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Research Paper

MDR1 Haplotypes Conferring an Increased Expression of Intestinal CYP3A4 Rather than MDR1 in Female Living-Donor Liver Transplant Patients

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Purpose. This study investigated whether haplotypes in the multidrug resistance 1 (*MDR1*) gene had effects on mRNA expression levels of MDR1 and cytochrome *P*450 (CYP) 3A4, and on the pharmacokinetics of tacrolimus in living-donor liver transplant (LDLT) patients, considering the gender difference.

Methods. Haplotype analysis of *MDR1* with G2677T/A and C3435T was performed in 63 *de novo* Japanese LDLT patients (17 to 55 years; 44.4% women). The expression levels of MDR1 and CYP3A4 mRNAs in jejunal biopsy specimens were quantified by real-time PCR.

Results. Intestinal CYP3A4 mRNA expression levels (amol/μg total RNA) showed significantly higher values in women carrying the 2677TT-3435TT haplotype (median, 10.7; range, 5.92–15.2) than those with 2677GG-3435CC (3.03; range 1.38–4.68) and 2677GT-3435CT (median, 4.31; range, 0.07–9.42) (P=0.022), but not in men (P=0.81). However, *MDR1* haplotype did not influence mRNA expression levels of MDR1 nor the concentration/dose ratio [(ng/mL)/(mg/day)] of oral tacrolimus for the postoperative 7 days, irrespective of gender.

Conclusion. *MDR1* haplotype may have a minor association with the tacrolimus pharmacokinetics after LDLT, but could be a good predictor of the inter-individual variation of intestinal expression of CYP3A4 in women.

KEY WORDS: ABCB1; P-glycoprotein; small intestine; tacrolimus.

INTRODUCTION

P-glycoprotein (Pgp), encoded by the multidrug resistance 1 (*MDR1,* also known as *ABCB1*) gene, is expressed in several organs, including the intestine, liver, and kidney, and mediates the detoxification of numerous drugs (1–3). To date, more than 50 single nucleotide polymorphisms (SNPs) have been reported for the *MDR1* gene (4–6). Among them, the most frequently studied SNPs are the G2677T/A transversion (A893S) in exon 21 and the synonymous C3435T transition in exon 26. *MDR1* SNPs C3435T and G2677T/A have been shown to affect the expression of Pgp as well as cytochrome P450 (CYP) 3A4 (7,8). We previously demonstrated that 3435TT was associated with a lower expression of enterocyte CYP3A4 mRNA than 3435CC in living-donor liver transplant (LDLT) patients, mainly pediatric patients (7). On the other hand, Lamba *et al.* (8) found increased expression of enterocyte CYP3A4 in 2677TT genotype, although not significantly.

Conflicting results have also been reported for the association of *MDR1* SNPs with the pharmacokinetics of tacrolimus, a substrate of both Pgp and CYP3A4 (9–11). Kim *et al.* (12) showed that 3435TT had significantly higher dose-normalized tacrolimus concentrations than 3435CC in renal transplant patients. In contrast, others have shown that this SNP had no significant influence on tacrolimus dose requirements in renal transplant patients (11). Meanwhile, G2677T/A SNPs have been shown to influence blood concentrations of tacrolimus in pediatric heart transplant patients (10), but not in liver transplant patients (7).

Although there are discrepancies in these clinical findings, some studies have shown that G2677T/A and C3435T were linked at the *MDR1* gene (12,13), and it has been suggested that *MDR1* haplotype derived from G2677T/A and C3435T may be a more useful marker of Pgp activity than individual SNPs (14). Furthermore, the remarkable gender differences have been reported in the expression of CYPs and transporters (15,16).

In the present study, we examined whether *MDR1* haplotype derived from the G2677T/A and C3435T could affect the mRNA expression levels of MDR1 and CYP3A4 in the native intestine as well as the graft liver, and the pharmacokinetics of tacrolimus in LDLT patients, considering the influence of gender.

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ABBREVIATIONS: C/D, Concentration/dose; CYP, Cytochrome *P*450; LDLT, Living-donor liver transplantation; MDR, Multidrug resistance; Pgp, P-glycoprotein; SNP, Single nucleotide polymorphism.

Table I. Characteristics of Patients

Variables	All	Men	Women
No. of LDLT patients, *n*	63	35	28
Age, years	43.3±10.5	45.2±9.3	40.9±11.7
Body weight, kg	62.3±12.4	68.3±10.8	54.7±10.0
ABO blood group match (identical/compatible/incompatible), n	41/ 6 / 16	25 / 6 / 4	16 / 0 / 12
Primary disease, *n*			
Cirrhosis	43	30	13
Primary biliary cirrhosis	8	1	7
Primary sclerosing cholangitis	3	3	0
Biliary atresia	3	0	3
Fulminant hepatic failure	1	0	1
Others[a]	5	1	4
No. of donors, *n*	63	33	30
Donor age, y	43.3±10.5	40.7±13.5	47.3±10.7

Data are expressed as number or mean±SD
[a] The primary disease was Budd–Chiari syndrome, nonalcoholic steatohepatitis, somatostatinoma, Wilson, or Caroli disease

MATERIALS AND METHODS

Patients and Clinical Samples

This study included 63 *de novo* LDLT patients (all Japanese), aged 17 to 55 years, who were treated with tacrolimus (Prograf®, Astellas Pharma, Tokyo, Japan), and their corresponding donors (Table I). Both the patients and their donors, having first provided written informed consent, were enrolled consecutively between April 2004 and December 2007.

Clinical samples of the upper jejunum were obtained from a part of the Roux-en-Y limb for biliary reconstruction, and liver samples (2 mm cubic) were obtained from biopsy specimens for pathological testing of the graft at surgery (zero biopsy) (17). This study was conducted in accordance with the Declaration of Helsinki and its amendments, and was approved by *the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee*.

Quantitation of mRNA Expression

Clinical samples of the upper jejunum and liver were immediately frozen in liquid nitrogen and stored at -80°C until used (18). The mRNA expression levels of CYP3A4 and MDR1 were quantified as described previously (7,18). Briefly, total RNA was isolated from biopsy specimens of the intestinal mucosa and graft liver, using MagNAPure LC RNA Isolation kit II (Roche, Mannheim, Germany), and was reverse-transcribed by Superscript II® reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random primers (100 ng/reaction) and digested by RNase H (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster, CA, USA).

Genotyping

Genomic DNA was extracted from peripheral blood of transplant patients or donors with Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). Using this genomic DNA, the *MDR1* polymorphisms were detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method with specific primers (Table II), as described previously (7). *MDR1* haplotypes were analyzed using SNPAlyze ver. 5.0 (Dynacom, Chiba, Japan).

Dosage Regimen of Tacrolimus and Measurement of Tacrolimus Concentrations

The basic immunosuppressive regimen consisted of tacrolimus with low-dose steroids (18). Tacrolimus was administered orally at a dose of 0.075 mg/kg body weight every 12 h from the evening of postoperative day 1 (18,19). The target whole-blood trough concentration of tacrolimus was set at between 10 and 15 ng/mL during the first 2 weeks. Steroid treatment was started at graft reperfusion at a dose of 10 mg/kg, with a gradual reduction from 2 mg kg^{-1} day^{-1} to 0.3 mg kg^{-1} day^{-1} during the first 2 weeks after surgery. The dosage of tacrolimus was adjusted on the basis of whole-blood trough concentrations measured about 12 h after the evening dosage every day, by use of a semiautomated microparticle enzyme immunoassay (IMx®; Abbott, Tokyo, Japan) (19).

Statistical Analysis

Median values of the expression of MDR1 and CYP3A4 were compared among the haplotypes using the Kruskal–

Table II. Primer Sequences Used for Amplification of PCR Fragments

SNP	Primer sequence
Exon 21 G2677A	F: 5′-TGCAGGCTATAGGTTCCAGG-3′
	R: 5′-GTTTGACTCACCTTCCCAG-3′
Exon 21 G2677T	F: 5′-TGCAGGCTATAGGTTCCAGG-3′
	R: 5′-TTTAGTTTGACTCACCTTCCCG-3′
Exon 26 C3435T	F: 5′-TGTTTTCAGCTGCTTGATGG-3′
	R: 5′-AAGGCATGTATGTTGGCCTC-3′

Table III. Effects of *MDR1* Haplotypes (G2677T/A-C3435T) on the Expression of MDR1 and CYP3A4 in Native Intestine and Graft Liver

MDR1 haplotypes (G2677T/A-C3435T)	Native intestine (all recipients)			Graft liver (All donors)		
	n (%)	MDR1 mRNA	CYP3A4 mRNA	*n* (%)	MDR1 mRNA	CYP3A4 mRNA
GG-CC	6 (9.5)	0.23 (0.09–0.32)	3.8 (1.2–7.1)	10 (15.9)	0.84 (0.38–2.3)	70.6 (11.1–107.8)
GG-CT	2 (3.2)	0.09 (0.02–0.15)	3.3 (0.04–6.6)	1 (1.6)	2.9	129.6
GA-CC	9 (14.3)	0.19 (0.02–0.94)	4.8 (0.005–16.4)	7 (11.1)	0.79 (0.51–1.4)	56.7 (19.6–84.6)
GA-CT	1 (1.6)	0.27	4.0	3 (4.7)	0.42 (0.39–1.4)	54.1 (46.5–64.7)
GT-CC	2 (3.2)	0.33 (0.24–0.42)	14.7 (7.1–22.2)	3 (4.7)	0.97 (0.93–1.5)	132.0 (73.6–157.0)
GT-CT	24 (38.0)	0.24 (0.03–1.1)	4.1 (0.07–15.7)	18 (28.6)	0.89 (0.42–2.4)	55.5 (18.0–131.5)
GT-TT	1 (1.6)	0.05	2.4	1 (1.6)	1.1	40.3
AA-CC	1 (1.6)	0.12	0.93	1 (1.6)	0.60	31.6
AT-CC	0	–	–	2 (3.2)	0.53 (0.52–0.54)	35.5 (30.5–40.4)
AT-CT	5 (7.9)	0.12 (0.01–0.46)	4.3 (0.004–10.2)	4 (6.4)	0.92 (0.61–1.2)	64.7 (20.0–71.3)
TT-CT	1 (1.6)	0.84	22.7	3 (4.7)	0.88 (0.44–1.4)	70.5 (64.8–88.2)
TT-TT	10 (15.9)	0.29 (0.12–0.57)	6.3 (0.37–15.1)	10 (15.9)	0.96 (0.45–2.6)	43.9 (12.0–168.4)

Data are expressed as median (range). We excluded one intestinal sample (GG-CC) from the analysis for undetectable values in the mRNA expression

Wallis test, followed by the Dunn *post hoc* test for multiple comparisons. Data are expressed as the median and range or mean±SD, depending on type. For all analyses, $P<0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad PRISM, version 4 (GraphPad Software, San Diego, CA, USA).

RESULTS

Frequencies of MDR1 SNPs

Allele frequencies of the 2677G, 2677T and 2677A were 43.1%, 43.1% and 13.8% in recipients, and 43.1%, 42.5% and 14.4% in donors, respectively. For C3435T, the frequencies of the 3435C and 3435T were 56.2% and 43.8% in recipients, and 58.7% and 41.3% in donors, respectively. The frequencies of genotypes in both recipients (G2677T/A, $P=0.41$; C3435T, $P=0.80$) and donors (G2677T/A, $P=0.54$; C3435T, $P=0.93$) complied with Hardy-Weinberg equilibrium. Construction of haplotypes, via estimation of maximization, resulted in 4 major (GT-CT, TT-TT, GA-CC, and GG-CC) and 8 minor haplotypes (GG-CT, GA-CC, GA-CT, GT-CC, GT-TT, AA-CC, AT-CT, and TT-CT) (Table III).

Effects of *MDR1* Haplotypes on the mRNA Expression Levels of MDR1 and CYP3A4

We evaluated the relationships between the *MDR1* haplotypes (G2677T/A-C3435T) and the mRNA expression levels (amol/μg total RNA) of MDR1 as well as CYP3A4 (Table III). We excluded one intestinal sample (GG-CC) from the analysis for undetectable values in the mRNA expression. The TT-TT haplotype tended to have a higher mRNA expression of intestinal CYP3A4, but did not affect the expression levels of intestinal MDR1. Stratified by gender, 2677TT-3435TT haplotype in the native intestine conferred a significantly higher CYP3A4 mRNA expression levels (median, 10.7; range, 5.92–15.2) than 2677GG-3435CC (3.03; range 1.38–

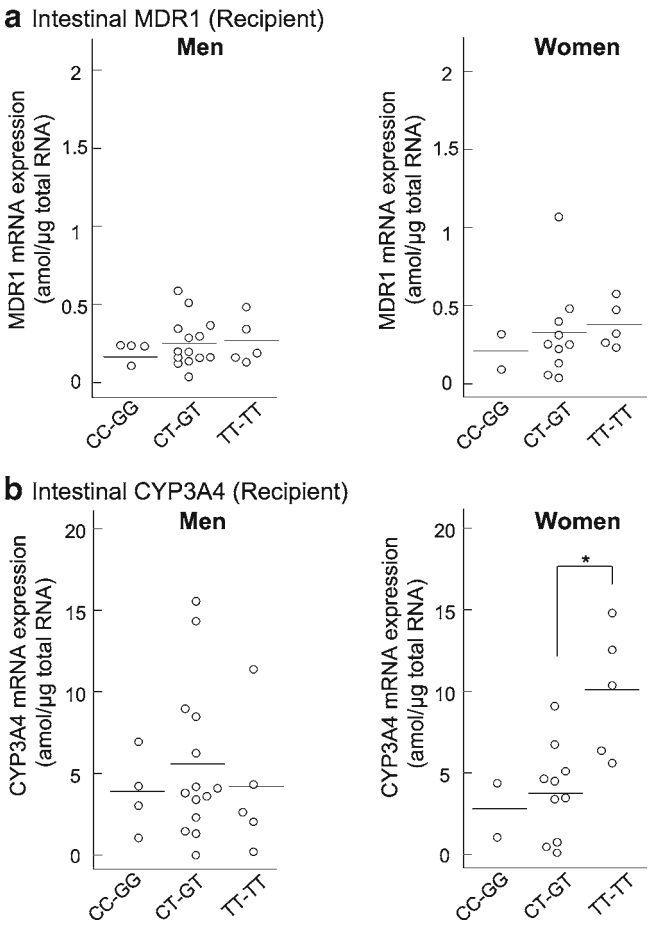

Fig. 1. Association between *MDR1* haplotypes (G2677T/A-C3435T) and mRNA levels of MDR1 (**a**) and CYP3A4 (**b**) in the native intestine (recipient). We excluded one intestinal sample (GG-CC) from the analysis for undetectable values in the mRNA expression. *$P<0.05$, significant difference between *MDR1* haplotype groups. The *bars* show the median mRNA expression levels in each haplotype.

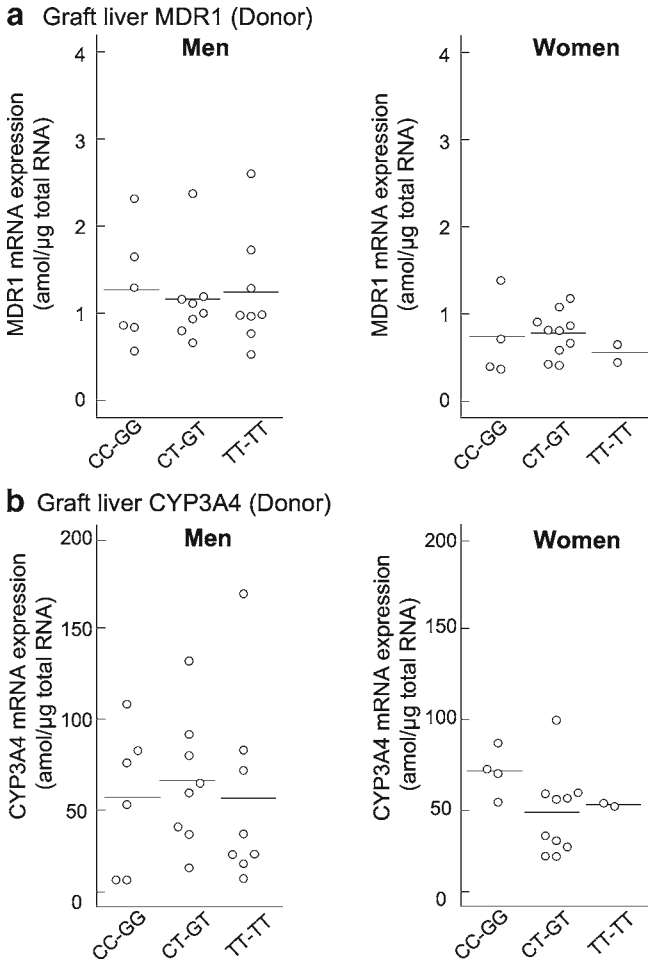

Fig. 2. Association between *MDR1* haplotypes (G2677T/A-C3435T) and mRNA levels of MDR1 (**a**) and CYP3A4 (**b**) in the graft liver (donor). The *bars* show the median mRNA expression levels in each haplotype.

4.68) and 2677GT-3435CT (median, 4.31; range, 0.07–9.42) haplotypes in women (P=0.022), but not in men (P=0.81) (Fig. 1 b). There were no significant differences in the mRNA expression levels of intestinal MDR1 among *MDR1* haplotypes (Fig. 1 a). In addition, we found no significant association between *MDR1* haplotypes and the expression of MDR1 and CYP3A4 in the graft liver, irrespective of gender (Fig. 2).

Effects of *MDR1* Haplotypes on the Pharmacokinetics of Tacrolimus

Next, to assess whether the *MDR1* haplotypes affect the pharmacokinetics of tacrolimus, we examined the concentration/dose (C/D) ratio of tacrolimus in LDLT patients for the first week after the surgery (Table IV). There was no significant difference in the C/D ratio of tacrolimus among the *MDR1* haplotypes.

DISCUSSION

Previously, we analyzed 10 common SNPs, including G2677T/A and C3435T in the *MDR1* gene in 46 LDLT recipients aged 0.6 to 59.6 years, and found that individual SNPs did not relate to either the intestinal expression of MDR1 mRNA or the C/D ratio of tacrolimus (7). In the present study, we restricted analysis to subjects aged 17 to 55 years in a larger cohort, and focused on *MDR1* haplotypes for the most common SNPs, G2677T/A and C3435T, in the mRNA expression of MDR1 as well as CYP3A4, and the pharmacokinetics of tacrolimus in 63 LDLT recipients, with consideration of the gender difference. Our results revealed that 2677TT-3435TT haplotype had significantly higher levels of intestinal CYP3A4 mRNA than those with 2677GT-3435CT haplotype in women (P=0.022), but not in men (P=0.81). To our knowledge, this is the first study to reveal the female-specific effect of *MDR1* haplotype on the intestinal expression of CYP3A4 mRNA.

To date, more than 30 allelic variants in the *CYP3A4* gene have been identified, but low variant frequencies, often combined with a lack of functional consequences, indicate their limited contribution to the large inter-individual variations in CYP3A4 expression (20,21). Hirota *et al.* (22) demonstrated using liver tissues from eight Caucasians that the whole CYP3A4 gene was sequenced, but none of the SNPs in the 5'-flanking region and 3'-UTR of the *CYP3A4* gene was associated with differences in total CYP3A4 mRNA levels and testosterone 6β-hydroxylation capability. Therefore, some genetic markers in addition to SNPs in the *CYP3A4* gene could be useful to explore the large inter-individual variation of CYP3A4 content, including its enzymatic capacity.

Previous studies reported that *MDR1* genotypes were associated with mRNA expression levels of enterocyte CYP3A4 (7,8). For example, Lamba *et al.* (8) found that carriers of variant alleles of *MDR1* gene tended to have

Table IV. Effects of intestinal *MDR1* Haplotypes (G2677T/A-C3435T) on the C/D Ratio of Tacrolimus in LDLT Patients for the Period of 1–7 Days Post Transplantation

	All		Men		Women	
MDR1 haplotypes (G2677T/A-C3435T)	*n*	C/D ratio of tacrolimus [(ng/mL)/(mg/day)]	*n*	C/D ratio of tacrolimus [(ng/mL)/(mg/day)]	*n*	C/D ratio of tacrolimus [(ng/mL)/(mg/day)]
GG-CC	7	2.0 (1.5–10.8)	4	1.7 (1.5–10.8)	3	3.7 (1.8–6.9)
GT-CT	24	4.2 (1.3–18.7)	14	3.6 (1.3–17.6)	10	4.7 (2.3–18.7)
TT-TT	10	3.9 (2.1–12.6)	5	2.5 (2.1–4.3)	5	8.6 (3.4–12.6)
P-value		0.37		0.51		0.36

Data are expressed as median (range)

increased expression of enterocyte CYP3A4, in the analysis of three separate cohorts (n=20, 27, and 10, respectively). However, their intestinal study populations were too small to detect differences among the *MDR1* polymorphisms by gender. Because the remarkable gender differences have been reported in the expression of CYPs and transporters (15,16), we hypothesized that the expression of CYP3A4 associated with *MDR1* genotypes would differ by gender. In the present study, we showed that *MDR1* G2677T/A-C3435T haplotypes significantly influenced the intestinal expression in women (Fig. 1). These results suggest that the *MDR1* haplotype may be a useful predictor of the inter-individual variability of the intestinal expression of CYP3A4 and the extent of some CYP3A4-mediated drug interactions in women.

The exact mechanism by which *MDR1* G2677T/A-C3435T haplotypes influence CYP3A4 expression in women remains unknown. In our results, we observed no significant difference in MDR1 mRNA expression among *MDR1* G2677T/A-C3435T haplotypes (Fig. 1 a), but it has been shown that "silent" polymorphisms (in particular, C3435T) in the *MDR1* gene can alter Pgp conformation and substrate specificity, especially when no change in MDR1 mRNA and protein levels has been reported (23). Furthermore, *in vitro* studies showed that G2677T/A-C3435T haplotypes can reduce the activity of Pgp (14). Therefore, reduced function of Pgp could possibly lead to high intracellular concentrations of endogenous regulators such as sex-steroid hormones, regulating the expression of CYP3A4 (24–27). In contrast, we found no significant differences in the hepatic expression of CYP3A4 among *MDR1* haplotypes for G2677T/A-C3435T in women. These opposing effects of the same haplotypes on hepatic and intestinal mRNA expressions of CYP3A4 in women could be partly due to differences in the underlying mechanism between the two types of organ.

Furthermore, we found that intestinal *MDR1* haplotypes had no effects on the C/D ratio of tacrolimus in LDLT patients (Table IV). Some studies have reported that 2677T or 3435T alleles affected the pharmacokinetics of tacrolimus in Caucasians (9,28,29), while others demonstrated contrary results in Asians (7,30). Similar discrepancies have been observed for digoxin (31). These might reflect disparities in different frequencies of *MDR1* haplotypes in different ethnicities (32,33). The 2677TT-3435TT haplotype is found in 42% of Caucasians and 8% of African-Americans (34), while 15.9% in the present Japanese population (Table III). Based on the present results and these previous findings, the *MDR1* haplotype is suggested to have a minor effect on the pharmacokinetics of tacrolimus in Asians compared with Caucasians.

CONCLUSION

In conclusion, the *MDR1* haplotype derived from G2677T/A and C3435T was significantly associated with intestinal CYP3A4 mRNA expression in women, but not in men, suggesting that it could be a good marker to predict the basal mRNA level of intestinal CYP3A4 in women. However, this effect was not observed for the pharmacokinetics of tacrolimus in LDLT patients. Therefore, extensive clinical pharmacokinetic studies are necessary to elucidate this effect on other drugs which are CYP3A4 substrates, in consideration of gender-differences in pharmacokinetics.

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Disclosures None.

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