**B**), and its variants P283L (**C**), R287G (**D**), and P341L (**E**). HEK293 cells transfected with wild-type hOCT1 and its variant (P283L, R287G and P341L) cDNAs were fixed and stained with antiserum for hOCT1.
hOCT: Human organic cation transporter; PVDF: Polyvinylidene difluoride.

and of the antidiabetic drug metformin [38,49]. It was implied that P283L and R287G mutants resulted in non-functional hOCT1 proteins in spite of the normal expression at the plasma membrane. Although these variants are very rare and further investigation is needed to clarify the importance of hOCT1 polymorphisms, these findings suggest that the genetic variation in hOCT1 may contribute to interindividual variability in the disposition of several organic cations. Thus, it is of interest to know whether a similar reduction is observed in individuals with hOCT1 polymorphisms. At present, data are still limited about the *in vivo* phenotypes of functionally deleterious mutations in the hOCT1 gene. Large clinical studies on the genotype–phenotype relationship may provide useful information concerning the contribution of hOCT1 gene polymorphism *in vivo*. Further studies are necessary to determine whether the identified variants contribute to diseases or to alterations in drug response.

ATP-binding cassette transporters

ABC proteins form one of the largest protein families in the human genome. Currently, > 48 human ABC protein genes have been identified and sequenced [50]. It has been reported that mutations of the genes encoding ABC protein are causative in several genetic disorders in humans [51]. Many of the human ABC proteins are involved in membrane transport of drugs, xenobiotics, endogenous substances, or ions, thereby exhibiting a wide spectrum of biological functions. Based on the arrangement of molecular structure components (i.e., nucleotide-binding domains and topologies of transmembrane domains), reported human ABC proteins were originally classified into seven different subfamilies (A to G). However, the Human Genome Organisation (HUGO) Human Gene Nomenclature Committee developed a new system of nomenclature for the human ABC-transporter family. The new nomenclature scheme was implemented in 1999, and detailed information is available on the Internet [201].

The primary structures of the transmembrane domains differ significantly among ABC transporters and have been recognized as the main determinants of substrate specificity. The ABCs, on the other hand, share an overall sequence

identity of ~ 30%. They contain three core consensus motifs, known as Walker A, Walker B, and Signature C [52], which are essential for ATP binding. The high conservation of the ATP-binding cassettes in the rapidly growing list of discovered ABC transporters suggests that these membrane proteins may use similar mechanisms to execute their transport activities.

ABCB1 (P-glycoprotein/MDR1)

Human ABCB1 (P-glycoprotein or multi-drug resistance 1 [MDR1]) was identified because of its overexpression in cultured cancer cells associated with an acquired cross-resistance to multiple anticancer drugs [53]. While 'P-glycoprotein' was initially thought to play a role in modulating cellular permeability ('P' stands for permeability) to drugs, it has later been demonstrated to be an ATP-dependent efflux pump of hydrophobic anticancer drugs, including colchitine, doxorubicin, daunorubicin, vincristiene, and VP16. Historically, P-glycoprotein provided one of the mechanistic explanations for the multi-drug resistance phenomenon. The function of human ABCB1 as a mechanism of multi-drug resistance has been extensively investigated [54]. It was assumed that ABCB1 functions as a membrane pore to export intracellularly located substrate. Subsequently, another model has been proposed where ABCB1 translocates a substrate from the inner leaflet side of the membrane to the outer leaflet side; thus, it functions as a flippase or membrane vacuum cleaner.

Molecular structure

Human ABCB1 and its orthologs in mammals are single peptide chains, integral membrane proteins of an approximate length of 1280 amino acid residues. The apparent molecular weights of mature ABCB1 are in the range of ~ 130–180 kDa, depending on the species and cell type in which they are expressed. ABCB1 is composed of two homologous halves each of which consist of an N-terminal, hydrophobic, membrane-associated domain (~ 250 amino acid residues) and a C-terminal, hydrophilic nucleotide-binding fold (~ 300 amino acid residues). The plasma membrane-associated domains in the two halves of ABCB1 each consist of six transmembrane domains, which are followed by an intracellular ABC.

In order to elucidate the transport mechanism and structure of ABCB1, mutational analysis was widely carried out using site-directed mutagenesis. A relatively large number of mutants alter the transporter's substrate specificity, in particular those in TMD5, -6, and -12. Accordingly, photo-affinity labeling studies indicated that such domains probably are of major importance in substrate binding.

Gene structure

Humans *ABCB1* and *ABCB4* genes are adjacently located on chromosome 7q21. *ABCB1* encodes a drug transporter directly associated multi-drug resistance of cancer, whereas *ABCB4* encodes the flippase translocating phospholipids. In rodents, three genes are present: *mdr1a*, *mdr1b*, and *mdr2*. In the mouse, the genes are clustered on chromosome 5, whereas they are located at chromosomal region 4q11-12 in the rat. In rodents, both Mdr1a and Mdr1b functionally correspond to ABCB1 (MDR1) in humans.

Transcription of the *ABCB1* gene appears to be regulated by multiple factors. For example, the proximal promoter region has a GC-rich region at ~ -100 to -120 bp from the transcriptional start codon, which contains a site responsible for the repression of transcription. Also, basal transcription appears to involve a consensus site that binds NF-Y transcription factors at a Y-box (inverted CCAAT box) between -70 and -80 bp. In addition, a binding site for specificity protein 1 (SP-1) and members of the early growth response (EGR) family of transcriptional factors is present, which overlaps with the NF-Y consensus site. A 13-bp region around the initiation site involved in accurate initiation of the transcription has also been identified. ABCB1 is expressed at a high frequency in tumor cells and both c-H-Ras and mutant forms of p53 have been shown to activate the *ABCB1* promoter. On the other hand, c- and N-Myc expression is apparently inversely correlated with ABCB1 expression. In addition to such transcriptional regulations, the stability of mRNA and post-translational regulation are also considered important in the regulation of ABCB1 expression.

ABCB1 in normal tissues

It is important to know that ABCB1 is expressed not only in cancer cells but also in many normal tissues. For example, ABCB1 is located in the apical domain of the enterocytes of the gastrointestinal tract (jejunum and duodenum) and limits the uptake and absorption of drugs and other substrates from the intestine into the systemic circulation by excreting substrates into the gastrointestinal tract. Likewise, the expression of ABCB1 on the lumenal

membrane of capillary endothelial cells of the brain restricts drug distribution into the CNS. This function appears to be very important in protecting the CNS from the attack of toxic compounds. Evidence for the protective role of ABCB1 in the blood–brain barrier has been demonstrated in several studies using *mdr1a*-knockout mice [55]. A similar protective role to limit the distribution of potentially toxic xenobiotics into tissues was suggested for ABCB1 expressed in the placenta and testis. ABCB1 expressed in the canalicular domain of the hepatocyte and the brush border of the proximal renal tubule plays a role in the biliary and urinary excretion of xenobiotics and endogenous compounds.

Acquired mutation of ABCB1 in cancer cells

Cells selected *in vitro* against a lipophilic cytotoxic compound usually develop cross-resistance to other drugs. Some multi-drug resistant cell lines are significantly more resistant to the drug used in their selection than to the other drugs. A single amino acid substitution, G185V, in the human ABCB1 protein was found to cause an altered pattern of drug resistance in cell lines transfected with the ABCB1 cDNA carrying this mutation [56]. It is suggested that the amino acid at position 185 is involved in colchicines and verapamil but not in vinblastine binding/transport. In addition, several recombinant variants have been generated either by *in vivo* drug selection or by site-directed mutagenesis techniques, which show altered substrate specificity or impaired function of a properly assembled protein [54].

Naturally occurring SNPs of ABCB1

To date, genetic variations of the human *ABCB1* gene have been the most extensively studied ([57,58] for recent reviews); 50 SNPs and 3 insertion/deletion polymorphisms in the *ABCB1* gene have been reported [59-62]. In addition, 12 novel SNPs of ABCB1 were reported in Japanese patients with ventricular tachycardia who were administered amiodarone [63]. Several preclinical and clinical studies have provided evidence for the naturally occurring polymorphisms in ABCB1 and their effects on drug absorption, distribution and elimination [64-97]. Hoffmeyer *et al.* [73] reported multiple polymorphisms in the *ABCB1* gene. One of those mutations, a C-to-T variant at position 3435 in the exon 26 of the *ABCB1* gene, reportedly correlated with ABCB1 expression and function. While this SNP does not alter the amino acid sequence of ABCB1, individuals homozygous for the polymorphism expressed significantly less duodenal ABCB1 and significantly more plasma digoxin [73]. The distribution of the SNP C3435T in exon 26 in the Chinese and Malay population was found to be similar to the Caucasians, whereas the Indians were different. The Asian population also differed significantly from the African and Caucasian population in the distribution of the C3435T SNP [74,75]. However, the association of the C3435T polymorphism with ABCB1 protein expression and function remains controversial. In fact, various investigators have reported that the T allele is associated with increased (or decreased) expression levels or has no clearly discernible effect [76-83]. Furthermore, the reported effects of C3435T SNP on the pharmacokinetic profiles of drugs are also controversial [84-97] (see also Table 3 in [58]).

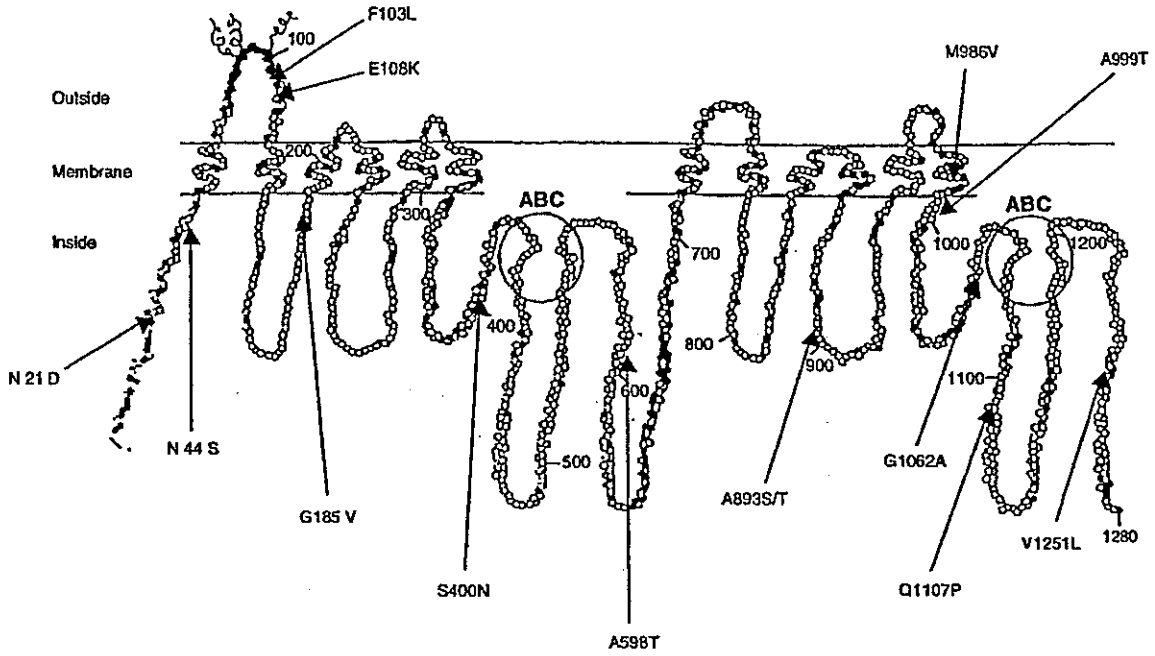
Recently, the five most common coding SNPs were assessed by a vaccinia virus-based transient expression system [98]. The study on cellular accumulation of several tested substrates indicated that the substrate specificity of the protein was not substantially affected by any of the SNPs, whereas cell surface expression and function of even double mutants showed no difference from the wild-type protein. The study suggests that these SNPs result in mutant proteins with a distribution and function similar to the wild-type protein. However, kinetic parameters for those variants were not assessed in the study.

On the other hand, the ABCB1 G2677T/A [99] contains a tri-allelic polymorphism (with G at nucleotide 2677 in the wild-type sequence, and A or T nucleotide 2677 in the two possible variants), which results in an amino acid change (A893S/T) in exon 21. Previous work has shown that the S893 substitution is associated with an altered drug-resistance pattern in AdrR MCF-7 cells as well as enhanced efflux transporting ability in stably transfected NIH3T3 GP+E86 cells [100].

Functional analysis of polymorphism of ABCB1

Figure 7 depicts the non-synonymous polymorphisms in the ABCB1 protein that have been identified to date. Quantitative studies are required to precisely evaluate functional changes associated with genetic polymorphisms of ABCB1. For this purpose, the cDNA of ABCB1 was cloned from the human liver cDNA library, and several variant forms (N21D, N44S, F103L,

Figure 7. Schematic representation of ABCB1 and non-synonymous polymorphism.



The positions of amino acid substitutions are indicated by arrows. Data are from [59-62,73,204]. The molecular structure of ABCB1 is modified from Gottesman MM, Pastan I: The multi-drug transporter, a double-edged sward. *J. Biol. Chem.* 263, 12153-12166 (1988).
ABC: ATP-binding cassette.

G185V, S400N, A893S, A893T, and M986V) were prepared by site-directed mutagenesis (see Figure 8A for primers). These variants and the wild type of ABCB1 were then expressed in Sf9 cells using the pFASTBAC1 vector and recombinant baculoviruses. ABCB1 variant proteins expressed in Sf9 cell membranes were detected by the western blot method using the C219 monoclonal antibody (Figure 8B).

Using membranes prepared from Sf9 cells expressing ABCB1 variants, ATPase activity was measured in the presence of various compounds. The ATPase activity of the isolated Sf9 cell membranes was determined by measuring inorganic phosphate liberation [101] according to the procedure reported by Sarkadi *et al.* [102], with some modifications. The authors have recently developed a high-throughput screening system using 96-well plates (Ishikawa *et al.* manuscript in preparation) (Figure 8). As demonstrated in Figure 8C, verapamil, one of the typical substrates of ABCB1, enhanced ATPase activity. Table 6 summarizes kinetic parameters observed for the variant forms as well as the wild type of ABCB1. The variant forms (N21D, N44S, F103L, G185V, S400N, A893S, A893T, and M986V) exhibited the verapamil-enhanced ATPase activity, as did the wild type of ABCB1. K_m values for verapamil were slightly different among those variants. The V_{max} values of the variants were normalized to that of the wild type by referring to the intensity of each variant protein on the western blotting (Figure 8B). The variant G185V (acquired mutation) was found to have the highest V_{max} value, which was followed by N21D (Table 6). Thus, it is critically important to quantitatively analyze the functional difference among such variants in evaluating naturally occurring non-synonymous polymorphisms. Kinetic parameters of those variants observed with different substrates will be reported elsewhere.

ABCC2 (cMOAT/MRP2)

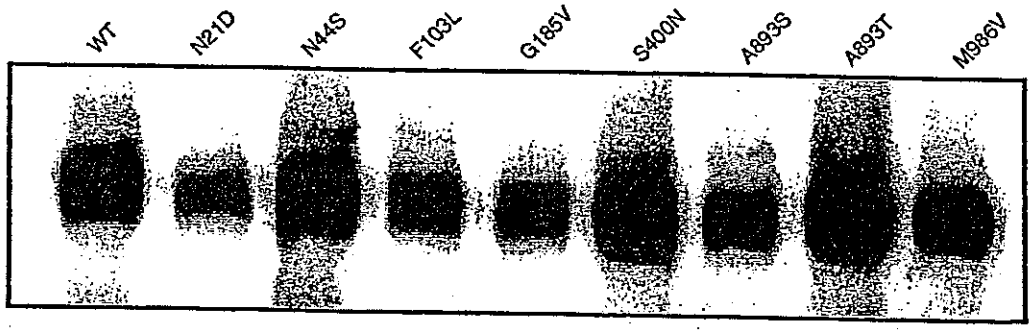
The ABCC2 protein (also designated canalicular multispecific organic anion transporter [cMOAT] or multi-drug resistance protein 2 [MRP2]) encodes a 190–200 kDa polytopic transmembrane protein comprising 1545 amino acids and belongs to the subfamily C of ABC transporter proteins

Figure 8A and 8B. Expression and functional analysis of ABCB1 variants.

A

N21D	Asn	taaactgaacaataaaagtgaa
	Asp	taaactgaacgataaaagtgaa
N44S	Asn	tcgctattcaaattggcttgaca
	Ser	tcgctattcaagttggcttgaca
F103L	Phe	atgatacagggttcttcatgaatct
	Leu	atgatacagggctcttcatgaatct
G185V	Gly	agattaatgaaggtattggtgaca
	Val	agattaatgaagttattggtgaca
S400N	Ser	aatgttcacttcagttacccatctcg
	Asn	aatgttcacttcaattacccatctcg
A893S/T	Ala	aactagaaggtgctgggaagatc
	Ser	aactagaaggttctgggaagatc
	Thr	aactagaaggtactgggaagatc
M986V	Met	gtctttggtgccatggccgtggggc
	Val	gtctttggtgccgtggccgtggggc

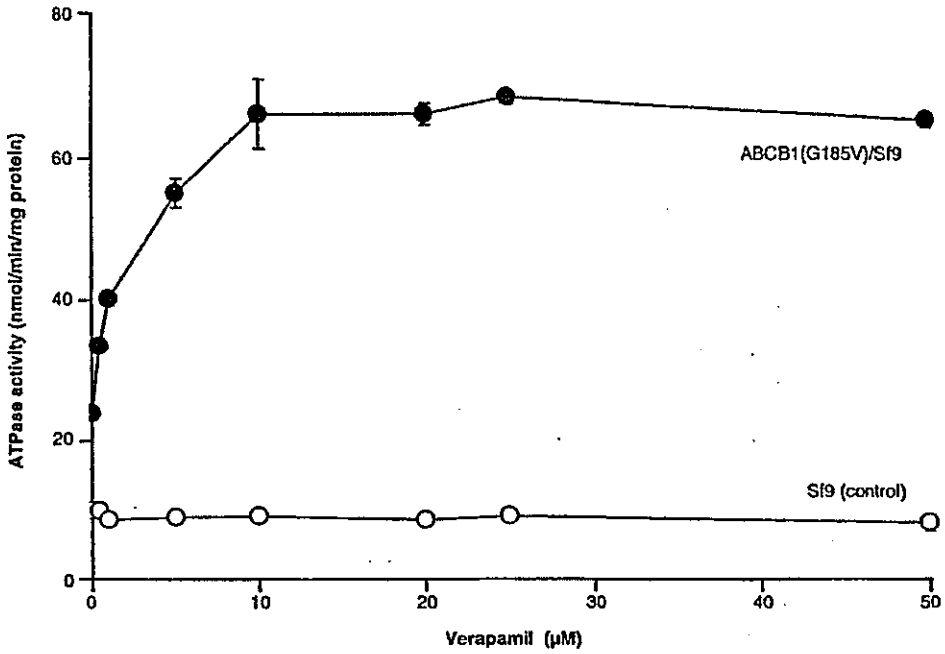
B



A. PCR primers used for site-directed mutagenesis. The sequences are compared with those of the wild-type ABCB1. The codons corresponding to amino acid substitutions have been highlighted in bold. **B.** Western blotting analysis for ABCB1 variants expressed in the plasma membrane fraction of Sf9 cells. ABC: ATP-binding cassette; PCR: Polymerase chain reaction.

[103-105]. The human *ABCC2* cDNA was originally isolated from a cisplatin-resistant human cancer cell line [106], suggesting that the transporter can provide resistance against anionic drugs, such as cisplatin. The ABCC2 was also identified as a multispecific organic anion-excreting transporter across the hepatocyte canalicular membrane [107-109]. Wada *et al.* and others have identified mutations in the *ABCC2* gene in Dubin–Johnson syndrome (DJS) patients (Table 7), an autosomal

Figure 8C. Expression and functional analysis of ABCB1 variants (continued).



C. Verapamil-enhanced ATPase activity in the plasma membrane fraction of Sf9 cells. Sf9 cell membranes (2 μg of protein per well) were suspended in 10 μl of the incubation medium containing 50 mM Tris-Mes (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM potassium chloride, 5 mM sodium azide, and 2 mM ouabain. This medium was mixed with 10 μl of verapamil solution and then pre-incubated at 37°C for 3 min. The ATPase reaction was started by adding 10 μl of 4 mM ATP-solution to the reaction mixture (30 μl) and the incubation was maintained at 37°C for 30 min. The reaction was stopped by the addition of 20 μl of 5% trichloroacetic acid and liberated inorganic phosphate was measured at wavelength of 630 nm [101] in a Multiskan JX system (Dainippon Pharmaceuticals Co., Osaka, Japan). Data are expressed as mean values ± standard deviation of triplicate experiments.

Table 6. Kinetic parameters of the wild type and SNP variants of ABCB1.

Variant	K_m (μM)	V_{max} (nmol/min/mg protein)
Wild type	2.190±0.15	13.14±1.95
N21D	0.502±0.126	45.26±11.33
N44S	0.580±0.148	31.03±4.65
F103L	1.100±0.078	36.34±8.33
G185V	0.831±0.102	56.76±6.76
S400N	0.327±0.025	13.74±2.08
A893S	0.441±0.042	17.24±6.72
A893T	0.904±0.244	10.77±1.35
M986V	0.419±0.062	22.69±6.84

The wild type and variants of ABCB1 were then expressed in Sf9 cells using the pFASTBAC1 vector and recombinant baculoviruses. Using membranes prepared from Sf9 cells expressing ABCB1 variants, ATPase activity was measured in the presence of verapamil at different concentrations. K_m and V_{max} values were calculated from Lineweaver–Burk plots. ABCB1 proteins expressed in Sf9 cell membranes were detected by the western blot method using the C219 monoclonal antibody. The V_{max} values of the variants were normalized to that of the wild type by referring to the intensity of each variant protein on the western blotting. Data are expressed as mean values ± standard deviation (n = 3).
ABC: ATP-binding cassette; K_m: Michaelis–Menten constants; SNP: Single nucleotide polymorphism; V_{max}: Maximal uptake rate.

recessive disorder characterized by conjugated hyperbilirubinemia, further suggesting that ABCC2 mediates the multispecific efflux of various types of organic anions. ABCC2 has a broad substrate specificity covering anticancer drugs [110-112], organic anions derived from phases I and II metabolism of xenobiotics [113-117], and endogenous compounds like cysteinyl leukotriene C4 (LTC_4) [118], bilirubin-glucuronides, glutathione disulfide (GSSG), estradiol-glucuronide ($E_2$17βG), and others [112,119-122]. These findings are consistent with the idea that ABCC2 plays a pivotal role in the phase III system of xenobiotics metabolism [113].

Genetic alterations and polymorphisms in the ABCC2 *gene*

Drugs are detoxified and conjugated *in vivo* and then exported out of the cells. The activity of the detoxification system affects the pharmacokinetics of drugs. Because the ABCC2 protein is responsible for the export of conjugated drugs from hepatocytes, interindividual variations on both activity and expression level of the transporter might be critical factors for considering pharmacokinetics, and clarifying molecular basis for the interindividual variation is necessary for the optimization of medication.

Laboratory data may elaborate on the possible relevance of ABCC2 mutations in pharmacokinetics in DJS carriers [123]. The urinary coproporphyrine I is an undesired byproduct of heme biosynthesis. All DJS patients showed > 80% of urinary coproporphyrine I fraction, while normal controls showed < 27% [124]. Interestingly, all family members examined, who carry a heterozygous mutation in the *ABCC2* gene, had a normal range of T- and D-bilirubin levels, whereas slightly higher levels of urinary coproporphyrine I fraction than the normal range (< 27%) was seen. The mechanisms for abnormal coproporphyrine I fraction in the urine is unknown, but a correlation may exist between the urinary coproporphyrine I level and the homozygous/heterozygous status of mutations in the *ABCC2* gene. In Japan, the expected number of people carrying a heterozygous mutation in *ABCC2* is at least 200,000, which is calculated by the frequency of DJS patients; that is, 121 patients out of 100 million people from a nationwide survey in Japan [125]. The transport and/or pharmacokinetics of some substrates may be affected by a heterozygous mutation in the *ABCC2* gene as observed in coproporphyrine levels, and a putative differential responsiveness to some drugs and their side effects in these carriers may also be an important clinical factor.

The genetic polymorphisms also have potential significance in drug disposition and pharmacokinetics in the same sense. Although numerous SNPs in *ABCC2* have been accumulated in several databases and publications [126-128], knowledge about biochemical consequences is very poor (Table 8) and their association with clinical phenotype, including drug disposition, remains to be clarified. Naturally occurring base substitutions of the *ABCC2* gene accompanied by amino acid substitutions reported to date are shown in Table 8.

Biochemical consequences of genetic defect in DJS patients

Absence of the ABCC2 protein in DJS patients was first reported by Kartenbeck *et al.* [129,130]. Subsequently, absence of ABCC2 was reported in hepatocytes of patients carrying nonsense or deletion mutations [131,132]. Splicing mutations that cause exon skipping and deleted mRNA molecules have been observed as well [123,133,134]. Besides possible instability of the mutant protein, the instability and degradation of the mRNA may also contribute to the decrease of the ABCC2 protein level in patients carrying nonsense, deletion, or splicing mutations, as seen in mutant rats [109]. Among these mutations, biochemical consequences of the deletion mutation RM1392-1393del (Table 7) were analyzed *in vitro* in detail [135]. The mutation is associated with absence of the ABCC2 protein from the apical membrane of hepatocytes. Transfection of mutated *ABCC2* cDNA led to a mutant protein that was only core glycosylated, sensitive to endoglycosidase H digestion, and located in the endoplasmic reticulum (ER) of transfected HEK293 and HepG2 cells, suggesting that the deletion leads to impaired maturation and trafficking of the protein from the ER to the Golgi complex. Inhibition of proteasome function resulted in a paranuclear accumulation of the mutant protein, suggesting the involvement of proteasomes in the degradation.

Analysis of the missense mutations as they occur in patients suffering DJS not only provide the knowledge about the molecular events leading to the pathogenesis of DJS, but also give hints about functional roles of certain amino acid residues in the ABCC2 protein. Mor-Cohen carried out *in vitro* analysis on two novel missense mutations identified in exon 25 in DJS (Tables 7 and 8) [136]. Continuous measurement

Table 7. Summary of mutations identified in Dubin–Johnson syndrome.

Mutation	Exon	IVS	Amino acid alteration	Ref.
1815+2T>A		13	Exon 13 skip	[121]
1967+2T>C		15	Exon 15 skip	[144]
2302C>T	18		R768W	[131]
2439+2T>C		18	Exon 18 skip	[121]
3196C>T	23		R1066X	[107]
3517A>T	25		I1173F	[134]
3449G>A	25		R1150H	[134]
3928C>T	28		R1310X	[132]
4145A>G	29		Q1382R	[121]
4175-4180del	30		RM1392-1393del	[130]

IVS: Intervening sequence (intron).

of probenecid-sensitive carboxyfluorescein efflux, a measure of ABCC2 activity, revealed that both mutations impaired the transport activity. Immunoblot analysis and immunocytochemistry showed that one mutant ABCC2 (R1150H) matured properly and localized at the plasma membrane of transfected cells. In contrast, expression of another mutant ABCC2 (I1173F) was low and mislocated to the ER of the transfected cells.

ABCC2 (R768W), being mutated in the C motif of nucleotide-binding domain 1 (NBD1), was localized in the cytoplasm with an ER-like distribution [137]. This result is consistent with immunohistochemical data showing that there was no apparent expression of ABCC2 protein in the canalicular membrane of hepatocytes in a DJS patient carrying the same mutation. The mutation in the NBD1 thus appeared to block the maturation process of the ABCC2 protein during membrane sorting, probably from the ER to the Golgi apparatus (Table 8). The mutations in the NBD1 might induce an inadequate conformational change, resulting in a defective sorting of the ABCC2 protein and a failure to reach the plasma membrane. MG132, an inhibitor of the cytosolic proteasome, blocked the degradation of the precursor form of ABCC2 (R768W), suggesting that ABCC2 (R768W) is degraded by the proteasome pathway, which is involved in the degradation of newly synthesized, misfolded and unassembled proteins in the ER [138].

Another missense mutation, Q1382R in NBD2, was found in one DJS patient with compound heterozygous mutations (Table 8) [123]. The precursor form of the ABCC2 (Q1382R) was rapidly converted to the mature form and sorted to the apical membrane of the LLC-PK_1 cells as the wild-type ABCC2. These results suggested that, unlike the R768W mutation, the Q1382R mutation does not affect either the maturation process or the subcellular localization of ABCC2. However, efflux of the glutathione conjugate of monochlorobimane (GS-MCLB) and ATP-dependent LTC_4 uptake into plasma membrane vesicles derived from HEK293 cells expressing ABCC2 (Q1382R) was markedly reduced compared to that from cells expressing wild-type ABCC2. This indicated that ABCC2 (Q1382R), although localized on the apical membrane, was non-functional. The ATP-binding site of ABCC1 can be specifically labeled when crude membranes containing ABCC1 are incubated with light-sensitive 8-azido-[α-^{32}P] ATP in the presence of excess vanadate, as established by Senior [139,140]. Labeling occurs because a stable inhibitory complex [ABCC1·MgADP·vanadate] is formed, which is an analog of the transition state complex [ABCC1·MgADP·Pi], formed after ATP hydrolysis [141,142]. Vanadate-induced nucleotide trapping in the wild-type ABCC2 was stimulated by the transporter substrate $E_2$17βG, but not in ABCC2 (Q1382R). In the absence of vanadate, $E_2$17βG did not enhance photoaffinity labeling of the wild-type ABCC2. ABCC2 was scarcely photoaffinity labeled with 8-azido-[γ-^{32}P]ATP. These results suggest that 8-azido-[α-^{32}P]ATP is trapped together with vanadate after hydrolysis, and that the Q1382R mutation impaired substrate-induced ATP hydrolysis (Table 8).

This glutamine located between the Walker A motif and the C motif is highly conserved among the ABC superfamily proteins. Crystal

Table 8. Naturally occurring base-change in *ABCC2* gene accompanied by amino acid substitution.

Location (exon)	Nucleic acid substitution	Amino acid substitution	Domain	Pathogenetic consequence (biochemical defect)	Frequency (%)		Ref.
					Jews	Japanese	
7	842G>A	S281N	Linker	Unknown	2.4	Not reported	[134]
10	1249G>A	V417I	MSD2	Unknown	22.7	10.9	[124,125,134]*
18	2302C>T	R768W	NBD1	DJS (protein maturation)	Not reported	0.4?	[124,131,135]
18	2366C>T	S789F	NBD1	(Transport activity)	Not reported	0.9	[124]*
25	3449G>A	R1150H	MSD3	DJS (transport activity)	0.3	Not reported	[134]
25	3517A>T	I1173F	MSD3	DJS (protein maturation)	1.4	Not reported	[134]
28	3895A>C	K1299Q	NBD2	Unknown	Not reported	1?	[125]
29	4145A>G	Q1382R	NBD2	DJS (ATP hydrilysis)	Not reported	0.1?	[121,135]
31	4348G>A	A1450T	NBD2	(Transport activity)	Not reported	0.4?	[124]*

**Ebihara, Kuwano and Wada, unpublished data.*

ABC: ATP-binding cassette; DJS: Dubin–Johnson syndrome; MSD: Membrane-spanning domain; NBD: Nucleotide-binding domain.

structure analysis of the ATP-binding domain of the bacterial histidine permease (HisP, an ABC transporter) suggests that the corresponding glutamine in the HisP molecule (Q100) is likely to form hydrogen bonds with a water molecule that interacts with the γ-phosphate of ATP [143]. This water molecule is the most likely candidate for attacking water during ATP hydrolysis [143]. The comparable amino acid substitution, Q1291R, in cystic fibrosis transmembrane regulator (CFTR) was observed in patients suffering from cystic fibrosis [144]. It has also been reported that the CFTR (Q1291R) shows no chloride channel function although it reaches the plasma membrane as a fully glycosylated mature protein. However, the role of the glutamine in the Q-loop has been controversial, since the corresponding glutamine residue in the bacterial maltose permease was suggested to be placed too far away from the nucleotide to coordinate Mg^{2+} and the water molecule that attacks the γ-phosphate bond [145]. In our study, the lack of substrate-induced vanadate trapping in ABCC2 (Q1382R) may suggest that Q1382 is directly involved in ATP hydrolysis [137].

ABCG2 (BCRP/MXR1/ABCP)

The breast cancer-resistant protein (BCRP) has recently been discovered in doxorubicin-resistant breast cancer cells [147]. Since the same transporter has also been found in the human placenta [148], as well as in drug-resistant cancer cells selected in mitoxantrone [149], the transporter was also called ABCP or MXR1. This ABC transporter protein is now named ABCG2 and has been classified in the G subfamily of human ABC transporter genes according to the new nomenclature. In the same subfamily, ABCG1, ABCG5, and ABCG8 have been reported to be critically involved in the regulation of lipid- and sterol-trafficking mechanisms in macrophages, hepatocytes, and intestinal mucosa cells [150]. As compared to the molecular structures of ABCB1 and ABCC2, the newly found ABCG2 transporter is a so-called 'half-transporter' bearing six transmembrane domains and one ABC. It has recently been demonstrated that the ABCG2 protein functions as a homodimer bound via a cysteinyl disulfide bond(s) [151].

The *ABCG2* gene is located on chromosome 4q22 and spans over 66 kb, consisting of 16 exons and 15 introns [152]. The *ABCG2* gene is transcribed by a TATA-less promoter with several Sp1 sites, which are downstream from a putative CpG island. The sequence 312 bp directly upstream from the *ABCG2* transcriptional start site conferred basal promoter activity.

ABCG2 in normal tissues

ABCG2 is expressed endogenously in placental trophoblast cells, the epithelium of the small intestine and liver canalicular membrane, as well as in ducts and lobules of the breast [36]. In

particular, the high expression of ABCG2 in trophoblast cells suggests that the pump is responsible either for transporting compounds into the fetal blood supply or removing toxic metabolites. The apical localization in the epithelium of the small intestine and colon indicates a possible role of ABCG2 in the regulation of the uptake of orally administered drugs.

In addition, expression of ABCG2 is detected in venous and capillary endothelium [153]. Furthermore, it has been reported that ABCG2 is expressed in a wide variety of stem cells, and its potential role in the regulation of hematopoetic development is suggested [154]. To date, however, physiological substrates of ABCG2 in stem cells have not yet been identified.

Jonker *et al.* most recently demonstrated that mouse Abcg2 protects against a major chlorophyll-derived dietary phototoxin and protoporphyria [155]. The wild type of mouse Abcg2 has been suggested to export chlorophyll and porphyrin metabolites in the small intestine.

ABCG2 in cancer

ABCG2 is amplified or involved in chromosomal translocations in cancer cell lines selected with mitoxantron, topotecan or doxorubicin treatment, and AGCG2 was shown to confer resistance to anticancer drugs [156-160]. Furthermore, relatively high expression of ABCG2 is observed in ~ 30% of acute myeloid leukemia patients [161] and is correlated with an immature immunophenotype as determined by expression of the surface marker CD34 [162]. Overexpression of ABCG2 is related to cancer cell resistance to camptothecin-based anticancer drugs, such as topotecan [158] and 7-ethyl-10-hydroxycamptothecin (SN-38; the active metabolite of irinotecan) [163]. SN-38-selected PC-6/SN2-5H human lung carcinoma cells were shown to overexpress ABCG2 with the reduced intracellular accumulation of SN-38 and its glucuronide metabolite [163]. Plasma membrane vesicles prepared from those cells transported both SN-38 and SN-38-glucuronide in an ATP-dependent manner, suggesting that ABCG2 is involved in the active extrusion of SN-38 and its metabolite from cancer cells [164].

Acquired mutations of ABCG2

Until now, several acquired mutations were documented for ABCG2 cloned from drug-resistant cell lines [165,166]. Drug resistance phenotypes vary among different cell lines expressing variant types of ABCG2. To date, at least three variant forms of ABCG2 have been documented on the basis of amino acid moieties at position 482, which resides within the third transmembrane domain. The wild-type form of ABCG2 has an arginine at that position [148], whereas other variants cloned from cancer cell lines [156,157] have glycine and threonine at position 482. It has recently been demonstrated that the substrate specificities and resistance profiles of ABCG2 greatly differ among those variant forms [158-160]. Transfectants with the wild type (R482) were reportedly not resistant to topotecan [160], while overexpression of the G482 and T482 variants conferred resistance to mitoxantrone, doxorubicin, daunorubicin, and various camptothecin analogs including topotecan [147,156-158]. HEK293 cells transfected with the wild type (R482), however, were recently found to be resistant to SN-38 (Ishikawa T, unpublished work). G482 and T482 variants mediated the efflux of rhodamine 123 and doxorubicin from cells, whereas R482 did not [165]. These findings strongly suggest that R482 has a critical role in the substrate specificity of ABCG2. Indeed, it has recently been reported that ABCG2 (R482) transports [^{3}H]methotrexate in an ATP-dependent manner; however, no transport activity was observed with the other variants (G482 and T482) [167-168]. Overexpression of ABCG2 (R482) may be related to methotrexate resistance [169]. The identification of mutations at 482 in ABCG2 may explain some discrepancies observed in the cross-resistance profiles of human cancer cell lines. Likewise, one mutation 'hot spot' was identified at the same amino acid position 482 in mouse Abcg2 [170]. In the case of mouse Abcg2, variants of arginine, serine, and methionine significantly affect the drug resistance profile in cancer cell lines [170].

Non-synonymous SNPs of ABCG2

There is accumulating information on the genetic polymorphism of ABCG2. To date, seven naturally occurring, non-synonymous SNPs have been reported in Japanese and Caucasian populations: V12M, Q126Stop, Q141K, I206L, F431L, F489L, and N590Y [171-177]. In addition, several other non-synonymous SNPs have also been registered in the SNP database of the NCBI.

Honjo *et al.* have identified 3 non-coding SNPs in the untranslated regions, 3 non-synonymous and 2 synonymous SNPs in the coding region, and 7 SNPs in the intron sequences adjacent to the 16 *ABCG2* exons [171]. Non-synonymous SNPs are located at nucleotides 238

(exon 2) and 625, resulting in amino acid substitutions V12M and Q141K, respectively. These SNPs showed a greater frequency of heterozygosity (22.2 and 10%, respectively) than the SNP at nucleotide 2062 (D620N, exon 16).

Recently, Imai *et al.* [173] identified three allelic variants in the *ABCG2* gene, of which two were non-synonymous SNPs (V12M and Q141K) and the third was a splice variant with deletion of nucleotides 944–949 that lacks A315 and T316 (Δ315-6). Compared with wild-type transfected cells, the ABCG2 Q141K variant-transfected cells showed low levels of drug resistance associated with decreased protein expression. The SNP (Q141K) was postulated to cause increased sensitivity of normal cells to anticancer agents that are ABCG2 substrates such as SN-38. Moreover, a novel SNP in exon 4, 376C>T, substituting a stop codon for Q124, was found in the Japanese population with an allelic frequency of 2.4% [171]. It has been postulated that the 376C>T SNP may have a higher impact than 421C>A polymorphism causing Q141K, because active ABCG2 protein will not be synthesized from the variant allele. More recently, Chowbay *et al.* [174] have investigated genetic polymorphisms in the *ABCG2* gene in three distinct Asian populations and compared them with the Japanese population. They have identified five SNPs at exons 2 (34G>A), 4 (376C>T), 5 (421C>A), and 12 (1444A>G and 1445G>C) in healthy Chinese, Malay and Indian subjects. SNPs at two loci, 34G>A at exon 2 and 421C>A at exon 5, showed genotypic and allelic variations in the three Asian populations. These two SNPs were also strongly associated and found to be in linkage disequilibrium in the Chinese and Malay populations, but not in the Indian population [174].

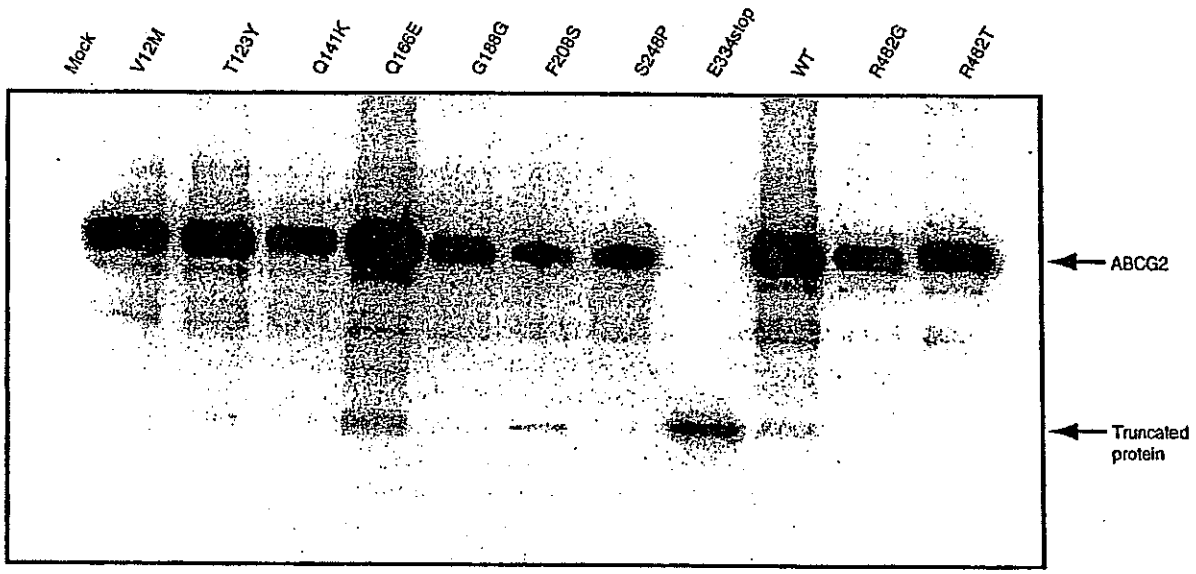
Functional analysis of ABCG2 polymorphisms

Quantitative studies were carried out to precisely evaluate functional changes associated with genetic polymorphisms and acquired mutations of ABCG2. For this purpose, a pFASTBAC1 expression vector carrying the ABCG2 wild-type cDNA was used as the template, and variant forms (V12M, T123Y, Q141K, Q166E, G188G, F208S, S248P, E334stop, R482G, and R482T) were created by the site-directed mutagenesis using the QuickChange Site-directed Mutagensis Kit (Stratagene). Recombinant baculoviruses to express the above-mentioned variant forms of ABCG2 in insect cells were generated with the BAC-TO-BAC® Baculovirus Expression Systems (Invitrogen). Insect Sf9 cells (1×10^6 cell/ml) were infected with the recombinant baculoviruses and cultured in the EX-CELL™ 420 Insect serum-free medium (JRH Bioscience, Levexa, KS, USA) at 26°C with gentle shaking. Cells were harvested by centrifugation 48 h after the infection. Cell membranes were prepared as described previously [178]. Expression of ABCG2 in Sf9 cell membranes was determined by immunoblotting with BXP-21 (Signet Laboratories, Dedham, MA, USA) as the first antibody, and an anti-mouse IgG-horseradish peroxidase (HRP)-conjugate (Cell Signaling Technology, Beverly, MA, USA) as the secondary antibody. HRP-dependent luminescence was developed by using Western Lighting Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) and detected by Lumino Imaging Analyzer FAS-1000 (TOYOBO, Osaka, Japan).

The frozen stocked cell membrane was thawed quickly at 37°C, and vesicles were formed by passing the membrane suspension through a 27-gauge needle. The standard incubation medium contained plasma membrane vesicles (40 or 80 μg of protein), 10 mM [3′,5′,7′-^{3}H]methotrexate (Amersham, Buckinghamshire, UK), 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate, and 100 μg/ml creatine kinase in a final volume of 110 μl. The reaction was started by adding [^{3}H]methotrexate to the incubation medium. The reaction was carried out at 37°C and the amount of [^{3}H]methotrexate incorporated into the vesicles was measured by a rapid filtration technique as previously described [178,179]. The ATPase activity of the isolated Sf9 cell membrane was determined by measuring inorganic phosphate liberation [178] according to the procedure as described for the functional analysis of ABCB1 variants.

Figure 9A illustrates the levels of ABCG2 variants expressed in Sf9 cells. The molecular size of the expressed ABCG2 variants was ~ 65 kDa except for the E334stop variant. The E334stop variant carried the ABC but lacked the transmembrane domains of ABCG2. Nevertheless, this truncated protein was weakly associated with the plasma membrane of Sf9 cells (Figure 9A). As shown in Figure 9B, there was a linear relationship between the signal intensity of western blotting and the logarithmic value of the membrane protein amount. Based on the linear relationship, the expression level of ABCG2 variants in the plasma membrane of Sf9 cells could be quantitatively estimated and

Figure 9A. Expression of the wild type and variants of ABCG2 in Sf9 cells.



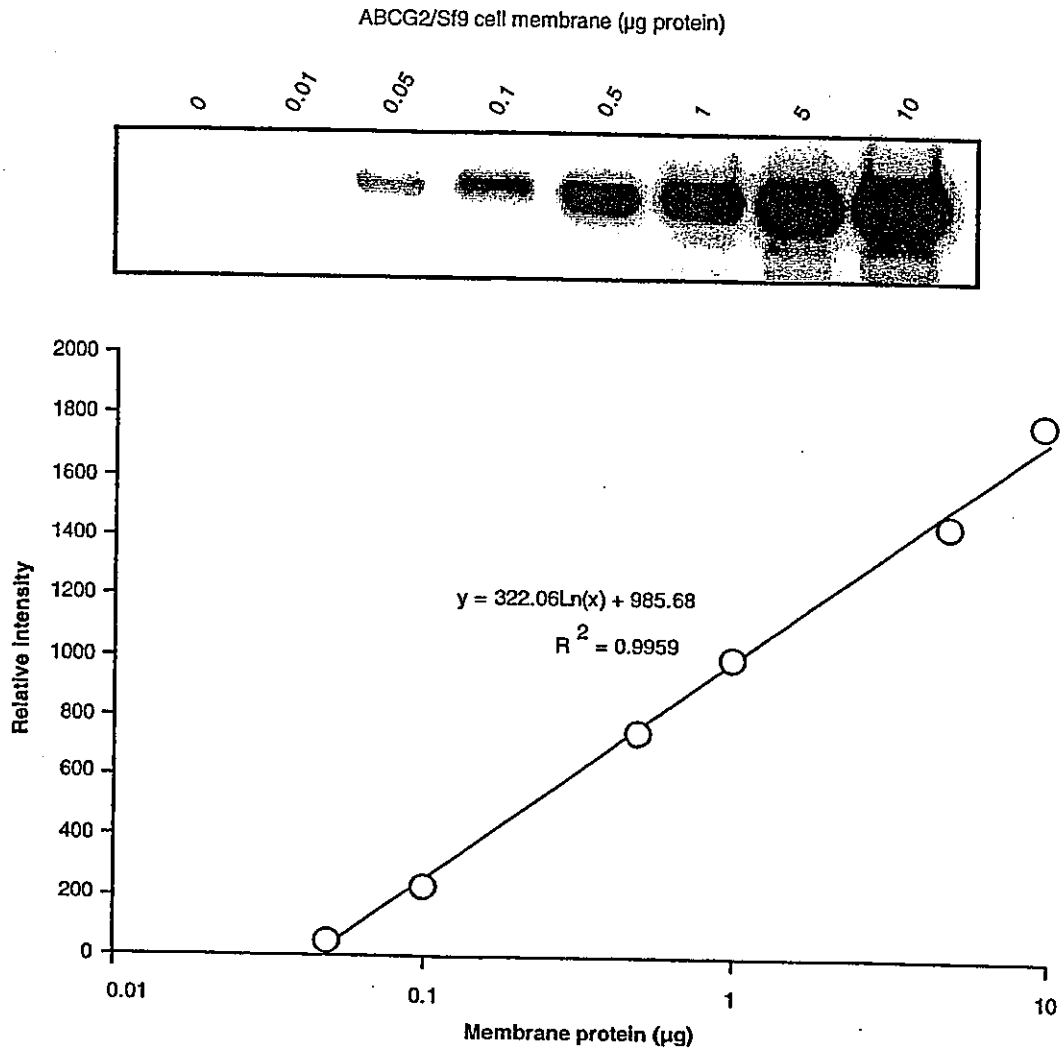
SNP ID	Amino acid position	Translation	SNP detail
2231137	12	Val → Met	G/A
2231139	123	Tyr → Tyr	C/T
2231142	141	Gln → Lys	C/A
1061017	166	Gln → Glu	C/G
3116439	188	Gly → Gly	G/A
1061018	208	Phe → Ser	T/C
3116448	248	Ser → Pro	C/T
3201997	334	Glu → stop	G/C

A. Western blotting analysis of ABCG2 and its variants in the plasma membrane fraction of Sf9 cells. ABCG2 proteins were immunologically detected using the BXP-21 antibody (Signet Laboratories, Dedham, MA, USA) as the first antibody.
ABC: ATP-binding cassette; SNP: Single nucleotide polymorphism; WT: Wild type.

normalized with the amount of the wild type. Figure 10 demonstrates methotrexate transport and prazosin-enhanced ATPase activities after normalization. The ABCG2 variants V12M, T123Y, Q141K, Q166E, G188G, F208, and S248, as well as the wild type transported [^{3}H]methotrexate; however, they did not exhibit prazosin-enhanced ATPase activity. The G181G (synonymous SNP) showed the same profile of activities as the wild type. In contrast, acquired mutants (i.e., R482G and R482T) displayed prazosin-enhanced ATPase activity, but lacked methotrexate transport activity. The K_m values of both R482G and R482T variants were estimated to be 1 μM for prazosin. The E334stop variant exhibited neither the activity of methotrexate transport nor prazosin-enhanced ATPase activity. Based on these data, it can be concluded that R482 is a critical amino acid moiety in substrate specificity and transport of ABCG2 for certain drugs, such as methotrexate.

The wild type (Arg-482) and acquired mutants (R482G, and R482T) of ABCG2 were stably transfected in HEK293 cells and exhibited resistance to SN-38 by 15-, 5-, and 5.3-fold, respectively [180]. In plasma membrane vesicles prepared from the transfected HEK293 cells, wild-type ABCG2 transported SN-38 and its glucuronide conjugate in an ATP-dependent manner with K_m values of 3 and 22 μM, respectively; however, only minimal transport activities were observed with the mutants (R482G and R482T) [180]. These results strongly suggest that the wild-type ABCG2 plays a pivotal role in confering drug resistance by extruding SN-38 and its glucuronide metabolite from cells.

Figure 9B. Expression of the wild type and variants of ABCG2 in Sf9 cells (Continued).



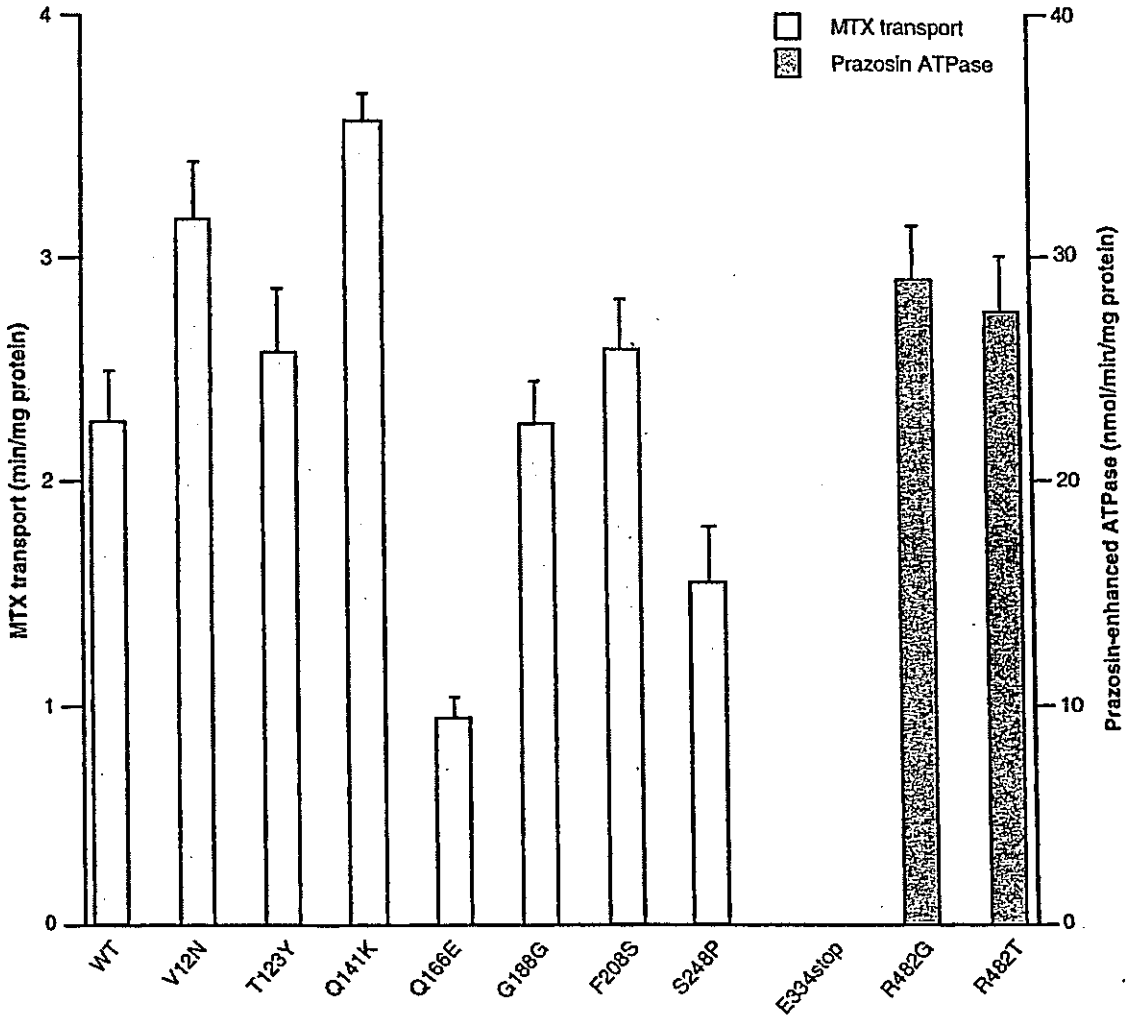
B. Quantitative analysis for the expression levels of the ABCG2 protein. ABCG2-expressing plasma membrane was applied for SDS–PAGE and detected by western blotting. Expression levels were measured by using Western Lighting Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) and Lumino Imaging Analyzer FAS-1000 (TOYOBO, Osaka, Japan). The figure depicts a relationship between the chemiluminescence intensity of the western blot and the amount of the plasma membrane protein applied.
ABC: ATP-binding cassette.

Outlook and conclusions

There is an increasing number of literature that addresses genetic polymorphisms of drug transporters. At present, concerning the effects of genetic polymorphisms on pharmacokinetic profiles, the best characterized transporter is ABCB1 [73-97,181-187]. However, it is also true that there is still considerable discrepancy among all reported results to date (see Table 3 in [58]). As described in this review, detailed functional analyses *in vitro* may provide clear insight into the biochemical significance of genetic polymorphisms. In addition, more information on SNPs and haplotype maps will be available for a variety of drug transporters. It will become important to determine which polymorphism has influence on the function and gene expression of drug transporters. The effect of SNPs on transport activity may depend on substrates tested, and, therefore, the functional analysis of SNPs using a wide variety of substrates is of great interest.

There are many factors that can affect not only the function but also the expression levels of drug transporters. Numerous environmental

Figure 10. ATP-dependent transport of methotrexate and prazosin-enhanced ATPase activity in the plasma membrane prepared from Sf9 cells expressing the wild type and variant forms of ABCG2.



ATP-dependent MTX transport was measured as described previously [167,178,179]. The reaction was carried out at 37°C in the presence of 200 µM [^{3}H]methotrexate for 30 min. The amount of [^{3}H]methotrexate incorporated into the vesicles was measured by the rapid filtration technique [179]. The prazosin-enhanced ATPase activity was measured by incubating the isolated Sf9 cell membrane in the absence or presence of 50 µM prazosin at 37°C for 30 min. Liberated inorganic phosphate was determined as previously described [178]. Data are expressed as mean values ± standard deviation of triplicate experiments.
ABC: ATP-binding cassette; MTX: Methotrexate; WT: Wild type.

factors affecting the phenotypical activity of drug transporters must be considered, which may include exogenous chemicals, food constituents, herbal preparations, and/or therapeutic drug use that may induce or inhibit the function or expression of drug transporters, as exemplified by the cases of ABCB1 [181,188,189].

Variation in the pharmacokinetic behavior of a drug among different patients is the net result of complex interactions between genetic, physiologic and environmental factors. If the function or expression level of drug transporters is altered due to genetic factors, intestinal secretion of the drug into the gut lumen may change. Such information may be valuable in predicting an increase or decrease in bioavailability or orally administered substrate drugs in individual patients. In the case of organ transplantation, it is critically important to maintain the concentration of an immunosuppressive drug at sufficient levels in the blood circulation. The success of the transplantation depends on a delicate balance

Highlights

- The drug transporters are one of the major determinants governing the pharmacokinetic profiles of drugs.
- Many factors can affect the function as well as the expression of drug transporters. These factors include genetic mutations, SNPs, splicing, transcriptional regulation, stability of mRNA, post-translational modification, and intracellular localization.
- There is accumulating evidence for the existence of genetic polymorphisms in drug transporters such as SLC and ABC transporters.
- Functional analysis of the polymorphism of drug transporters is an important approach to understand the molecular mechanisms underlying individual differences of drug response.
- It is critically important to identify the function-related SNPs that have potential impact on the substrate specificity and/or activity of drug transporters.
- Since the effect of SNPs on the transport activity may depend on substrates tested, the functional analysis of SNPs using a wide variety of substrates is of great interest.
- Certain SNPs in introns may affect the expression of transporter genes. Thus, pharmacogenomics studies on the molecular mechanisms underlying the induction and/or downregulation of drug transporters would significantly contribute to our understanding of individual difference in drug response.

between immunosuppression and rejection. For instance, tacrolimus (FK506) is a widely used immunosuppressor, whereas large variability has been noted in its bioavailability after oral administration of this drug. Recent studies have provided evidence that intestinal expression of ABCB1 is a good probe for prediction of the interindividual variation in tacrolimus pharmacokinetics after organ transplantation and also a powerful prognostic indicator for the outcome of organ transplantation [190,191].

The expression and phenotypical activity of drug transporters in patients may be strongly influenced by medication. Genetic variations in the promoter region or introns of drug transporter genes may alter the gene expression. In addition, non-genetic and/or epigenetic factors are also considered to have potential effects. Gene regulation of drug transporters as well as drug-metabolizing enzymes is of great interest in order to understand the molecular mechanisms of drug response and toxic events. It has been documented that hydrophobic ligands and several nuclear receptors are involved in induction or downregulation of cytochrome P450 isoforms and ABC transporters [16,192]. While the gene regulation of drug-metabolizing enzymes, such as cytochrome P450, glucuronide transferases, and glutathione transferases, have been well-characterized, pharmacogenomic studies on the molecular mechanisms underlying the induction and/or downregulation of drug transporters would significantly contribute to our understanding of individual differences in drug response.

Acknowledgments

The authors thank the Pharma SNP Consortium for their generous support. In particular, our cordial thanks go to Yoshinobu Yoshimura, Kimitoshi Saito, Akio Yoshida, Toshikazu Miyagishima, and Hiroshi Gushima. In addition, we thank Naoyuki Kamatani (Tokyo Women's Medical University) and Yusuke Nakamura (University of Tokyo) for their support in DNA sample collection and SNP analysis of Japanese volunteers. The studies of the authors were supported, in part, by a research grant entitled 'Studies on the genetic polymorphism and function of pharmacokinetics-related proteins in Japanese population' (H12-Genome-026) from the Japanese Ministry of Health and Welfare.

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