Significant Genetic Linkage of *MDR1* Polymorphisms at Positions 3435 and 2677: Functional Relevance to Pharmacokinetics of Digoxin

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INTRODUCTION

The human *MDR1* gene encodes MDR1, also called P-glycoprotein (P-gp), which functions in the energy-dependent export of substances from the inside of cells to the outside. MDR1 was originally isolated from resistant tumor cells as the protein responsible for conferring resistance against antitumor agents (1). Human MDR1 is also expressed in normal tissues including the mucosal cells in the small and large intestine, the epithelial cells of renal proximal tubules, the biliary canalicular side of hepatocytes, capillary endothelial cells of the brain and testis, and cells of the leukocyte lineage (2). MDR1 contributes to the limitation of drug absorption from the gastrointestinal tract, secretion of the drugs into bile and urine, and the prevention of penetration of drugs across the blood-brain barrier. Thus, MDR1 in these normal tissues defines the pharmacokinetics of many drugs, which are substrates for MDR1.

In 1989, nine nucleotide differences on the human *MDR1* gene were found from drug-selected multidrug-resistant cultured cells (3). To date, more than 20 single nucleotide polymorphisms (SNPs) in the exonic regions have been identified (4–7). Among them, the mutation in exon 26, position 3435 (C3435T), neighboring on the ATP binding domain, has been focused due to its suppressive effect on the expression of MDR1 protein in duodenal biopsies and resultant increase of plasma concentration of digoxin under rifampin induction or at steady-state in Caucasian subjects, although this is a silent mutation (5). C3435T mutation was shown to decrease efflux of the MDR1 substrate rhodamine from $CD56^+$ natural killer cells and lower MDR1 mRNA expression in leukocytes (8). However, recent investigations have suggested that C3435T has no effect on placental MDR1 expression (7) and moreover that C3435T mutation is related to a higher level of MDR1 mRNA expression in duodenal biopsies in healthy Japanese subjects (9). The C3435T mutation has been reported to have no effect on the plasma concentration of digoxin (10) and to be lower in subjects with T/T genotype (11). As for other MDR1 substrates, the C3435T mutation has been reported to have no effect on the cyclosporin A trough concentration (12) and to be lower plasma concentration of fexofenadine (13).

These investigations suggested the importance of *MDR1* genotyping, especially for C3435T. However, the molecular mechanisms underlying the effects of this polymorphism remain unclear, and further investigations should be addressed to elucidate these discrepancies. The C3435T SNP has been suggested to be linked with the SNP at exon 21, position 2677 (G2677(A,T)) producing Ala893Thr and Ala893Ser, respectively (7,13), and haplotype analysis might provide a rational explanation for these discrepancies. This study was, therefore, designed to elucidate the linkage of SNPs at positions 3435, 2677, and -129 in 117 healthy Japanese subjects. Position 2677 locates in the intracellular domain between the 10th and 11th transmembrane spanning domains. Position –129 is in the promoter region, exon 1b. In addition, the effects of SNPs at positions 3435 and 2677 on the serum concentration-time profiles of digoxin after single oral administration were examined in healthy Japanese subjects.

MATERIALS AND METHODS

Subjects

One hundred and seventeen healthy Japanese subjects living in Kobe city and neighboring areas, consisting of 65 males and 52 females, 19 to 76 years old were evaluated for *MDR1* genotypes at positions 3435, 2677, and –129. The aims of this study were fully explained to all subjects, and written informed consent was obtained. The protocol for this study was approved by the Institutional Review Board of Kobe University Hospital, Kobe University, Japan.

MDR1 Genotyping

Genomic DNA was extracted from 0.5 mL of whole blood using a DNA Extractor WB Kit (Wako Pure Chemical Industries Ltd.; Osaka, Japan) as described previously (11). The genotypes at positions 3435, 2677, and –129 of the *MDR1* gene were determined by polymerase chain reaction (PCR), restriction fragment length polymorphism (PCR-RFLP) analysis and by direct sequencing. For amplification of specific DNA fragments, primer for T-129C was designed based on the known sequence of MDR1 mRNA (GenBank accession number M14758) (forward primer: 5′-TCA GCA TTC AGT CAA TCC GG-3′, reverse primer: 5′-TTT GCG TGC CCC TAC CTC-3′), and previously reported primers were used for C3435T (11), G2677A, and G2677T (6,14). PCR was carried out under similar conditions for all reactions as described previously (11). After amplification, PCR products were digested with restriction enzymes (*Mbo* I for C3435T, *Bsr* I for G2677A, *Ban* I for G2677T, and *Tse* I for T-129C). DNA fragments digested with appropriate restriction en-

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zymes were separated on 3% agarose gels for C3435T and T-129C or 5% gels for G2677A and G2677T. The genotype determined by PCR-RFLP was confirmed by direct sequencing on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems; Foster City, CA) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sense primers for PCR amplification were used as sequencing primers.

Serum Concentration-Time Profiles of Digoxin after Single Oral Administration

Fifteen healthy Japanese subjects participated in the pharmacokinetic study of digoxin. The genotypes at position 3435 of the subjects were C/C^{3435} (n = 5), C/T^{3435} (n = 4), and T/T^{3435} (n = 6). Those at position 2677 were G/G^{2677} (n = 5), G/A^{2677} (n = 1), G/T^{2677} (n = 2), A/T^{2677} (n = 3), and T/T^{2677} (n = 4). Those at position –129 were T/T^{-129} (n = 12) and T/C^{-129} (n = 3). The subjects were prohibited from taking any drugs or alcoholic beverages for at least 1 week before administration of digoxin until the end of the study. After overnight fasting, each subject received a single oral dose of digoxin (0.25 mg) as a tablet (Digosin®, Chugai Pharmaceutical Co., Ltd.; Japan). They were cautioned not to eat or drink anything for 3 h after administration. Venous blood samples were obtained through an indwelling cannula at 0.5, 1, 2, 4, 6, 8, 12, and 24 h after administration, and serum samples were prepared. Digoxin serum concentrations were determined by fluorogenic enzyme immunoassay (FEIA) using an OPUS analyzer (Dade Behring, Inc.; Newark, DE). The determination was routinely validated to confirm accuracy and precision. All assays were performed in duplicate for each serum sample, and the mean value was calculated as the data. The limit of quantification in this assay was 0.25 ng/mL.

Statistical Analysis

The χ^2 test was used for analysis of linkage between SNPs at positions 3435, 2677, and –129. The unpaired Student's *t* test and Fisher's exact probability test were used for evaluation of the significance of differences in kinetic parameters and serum concentrations, respectively, between various genotype groups. A *P* value of less than .05 was considered statistically significant.

RESULTS

MDR1 Genotype Distribution in the Japanese Population

The genotypic and allelic frequencies of the C3435T, G2677(A,T), and T-129C SNPs in 117 Japanese subjects are shown in Table I. C3435T, G2677A, and G2677T mutations largely occurred with variant allele frequencies of 38.5%, 20.5%, and 35.5%, respectively, whereas T-129C mutation appeared with a frequency of 8.1%. Gender and age showed no effect on the genotype or allele distribution.

Genetic Linkage of *MDR1* SNPs

The genotype frequencies were tabulated to analyze the genetic linkage of *MDR1* SNPs (Table II). Significant linkage was detected between *MDR1* SNPs at positions 3435 and 2677 ($P < .0001$), but there was no linkage between positions –129 and 3435 or 2677. Of the subjects with C/C^{3435}, 31.7% (13/41) showed G/G^{2677}, and this was higher than the overall G/G^{2677} frequency of 12.8% (15/117). In contrast, 71.4% (10/14) of the subjects with T/T^{3435} showed T/T^{2677} genotype, which was markedly higher than the T/T^{2677} frequency of 9.4% (11/117). Compared with variant T-allele at position 2677, variant A-allele tended to be accompanied by the C-allele at position 3435: 80.0% (36/45) of subjects with G/T^{2677} were accompanied with C/T^{3435}, but 67.9% (19/28) of G/A^{2677} were accompanied with C/C^{3435}; 90.9% (10/11) of subjects with T/T^{2677} were accompanied with T/T^{3435}, but 87.5% (14/16) of A/T^{2677} were accompanied with C/T^{3435}; all with A/A^{2677} also showed C/C^{3435}.

Serum Concentration-Time Profiles of Digoxin following Single Oral Administration

Fig. 1 shows the effects of *MDR1* SNPs at positions 3435 and 2677 on the serum concentration-time profiles of digoxin after single oral administration in healthy Japanese subjects. The observed maximal serum concentration of digoxin was 1.98 ± 0.23 for the subjects with both C/C^{3435} and G/G^{2677}, 1.39 ± 0.23 for those with both C/T^{3435} and A/T^{2677} and 1.44 ± 0.23 ng/mL for those with both T/T^{3435} and T/T^{2677}; the value for the former group was significantly higher than those for the other groups ($P < .05$). The areas under the concentration-time curves after single oral administration of digoxin at time zero to 4 h were 4.00 ± 0.30, 2.99 ± 0.17 and 3.34 ± 0.29 ng h/mL, respectively, and the value for the C/C^{3435} and G/G^{2677} group was significantly higher than that for the C/T^{3435} and A/T^{2677} group (Table III).

DISCUSSION

There occurs increasing report on the correlation of *MDR1* genotype and phenotype for high relevance of MDR1 to drug disposition. It has been suggested that C3435T SNP in

Table I. Positions, Effects, and Genotype and Allele Frequencies of *MDR1* Single Nucleotide Polymorphisms (SNPs) in 117 Healthy Japanese Subjects

SNP	MDR1 resion/position	Effect	Genotype frequency (%)			Allele frequency[a] (%)	
			wt/wt	wt/mut	mut/mut	wt	mut
C3435T	Exon26/3435	Ile1145Ile	35.0	53.0	12.0	61.5	38.5
G2677A	Exon21/2677	Ala893Thr	12.8	23.9(G/A)	1.7(A/A)	44.0	20.5(A)
G2677T		Ala893Ser		38.5(G/T)	9.4(T/T)		35.5(T)
					13.7(A/T)		
T-129C	Exon1b/-129	Noncoding	84.6	14.5	0.9	91.9	8.1

Note. In all SNPs, no effects of gender or age were present on the genotype or allele distribution.
[a] Allele frequency was calculated on the basis of the Hardy-Weinberg distribution.

Table II. Two-Dimensional Graph of *MDR1* Genotypes at Positions 3435, 2677, and -129 in 117 Healthy Japanese Subjects

A							
3435 \ 2677		G/G	G/A	G/T	A/A	A/T	T/T
		15	28	45	2	16	11
C/C	41	13	19	5	2	2	0
C/T	62	2	9	36	0	14	1
T/T	14	0	0	4	0	0	10
B							
3435 \ −129		T/T	T/C	C/C			
		99	12	1			
C/C	41	33	7	1			
C/T	62	52	10	0			
T/T	14	14	0	0			
C							
−129 \ 2677		G/G	G/A	G/T	A/A	A/T	T/T
		15	28	45	2	16	11
T/T	99	14	20	41	1	12	11
T/C	17	1	7	4	1	4	0
C/C	1	0	1	0	0	0	0

Note. The values represent the numbers of subjects with corresponding genotype. A, 3435 versus 2677; B, 3435 versus −129; C, −129 versus 2677.
Statistical analyses of the correlation of the genotypes were performed by the χ^2 test.
A P value of less than .05 was considered statistically significant.

exon 26 of the *MDR1* gene influences MDR expression and function (5). However, recent investigations have suggested that it might be difficult to account for the correlation of the genotype and phenotype solely by C3435T SNP (7,13). The SNP in exon 21, G2677(A,T), has been shown to be correlated with MDR1 activity in variant allele transfected cells and with altered MDR1 function in humans with the probe drug, fexofenadine (13). T-129C SNP in exon 1b of the *MDR1* gene has been suggested to affect placental MDR1 expression (7). Therefore, the present study was performed to elucidate the linkage between SNPs at positions 3435, 2677, and -129 of the *MDR1* gene in 117 healthy Japanese subjects. In addition, the effects of the SNPs at positions 3435 and 2677 on the serum concentration-time profiles of digoxin after single oral administration were examined in healthy Japanese subjects.

The Japanese population showed a similar allele frequency at position 3435 to other Asian populations (Chinese, Filipino, and Saudi), and the variant T-allele frequency was higher than that in African populations (15). The allele frequency at position 2677 was lower for the wild G-allele and higher for the variant A-allele in Japanese compared with Caucasian populations (14). The T-129C genotype frequency was similar between Japanese and Caucasian populations (5).

Significant genetic linkage was detected between *MDR1* SNPs at positions 3435 and 2677 ($P < .0001$), but there was no linkage between positions −129 and 3435 or 2677. Of the subjects with C/C^{3435}, 31.7% (13/41) showed G/G^{2677}, and 71.4% (10/14) of the subjects with T/T^{3435} showed T/T^{2677}. Variant A-allele at position 2677 tended to be accompanied by the C-allele at position 3435, as compared with variant T-allele. The reason for the linkage is not clear, but the discrepancies in clinical reports indicating that the pharmacokinetics of drugs are dependent on the genotype at position 3435 might be resolved by analysis of the genotype at position 2677 or by haplotype analysis, since the mutation at position 3435 is a silent mutation, and the mutations at position 2677 cause amino acid substitutions.

Pharmacokinetic profiles of digoxin after single oral ad-

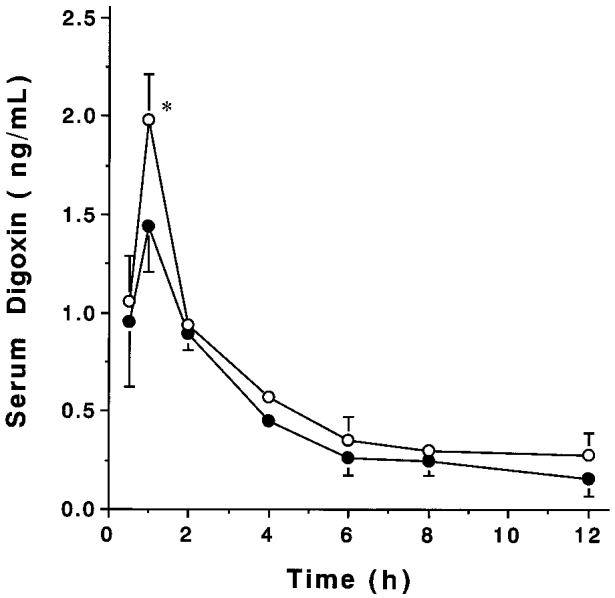

Fig. 1. The relationship between *MDR1* genotypes and serum concentration-time profiles of digoxin after single oral administration in healthy Japanese subjects. Open circles; C/C^{3435} and G/G^{2677} group (n = 4); closed circles, T/T^{3435} and T/T^{2677} group (n = 4). The data are expressed as the mean ± SE. * Statistically significant difference ($P < .05$) between both genotype groups.

Table III. Pharmacokinetic Parameters according to the Genotypes at Positions 3435 and 2677 after a Single Oral Administration of Digoxin

Genotype			Parameter	
C3435T	G2677(A,T)	N	C_{1h} (ng/mL)	AUC_{0-4h} (ng h/mL)
C/C	G/G	4	1.98 ± 0.23	4.00 ± 0.30
C/C	G/A	1	2.05	4.55
C/T	G/G	1	2.08	3.83
C/T	A/T	3	1.39 ± 0.23[a]	2.99 ± 0.17[a]
T/T	G/T	2	1.58	3.13
T/T	T/T	4	1.44 ± 0.23[a]	3.34 ± 0.29

Note. C_{1h}, serum concentration of digoxin at 1 h after administration; AUC_{0-4h}, area under the serum concentration-time curve from time zero to 4 h; N, number of subjects.
The values are expressed as the mean ± SE.
[a] Statistically significant difference ($P < .05$) compared with C/C^{3435} and G/G^{2677} group.

ministration depended significantly on the genotype at positions 3435 or 2677, but not at position –129. Subjects with both C/C^{3435} and G/G^{2677} showed higher serum concentrations of digoxin than those with both T/T^{3435} and T/T^{2677}. We also examined the relevance of *MDR1* SNPs to MDR1 mRNA expression, and it has also been suggested that C3435T and G2677(A,T) mutations are related with a higher level of expression of MDR1 mRNA in duodenal biopsies in healthy Japanese subjects (9). These results could reasonably explain the present pharmacokinetic observations. However, much more research is needed to determine the therapeutic significance of these mutations. Digoxin shows the narrow therapeutic range of serum concentration, and the stratification of the patients based on the *MDR1* genotype might decrease the frequency of adverse events.

In summary, the results of the present study indicated significant linkage between the *MDR1* SNPs at positions 3435 and 2677 in the Japanese population, and it was suggested that subjects with both C/C^{3435} and G/G^{2677} genotype showed higher serum concentrations than those with both T/T^{3435} and T/T^{2677} genotype. Future studies will need to evaluate the phenotype and the genetic linkage of heterozygous genotypes at positions 3435 and 2677, since C/T^{3435} and G/T^{2677} heterozygous genotypes were the most predominant in Asian and Caucasian populations in each position. The findings described here will help to understand the genotype distribution and genetic linkage of *MDR1* C3435T and G2677(A,T) SNPs, and the functional relevance of these SNPs to digoxin pharmacokinetics in the Japanese population.

NOTATION

In this article, the genotype of *MDR1* gene could be defined as C/C^{3435}, for example, when the genotype at position 3435 was homozygous for the C-allele.

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