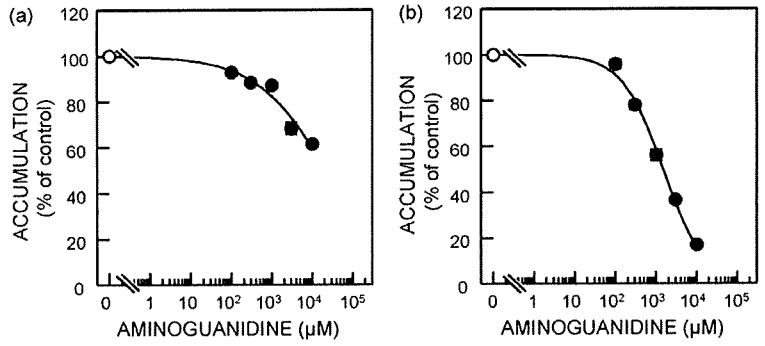

Fig. 8. Effects of aminoguanidine on [^{14}C]metformin transport by hOCT1 (a) and hOCT2 (b). HEK 293 cells transfected with hOCT1 and hOCT2 were incubated at 37 °C for 2 min with 10 μM [^{14}C]metformin (pH 7.4) in the absence (open circle) or presence of aminoguanidine (closed circle). Each point represents the mean ± S.E. of three independent experiments.

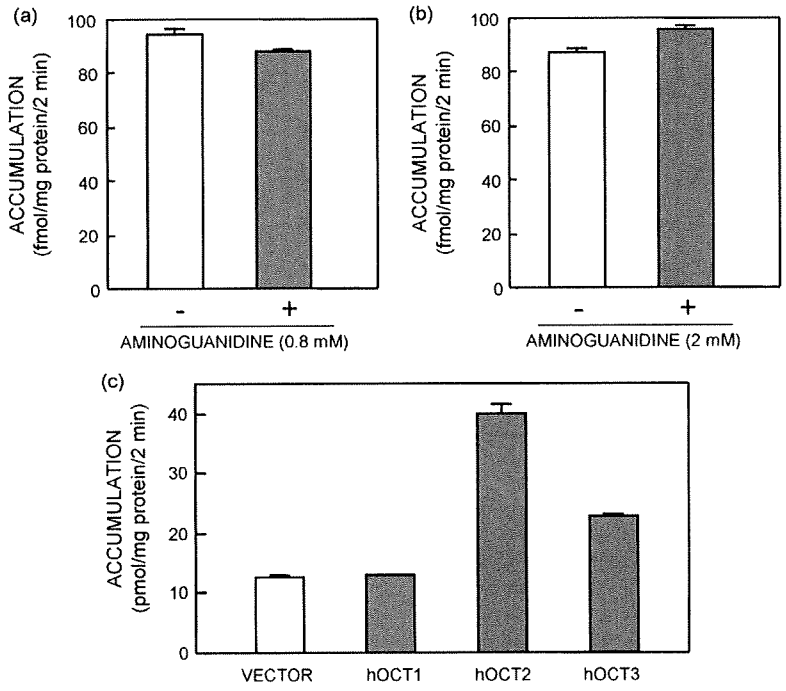

Fig. 9. Influence of *cis*-inhibition (a) and *trans*-stimulation (b) of aminoguanidine on the [^{3}H]MPP transport by hOCT3, and [^{14}C]aminoguanidine transport by hOCT1, hOCT2 and hOCT3 (c). (a) HEK293 cells transiently expressing hOCT3 were incubated at 37 °C for 2 min with 13.7 nM [^{3}C]MPP (pH 7.4) in the absence (−) or presence (+) of aminoguanidine (0.8 mM). (b) HEK293 cells transiently expressing hOCT3 were incubated for 2 min at 37 °C with 13.7 nM [^{3}H]MPP after preincubation with incubation medium (−) or incubation medium containing aminoguanidine (2 mM) (+) for 30 min at 37 °C. (c) HEK293 cells transfected with empty vector, hOCT1, hOCT2 or hOCT3 were incubated for 2 min at 37 °C with 5 μM [^{14}C]aminoguanidine (pH 7.4). Each column represents the mean ± S.E. of three monolayers.

examined in comparison with hOCT2 (Fig. 9a and b). The hOCT3-mediated uptake of [^{3}H]MPP was little affected by aminoguanidine in both conditions of *cis*-inhibition and *trans*-stimulation. In addition, the transport of [^{14}C]aminoguanidine by hOCT2 was the highest among three OCT isoforms (Fig. 9c).

4. Discussion

Previous reports suggested that guanidine and creatinine, which had a guadinino group, were predominantly transported by OCT2 rather than OCT1 [6,7]. We tested the hypothesis that the guanidino group was a decisive factor in being recognized by hOCT2, but could not found such selectivity simply by this group. At the same time, we discovered that aminoguanidine was a new superior substrate for hOCT2 than hOCT1.

Several guanidine compounds were reported to accumulate in blood with renal insufficiency, some being described as uremic toxins [9–14]. Guanidinosuccinic acid and methylguanidine had the two highest scores for the uremic concentration (C_U)/normal concentration (C_N) ratio, and there were also significant differences between the C_U and C_N of guanidine and creatinine [14]. The plasma concentrations of many cationic drugs increase with renal failure. It has been considered that the tubular secretion of organic cations is impaired and the elevated plasma level of alpha1 acid glycoprotein prevents the renal excretion in renal failure [21–25]. Based on the present results, it is also possible that the uremic guanidine compounds inhibit the excretion of cationic drugs mediated by hOCT.

Fig. 3 shows the relationship between the inhibitory patterns and the $C\log P$ values of guanidine compounds. In guanidine

compounds, hydrophobicity was not the major factor in determining the affinity for hOCT as it was, for example, in n-tetraalkylammonium [5,26,27].

In the *trans*-stimulation study, we showed that the [^{14}C]TEA uptake by hOCT2, but not hOCT1, were increased by preincubation with unlabeled guanidine, methylguanidine, creatinine, aminoguanidine and phenylguanidine. Possibly, these compounds are transported by hOCT2 and the dysfunction of hOCT2 with renal failure decreases the excretion of guanidine, methylguanidine, and creatinine as uremic toxins.

Because the three uremic guanidine compounds, guanidinosuccinic acid, methylguanidine and guanidinovaleric acid inhibited [^{14}C]TEA uptake by hOCT1 as well as hOCT2, the pharmacokinetics of the cationic drugs may be affected in the patients with renal failure. The [^{14}C]TEA uptake by hOCT1 was *trans*-stimulated by guanidinovaleric acid, suggesting the hOCT1-mediated transport of guanidinovaleric acid compensating the impaired renal function. It might relate to the fact that the serum level of guanidinovaleric acid in the patients with renal insufficiency was similar to normal values [10,13].

Among 14 guanidine compounds, aminoguanidine was found to be a selective substrate for hOCT2 compared to hOCT1 and hOCT3. A guanidine compound agmatine (1-amino-4-guanidobutane) was reported as a substrate for hOCT2 and hOCT3, but not for hOCT1 [28], while guanidine was transported by rOCT2, but not by rOCT1 and hOCT3 [6]. Therefore, aminoguanidine as well as agmatin and guanidine can be a good probe to examine the transport activity of hOCT2 in comparison with hOCT1 and hOCT3.

The apparent affinity of aminoguanidine for hOCT2 was similar to that of creatinine (K_m = 4.0 mM) [7] and lower than that of metformin (K_m = 1.4 mM) [19]. Aminoguanidine, which inhibits many diabetes-related complications, remains under therapeutic testing [16,17,29]. Because aminoguanidine was excreted into urine by tubular secretion as well as glomerular filtration and hOCT2 was the most abundant organic cation transporter in the basolateral membranes of human kidney [3,15], the secretion of aminoguanidine may be predominantly mediated by hOCT2.

In ACTION I trial (*A* Clinical *T*rial *I*n *O*vert *N*ephropathy of Type *1* Diabetics), which included patients with type 1 diabetes mellitus [17], aminoguanidine reduced significantly secondary measures of outcome such as proteinuria and had additional effects on diabetic retinopathy and circulating lipid levels. However, the reduction in the primary end point of time to doubling of the serum creatinine concentration was not statistically significant. Although creatinine clearance is often used for the estimation of GFR, creatinine is also excreted via tubular secretion mediated by hOCT2 [7,30,31]. Aminoguanidine might inhibit the transport of creatinine by hOCT2 and increase the serum concentration of creatinine without inducing renal impairment. Therefore, the other parameters whose elimination was unaffected by aminoguanidine should have been used.

Although creatinine and metformin were also excreted into urine through transport by hOCT2 [7,19], their IC_{50} values for aminoguanidine uptake by hOCT2 (creatinine, 42.4 mM; metformin, 2.37 mM) were much higher than the physiological concentrations of creatinine (about 45–85 μM for male and 30–60 μM for female) and metformin (about 15–25 μM) (Table 2, Fig. 7) [24,32–34]. Therefore, the transport of aminoguanidine mediated by hOCT2 is not likely to be affected by creatinine and metformin, and diabetic patients whose plasma creatinine concentrations are increased or who use metformin may be able to use aminoguanidine safely. It was reported that the maximum aminoguanidine concentration was only 40 μM, during the interdialytic period [15]. It is also probable that aminoguanidine has little effect on the transport of metformin mediated by hOCT2, at the physiological concentrations (Fig. 8).

In this study, we demonstrated that many guanidine compounds examined had relatively equal affinity to hOCT1 and hOCT2 and could not found the selectivity for hOCT2 simply by guanidino group. Among guanidine compounds, we newly discovered that aminoguanidine had greater affinity for hOCT2 than hOCT1, in addition to guanidine and creatinine. Therefore hOCT2 could function as a transporter for aminoguanidine at the basolateral membranes of renal proximal tubules. These findings will be helpful to elucidate the specificity of hOCT2, and clarify the pharmacokinetics of aminoguanidine.

Acknowledgements

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ORIGINAL ARTICLE

Identification of multidrug and toxin extrusion (MATE1 and MATE2-K) variants with complete loss of transport activity

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H^+/organic cation antiporters (multidrug and toxin extrusion: MATE1 and MATE2-K) play important roles in the renal tubular secretion of cationic drugs. We have recently identified a regulatory single nucleotide polymorphism (SNP) of the *MATE1* gene (−32G>A). There is no other information about SNPs of the *MATE* gene. In this study, we evaluated the functional significance of genetic polymorphisms in *MATE1* and *MATE2-K*. We sequenced all exons of *MATE1* and *MATE2-K* genes in 89 Japanese subjects and identified coding SNPs (cSNPs) encoding MATE1 (V10L, G64D, A310V, D328A and N474S) and MATE2-K (K64N and G211V). All the variants except for MATE1 V10L showed significant decrease in transport activity. In particular, MATE1 G64D and MATE2-K G211V variants completely lost transport activities. When membrane expression level was evaluated by cell surface biotinylation, those of MATE1 (G64D and D328A) and MATE2-K (K64N and G211V) were significantly decreased compared with that of wild type. These findings suggested that the loss of transport activities of the MATE1 G64D and MATE2-K G211V variants were due to the alteration of protein expression in cell surface membranes. This is the first demonstration of functional impairment of the MATE family induced by cSNPs.
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Keywords: cSNP; H^+/organic cation antiporter; MATE1; MATE2-K; pharmacogenomics; polymorphism; transporter

INTRODUCTION

In the proximal tubules of the mammalian kidney, organic ion transporters limit or prevent the toxicity of organic anions and cations by actively secreting these substances from the circulation into the urine.[1–4] Among human organic ion transporters located at the basolateral membranes, organic cation transporter 2 (OCT2), organic anion transporter 1 (OAT1) and OAT3 were isolated a decade ago, and have been well characterized as key transporters to regulate the renal handling of ionic drugs.[4,5] In contrast, the molecular functions of apical transporters have been only recently characterized. For example, multidrug resistance-associated protein 4 (MRP4) was demonstrated to be responsible for the renal elimination of antiviral drugs,[6] diuretics[7] and cephalosporin antibiotics.[8] Human orthologs of the multidrug and toxin extrusion (MATE) family, members of which confer multidrug resistance on bacteria, were identified most recently,[9,10] and named MATE1 (SLC47A1) and MATE2-K (SLC47A2). Both transporters are expressed mainly in the renal brush border membranes, and are able to transport tetraethylammonium (TEA) utilizing an oppositely directed H^+ gradient as a driving force,[11] indicating that MATE1 and MATE2-K are H^+/organic cation antiporters. These findings have improved the molecular understanding of the transcellular transport of ionic drugs in the renal tubules.

It is widely recognized that there is a large variation in the responses to drugs among individuals. Many enzymes involved in drug metabolism, such as cytochrome *P*450 and uridine diphosphate-glucuronosyltransferase are known to be polymorphic and have been associated with variations in blood concentrations of drugs.[12] In addition to drug-metabolizing enzymes, the clinical significance of genetic variation of drug transporters has been demonstrated.[13] For example, polymorphisms of *SLCO1B1*, which encodes the organic anion transporting polypeptide 1B1 to mediate the hepatic uptake of pravastatin, contribute to the interindividual variability in the disposition of pravastatin.[14] Recent studies of OCT have demonstrated that polymorphisms of the *OCT1* gene in Caucasians and the renal *OCT2* gene in Koreans are responsible for the interindividual differences in the therapeutic efficacy and pharmacokinetics of metformin, an anti-diabetic agent.[15–17]

Metformin showed large interindividual variation in renal clearance, and a potential genetic contribution by the renal transporter was speculated.[18] Because metformin is also a superior substrate for

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MATE1 and MATE2-K,[10,19] polymorphisms of *MATE1* and *MATE2-K* genes may be involved in the interindividual difference in the renal clearance. We have recently identified a single nucleotide polymorphism (SNP) in the promoter region of *MATE1* (−32G>A), which causes a decrease in Sp1 binding and promoter activity of approximately 50%.[20] However, other genetic information for these transporters, especially the polymorphisms in the coding region, and their effect on functional properties, have not been well evaluated. In this study, therefore, we screened for polymorphisms in all exons of *MATE1* and *MATE2-K* genes, and examined their transport activities by *in vitro* transient expression system.

MATERIALS AND METHODS

Materials

[^{14}C]TEA bromide (2.035 GBq mmol^{-1}) and [^{14}C]metformin (1.998 GBq mmol^{-1}) were obtained from American Radiolabeled Chemicals Inc. (St Louis, MO, USA) and Moravek Biochemicals Inc. (Brea, CA, USA), respectively. All other chemicals used were of the highest purity available.

Identification of SNPs of *MATE1* and *MATE2-K* genes

Genomic DNA was isolated from peripheral blood from 89 Japanese subjects with renal diseases using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Genotyping was investigated by direct sequencing. PCR primers were designed to span all 17 exons of *MATE1* and *MATE2-K* (GenBank accession number NT_010718) (Table 1). The PCR conditions were 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and then a final extension at 72 °C for 10 min, except for MATE1 exon 1. The condition for MATE1 exon 1 was 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s and 68 °C for 3 min, and then a final extension at 68 °C for 3 min. The PCR products were sequenced using a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan). This study was conducted in accordance with the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine. All subjects gave their written informed consents.

Construction of non-synonymous variants of MATE1 and MATE2-K

MATE1 and MATE2-K cDNA were excised from MATE1/pcDNA3.1 and MATE2-K/pcDNA3.1,[10] and were subcloned into pcDNA3.1/nV5-DEST (Invitrogen, Carsbad, CA, USA) to yield nV5-MATE1 and nV5-MATE2-K. Non-synonymous variants were constructed by the site-directed mutagenesis of nV5-MATE1 and nV5-MATE2-K, using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the primers listed in Table 2. The nucleotide sequences of these constructs were confirmed using a multicapillary DNA sequencer RISA384 system (Shimadzu).

Transport studies

HEK293 cells (ATCC CRL-1573; American Type Culture Collection) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Sigma Chemical Co., St Louis, MO, USA) with 10% fetal bovine serum (Invitrogen) in an atmosphere of 5% CO_2 and 95% air at 37 °C. cDNA plasmid transfection (Figure 2: 25 ng; Figure 3: 100 ng; Figure 6: 100 ng for MATE1 and 200 ng for MATE2-K) and cellular uptake of [^{14}C]TEA and [^{14}C]metformin were reported earlier.[11,19,21,22]

Table 1 Primers used for direct sequencing

Gene	*Location*	*Forward primer (5′ to 3′)*	*Reverse primer (5′ to 3′)*	*Amplified length (bp)*
MATE1	Exon 1	CGCAGTGGTGCAGAGAGAGGTGCAA	AGTCACCCGCGGAGGCAGAAATCAC	451
	Exon 2	AAGGTGGCAGAGGCTCACTGAAGTT	TCTGTGTAGGTTTCAGCCACTACAT	339
	Exon 3	TGAAGGAGGAGCTTTGCAGGCTCTT	CCTGCCAGTGGAGCTCTTCCATCTA	248
	Exon 4	CTTTGTGTGGCACAATTGAAGGCTT	CACCCAGACAGGATAATCTTTCCGT	303
	Exon 5	CTTCTGCCTAACTTTCCCTGGAAAC	CTGAGCTCACAGATATGGTGGCTAC	192
	Exon 6	CTGCCGTGTGACCTCACTTCTGTGT	GGTCCCTGGTCCTGGAGTATCTTCA	208
	Exon 7	GCCTGTGTGTGCTTGGGTAGCAGAA	CGCATGGACACAAGAACCAGCTGAA	279
	Exon 8, 9, 10	ATGAGTCTCCCCTCCTCACTGAGTT	TGCCTGTGCTCATCCATAGACTCTT	633
	Exon 11, 12	ATGAGGCTGCTTCTCTGCACGTGTT	CAGCAATGTTTCTGAACAGCCTGAT	481
	Exon 13	CCACTGCGCCTAGCCAGAAAGCTAT	CCCTCCTCTCAGCTGAAATTTACCA	224
	Exon 14	CTCGGGAGATGGGAGTGTTTCAAGA	AAGACCCGTGTGCTCCGACGGTCAT	276
	Exon 15	CTCCACCTCAGCCATGAAAGCAGAT	AGGGAGAGCCAGATCAGATCCTGTT	289
	Exon 16	TGGCTTGGCTCTTCCTAAACTAGGT	TAGCAGCAAATCTAGCTGTGTCTCA	258
	Exon 17	CTCTCCACTATTAGCACATATTCCTT	ATCCATGGGCACACCTGAATGACAT	436
MATE2-K	Exon 1	CTCATCCCACAAGTTGCCATGGTAG	GCACATTTCTGGATCCTGCCTGCAA	369
	Exon 2	CCTCAAAGCTGGAGAGGCCTGTCTT	GGCTGTGTCTTCCCATCCCTGACCA	297
	Exon 3	GGCACACAGCACATGAGGCTGCTGA	TGCCATCTCCATGGCACCTGTGGAA	292
	Exon 4	TCAGGAAGGCCGCTGTGCCATTACA	TGAGGGCTGGGCATCTTCAGGGTTT	400
	Exon 5	GAGGTTTCACAGTCCTGGCTGAGAC	AGGGATCTTCCGCAGCAGATAGAGT	262
	Exon 6	CAATCTGGGGTACTATGTCCTGGAA	GCTGGTTCACAGATGGTGGAGAGAA	252
	Exon 7	CCTTCTCTCCACCATCTGTGAACCA	CAGGATGGTGACTGATCTGTCTCCA	422
	Exon 8	CCCTGGTTGAGTCTGATCCCAGGAT	TCCAACAGGCTCTACTGCACCCTCT	351
	Exon 9	AATGCCCAGTGCCTGAGCCTGCTAA	TGAGGGCCTGGCCAGTGAAGCTGGAA	403
	Exon 10	TCCCCAAAGCAAAGCAGCGTCCTGT	GGGAGACAGAGATAGCTTCAGGTGA	254
	Exon 11	CTCTTACACTGCATGCCTGAGATCT	TCACAGCAGCAGGGAAGGAGTGAGT	488
	Exon 12	GGCTGGGCTGACTTGCACTGACATA	CCCAGCACTGAGCCAGGAATGTGAT	275
	Exon 13	CTCTGGGCTAGCAGTGCCAGTTACA	CAAGTTCATCCTCACAGCCCTGCGA	317
	Exon 14, 15	TGCCATGCGAATGGCTTAGCACAGT	CTGGGCATTTCTGGCTGAGTAGTCA	483
	Exon 16	CAGTGAAGGGGTGAACTGTTGAGCT	CACAGAGGGCAGACAAGAGCAACAT	225
	Exon 17	CACAGCCAGGTGGTTAACCTAGGTT	ACCTGCACTAGACCCCATTGGTGTT	416

Table 2 Primers used for site-directed mutagenesis

Gene	*Name*	*Direction*	*Sequence (5′-3′)*	*Position*
			Primers for the site-directed mutagenesis	
MATE1	V10L	F	GGAGCCCGCGCCA**T**TGCGCGGAGGCC	15/40
		R	GGCCTCCGCGCA**A**TGGCGCGGGCTCC	40/15
	G64D	F	CCGTGTTCTGTG**A**CCACCTGGGCAAGCTGG	179/208
		R	CCAGCTTGCCCAGGTGG**T**CACAGAACACGG	208/179
	A310V	F	CATGGTCCCTG**T**AGGCTTCAGTGTGGCTGCC	918/948
		R	GGCAGCCACACTGAAGCCT**A**CAGGGACCATG	948/918
	D328A	F	CGCTCTGGGTGCTGGAG**C**CATGGAGCAGG	966/994
		R	CCTGCTCCATG**G**CTCCAGCACCCAGAGCG	994/966
	N474S	F	GGCTCAGGTACACGCCA**G**TTTGAAAGTAAACAACGTGCC	1404/1442
		R	GGCACGTTGTTTACTTTCAAA**C**TGGCGTGTACCTGAGCC	1442/1404
MATE2-K	K64N	F	GGCACCTGGGCAA**T**GTGGAGCTGGC	179/203
		R	GCCAGCTCCAC**A**TTGCCCAGGTGCC	203/179
	G211V	F	GGGGGTCAGGG**T**CTCCGCCTATGCC	621/645
		R	GGCATAGGCGGAG**A**CCCTGACCCCC	645/621

Abbreviations: F, forward; R, reverse.
Mutations introduced into the oligonucleotides are shown in bold.

Table 3 cSNPs of the *MATE1* and *MATE2-K* in 89 Japanese subjects

Location	*SNP*	*dbSNP (NCBI)*	*Effects*	*Allelic frequency (%)*	*Genotype (n)*
MATE1					
Exon1	28G>T	ss104806851	V10L	2.2	G/G 85, G/T 4, T/T 0
Exon1	33C>T	ss104806852	R11R	0.6	C/C 88, C/T 1, T/T 0
Exon1	126T>C	ss104806853	A42A	0.6	T/T 88, T/C 1, C/C 0
Exon2	191G>A	ss104806854	G64D	0.6	G/G 88, G/A 1, A/A 0
Exon8	708C>T	ss104806855	L236L	9.6	C/C 74, C/T 13, T/T 2
Exon11	929C>T	ss104806856	A310V	2.2	C/C 85, C/T 4, T/T 0
Exon11	983A>C	ss104806857	D328A	0.6	A/A 88, A/C 1, C/C 0
Exon16	1421A>G	ss104806858	N474S	0.6	A/A 88, A/G 1, G/G 0
MATE2-K					
Exon2	192G>T	ss104806859	K64N	0.6	G/G 88, G/T 1, T/T 0
Exon2	207G>A	ss104806860	S69S	5.6	G/G 79, G/A 10, A/A 0
Exon4	345C>A	ss104806861	G115G	36.5	C/C 37, C/A 39, A/A 13
Exon8	632_633GC>TT	ss104806862	G211V	1.7	GC/GC 86, GC/TT 3, TT/TT 0
Exon10	885C>T	ss104806863	Y295Y	48.9	C/C 25, C/T 41, T/T 23

Abbreviations: cSNP, coding single nucleotide polymorphism; MATE, multidrug and toxin extrusion.

Cell surface biotinylation

Cell surface biotinylation was performed according to our earlier methods[22] with some modifications. HEK293 cells were grown on poly-D-lysine-coated 12-well plates and transfected with MATE1 or MATE2-K cDNA plasmids (50 ng for MATE1 and 200 ng for MATE2-K). At 48 h after the transfection, cells were washed three times with 1 ml ice-cold phosphate-buffered saline with Ca and Mg (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$, pH 7.3) and then treated with 400 µl of membrane-impermeable biotinylating agent, sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA) (1.5 mg ml^{-1}) at 4 °C for 1 h. Subsequently, the cells were washed three times with 1 ml ice-cold phosphate-buffered saline with Ca and Mg containing 100 mM glycine and then incubated for 20 min at 4 °C with the same buffer to remove the remaining labeling agent. After being washed with phosphate-buffered saline with Ca and Mg, cells were disrupted with 400 µl of lysis buffer (10 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 and 1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), pH 7.4) at 4 °C for 1 h with constant agitation. Following centrifugation, 50 µl of streptavidin agarose beads (Pierce) was added to 300 µl of cell lysate and incubated for 1 h at room temperature to isolate the biotinylated membrane proteins.

Western blot analysis and quantification of band density

Isolated biotinylated membrane proteins were subjected to western blot analysis according to NuPAGE manufacturer's instructions (Invitrogen). Monoclonal anti-V5 antibody (Invitrogen) (1:2500 dilution) or Na^+/K^+-ATPase antibody (1:10 000 dilution; Upstate Biotechnology, Lake Placid, NY, USA) was used as the primary antibody. A peroxidase-conjugated anti-mouse IgG antibody was used for the detection of bound antibodies, and the blots were visualized by chemiluminescence on X-ray film. Quantification of band density was performed on scanned images using ImageJ, a public domain image-processing program (W Rasband, National Institute of Mental Health, Bethesda, MD, USA). The optical density of each lane was plotted, and the area under the curve was measured.

Statistical analysis

Kinetic parameter data were statistically analyzed with unpaired *t*-test compared with the values for the wild type. The other experimental data were statistically analyzed with the one-way analysis of variance followed by Dunnett's test.

RESULTS

Identification of *MATE1* and *MATE2-K* SNPs

All 17 exons of the *MATE1* and *MATE2-K* genes were sequenced to find SNPs in 89 Japanese subjects. In this study, eight *MATE1* SNPs and five *MATE2-K* SNPs were identified in the coding region (Table 3). The allelic frequencies for the non-synonymous SNPs ranged from 0.6 to 2.2%. Figure 1 shows the position of mutated amino-acid residues in the predicted secondary structure of MATE1 (a) and MATE2-K (b), respectively. Only Ala310 was localized in the transmembrane domain (TMD), and other amino-acid residues are located at the intra- or extracellular domains.

Transport studies of the MATE1 and MATE2-K variants

To assess the functional alterations caused by the non-synonymous SNPs of both genes, [^{14}C]TEA transport activity by the variants was evaluated by *in vitro* transient expression system. As shown in Figure 2a, [^{14}C]TEA uptake by the MATE1 G64D variant was completely abolished. Other MATE1 variants except for the MATE1 V10L variant also showed a significant reduction in [^{14}C]TEA transport activity, and the order of the remaining transport activities were as follows: wild type=V10L>N474S>D328A=A310V. [^{14}C]Metformin uptake by various variants was similar to [^{14}C]TEA uptake (Figure 2b). Both the MATE2-K variants showed significant decrease in [^{14}C]TEA and [^{14}C]metformin uptake, and the transport activity of MATE2-K G211V was completely abolished (Figures 3a and b).

Cell surface expression levels of the MATE1 and MATE2-K variants

To determine whether the reduced transport activity of these variants was due to the decreased expression of transporter proteins in the plasma membranes, cell surface biotinylation followed by western blot analysis was carried out. Among the MATE1 variants, the cell surface expression level of MATE1 G64D and D328A showed a decrease to approximately 10 and 20% compared with that of the wild-type MATE1 (Figure 4), which are well correlated with the reduction ratios of the transport activity for these variants (Figures 2a and b). Other MATE1 variants exhibited similar cell surface expression level with wild-type MATE1. In the MATE2-K, both the MATE2-K K64N and MATE2-K G211V variants showed a decrease to approximately 50 and 1% compared with that of the wild-type MATE2-K, respectively (Figure 5). These reduction ratios were well correlated with those of transport activities of both the MATE2-K variants (Figures 3a and b). These findings suggested that the low transport activities of MATE1 G64D, D328A and two MATE2-K variants were due to the alteration of protein expression in cell surface membranes.

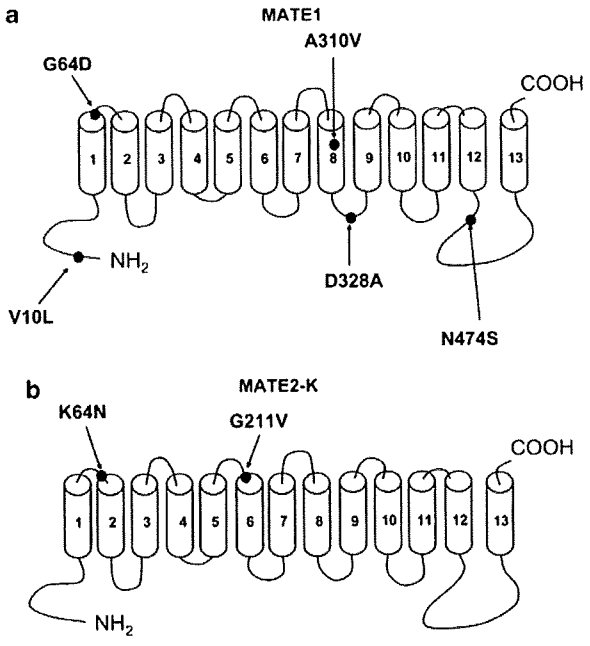

Figure 1 Locations of mutated amino-acid residues caused by non-synonymous single nucleotide polymorphisms (SNPs) in the secondary structure of multidrug and toxin extrusion 1 (MATE1) (**a**) or MATE2-K (**b**) protein. Amino-acid numbers are shown.

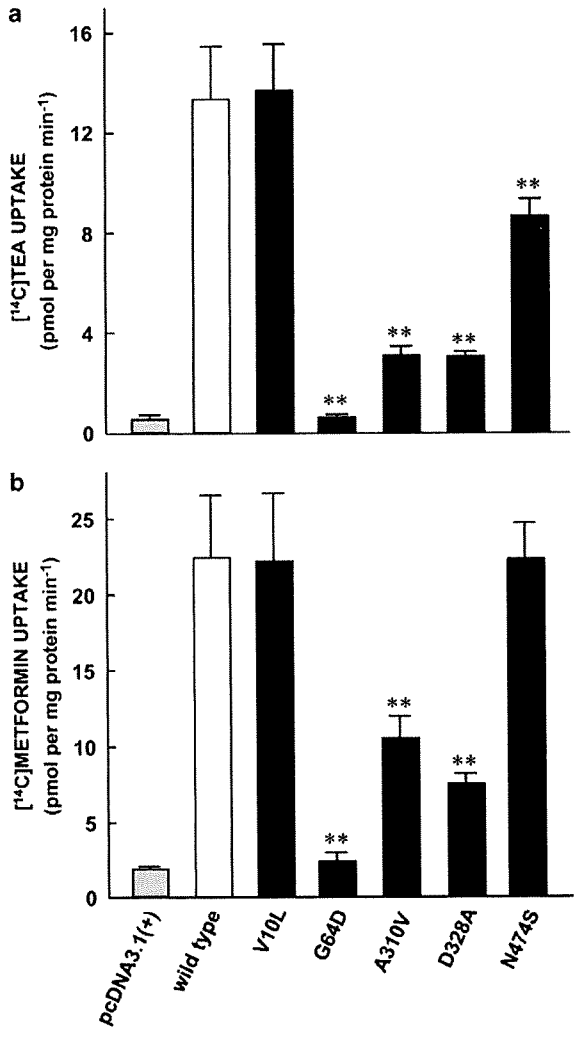

Figure 2 Uptake of [^{14}C]TEA (tetraethylammonium) (**a**) and [^{14}C]metformin (**b**) by HEK293 cells transiently expressing wild type or various multidrug and toxin extrusion 1 (MATE1) variants. The cells were preincubated with incubation medium (pH 7.4) in the presence of 30 mM ammonium chloride for 20 min. Then, the preincubation medium was removed, and the cells were incubated with 5 μM of [^{14}C]TEA or 10 μM of [^{14}C]metformin for 1 min at 37 °C. Each column represents the mean ± s.d. of six monolayers from two independent experiments. $^{**}P<0.01$, significantly different from the values for the wild type.

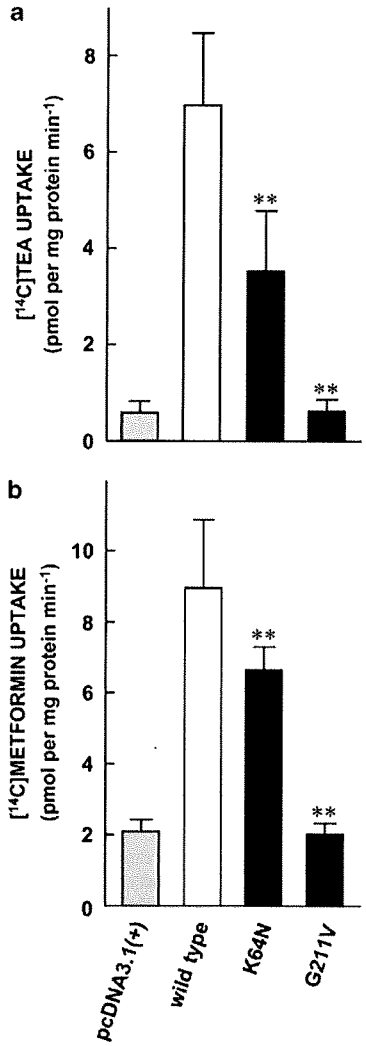

Figure 3 Uptake of [^{14}C]TEA (tetraethylammonium) (**a**) and [^{14}C]metformin (**b**) by HEK293 cells transiently expressing wild type or variants of multidrug and toxin extrusion 2-K (MATE2-K). The procedures are identical to those described in the legend of Figure 2. Each column represents the mean ± s.d. of six monolayers from two independent experiments. $^{**}P<0.01$, significantly different from the values for the wild type.

Comparison of functional characteristics between wild type and the variants of MATE1 and MATE2-K

To estimate kinetic parameters for [^{14}C]TEA uptake by several MATE1 and MATE2-K variants, concentration-dependent uptake was carried out (Figures 6a and b). The [^{14}C]TEA uptake by the MATE1 and MATE2-K variants exhibited saturable kinetics, following the Michaelis–Menten equation. The apparent maximal uptake velocity (V_{max}), Michaelis–Menten constant (K_m) and V_{max}/K_m values are summarized in Table 4. V_{max} values of MATE1 A310V, D328A and MATE2-K K64N were significantly decreased. K_m values of MATE1 A310V and N474S were significantly increased.

DISCUSSION

MATE1 and MATE2-K function as H^+/organic cation antiporters at the renal brush border membranes and play crucial roles in the renal handling of cationic drugs, such as cimetidine, metformin and oxaliplatin.[23] In this study, we identified five and two non-synonymous SNPs in their genes, all of which induced a reduction of transport activity except for MATE1 V10L. In MATE1, both the cell surface expression and transport activity of the G64D and D328A variants were significantly reduced to approximately 10 and 20% compared with that of the wild type. These findings suggest that reduced protein expression levels in the plasma membrane can account for the decrease in transport activity of MATE1 with G64D and D328A. Previously, we indicated that Cys63 of MATE1 plays an important role in substrate binding,[22] and Cys63 is the neighboring amino-acid residue of Gly64. Thus, regarding G64D, the change from the small side chain (Gly) to the bulkier and polar side chain (Asp) may inhibit the substrate binding and decrease transport activities, in addition to reduced protein expression levels in the plasma membrane.

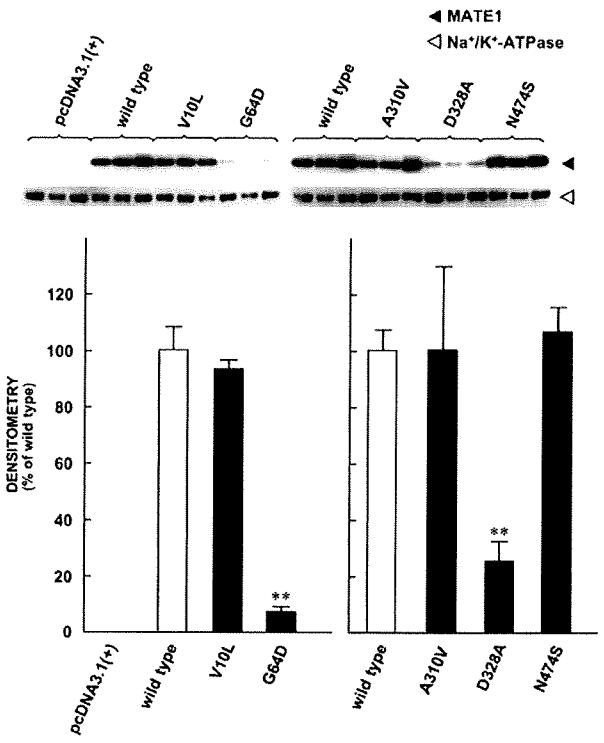

Figure 4 Western blot analysis of cell surface biotinylated proteins obtained from HEK293 cells transiently expressing wild type or variants of multidrug and toxin extrusion 1 (MATE1). Cell surface membrane fractions prepared by cell surface biotinylation were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (4–12%) and blotted onto polyvinylidene fluoride (PVDF) membranes. Each column represents the mean ± s.d. of three monolayers. $^{**}P<0.01$, significantly different from the values for the wild type.

On the other hand, N474S sustained a modest level of transport activity. The small impact of Asn474 on MATE1 function may be due to its position, in the intracellular loop between TMD12 and TMD13. Three-dimensional models of MATE1 will clarify the importance of these amino-acid residues.

In the MATE1 protein, Gly64 and Asn474 are conserved in the rat (AB248823), mouse (AAH31436) and rabbit (EF120627) orthologs, suggesting that these two amino-acid residues are essential. Ala310 of MATE1 is conserved in the rat ortholog only. The transport activity of A310V showed a decrease to approximately 20% compared with that of the wild type, though its membrane expression level was same as the wild type. Kinetic analysis indicated that K_m value for MATE1

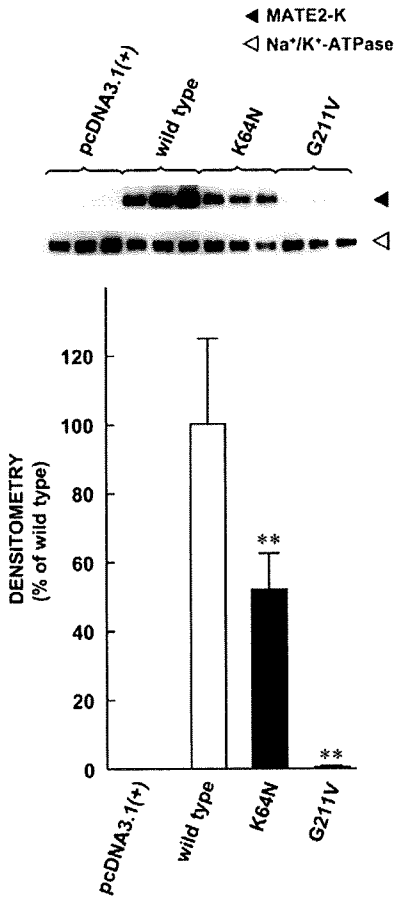

Figure 5 Western blot analysis of cell surface biotinylated proteins obtained from HEK293 cells transiently expressing wild type or variants of multidrug and toxin extrusion 2-K (MATE2-K). The procedures are identical to those described in the legend of Figure 4. Each column represents the mean ± s.d. of three monolayers. $^{**}P<0.01$, significantly different from the values for the wild type.

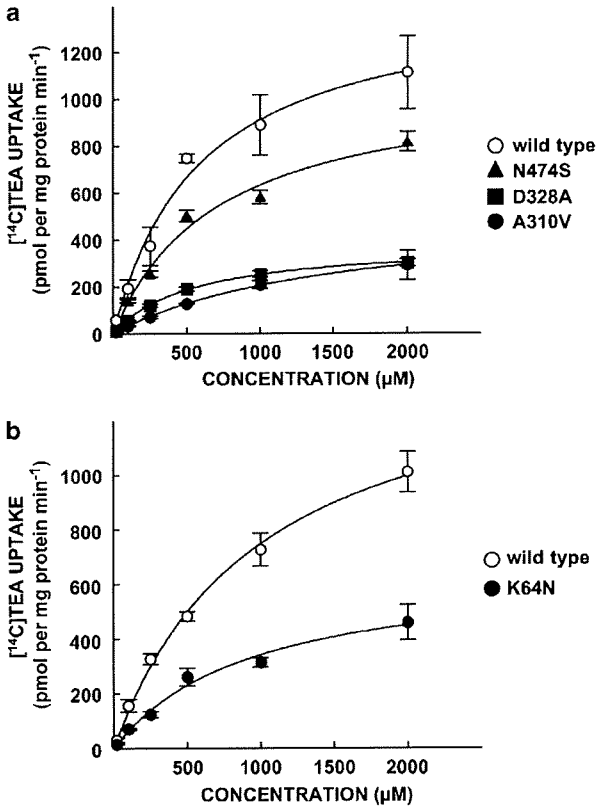

Figure 6 Concentration dependence of [^{14}C]TEA (tetraethylammonium) uptake by HEK293 cells transiently expressing wild type or variants of multidrug and toxin extrusion 1 (MATE1) (**a**) and MATE2-K (**b**). The figures show a specific uptake of [^{14}C]TEA obtained by subtracting the non-saturable components, which were estimated in the presence of 5 mM unlabeled compound. Each point represents the mean ± s.d. of three monolayers from a typical experiment.

A310V was significantly increased. These findings suggested that substitution of Ala for Val may inhibit the substrate binding or translocation because Ala310 was localized in the TMD.

In MATE2-K protein, Lys64 and Gly211 are conserved in the rabbit ortholog (EF121852). As for two MATE2-K SNPs, the alterations in transport activity were in accordance with the alterations in the cell surface expression of MATE2-K protein, indicating that the reduced function of MATE2-K K64N and MATE2-K G211V is mainly due to the decreased expression at the plasma membrane. Actually, it was demonstrated that V_{max} value of MATE2-K K64N was significantly decreased. These findings may provide important information to elucidate molecular mechanisms of membrane trafficking and stability of the MATE2-K protein in the plasma membrane.

There are several reports that investigated the cell surface expression level caused by coding SNPs (cSNPs) between HEK293 cells and tissues. For example, the human organic anion-transporting polypeptide 1B1 protein expression level was not changed by substitution of Leu643 to Phe in both transfected HEK293 cells and liver samples.[24] In this study, we examined the effect of cSNPs on cell surface expression level only *in vitro*. Our *in vitro* data are difficult to extrapolate to the case *in vivo* in renal proximal tubules; however, these data suggest that altered cell surface expression level may occur in individuals with MATE1 G64D, D328A and both the MATE2-K variants. Future studies on cell surface expression level of MATE1 and MATE2-K protein in human proximal tubules with these variants will elucidate whether *in vitro* data in this study are consistent with the case *in vivo*.

Metformin, a biguanide agent, is mainly excreted into the urine mostly through tubular secretion and shows large interindividual variation of renal clearance.[18] Recently, it has been demonstrated that SNPs of the hepatic *OCT1* gene in Caucasians and renal *OCT2* gene in Koreans are responsible for the interindividual differences in the therapeutic efficacy and pharmacokinetics of metformin.[15–17] On the other hand, Shikata *et al.*[25] reported that *OCT1* and *OCT2* polymorphisms contribute little to the clinical efficacy of metformin in Japanese. Previously, we demonstrated that metformin is a good substrate not only for OCT2 but also for MATE1 and MATE2-K.[19,26] Therefore, the SNPs of *MATE1* and *MATE2-K* genes identified in this study may be involved in the interindividual difference in the renal clearance of metformin in Japanese. However, as the allelic frequencies of *MATE1* and *MATE2-K* SNPs are not very high, these SNPs cannot fully account for the large interindividual variation in the renal clearance of metformin.

We reported that the kidney-specific expression of OCT2 is involved in the renal distribution and accumulation of the anticancer agent

Table 4 Kinetic parameters of [^{14}C]TEA uptake determined by HEK293 cells transiently expressing wild type or variants of MATE1 and MATE2-K

	V_{max} ± s.e. (nmol per mg protein min^{-1})	K_m ± s.e. (mM)	V_{max}/K_m ± s.e. (μl per mg protein min^{-1})
MATE1			
Wild type	1.94 ± 0.37	0.49 ± 0.05	4.10 ± 0.88
A310V	0.74 ± 0.17*	1.84 ± 0.38*	0.40 ± 0.03**
D328A	0.53 ± 0.06**	0.63 ± 0.04	0.84 ± 0.09*
N474S	1.36 ± 0.25	0.70 ± 0.06*	1.92 ± 0.20
MATE2-K			
Wild type	1.99 ± 0.44	1.39 ± 0.47	1.56 ± 0.14
K64N	0.73 ± 0.03*	0.79 ± 0.05	0.93 ± 0.08**

Abbreviations: MATE, multidrug and toxin extrusion; TEA, tetraethylammonium.
The values were calculated from four separate experiments performed in three monolayers.
*$P<0.05$, **$P<0.01$, significantly different from the values for the wild type.

cisplatin.[27,28] As there is little transport of cisplatin by MATE1 and MATE2-K, cisplatin is accumulated in the proximal tubular cells causing nephrotoxicity. A low-nephrotoxic platinum anticancer agent, oxaliplatin, was transported by OCT2 and MATE2-K,[27,28] suggesting that oxaliplatin does not accumulate in the renal proximal tubular cells. Therefore, loss of function of MATE2-K caused by cSNPs may lead to the accumulation of oxaliplatin in the kidney and the subsequent nephrotoxicity. Future study will be needed to clarify the clinical implications of the SNPs of both genes identified in this study.

In conclusion, five non-synonymous SNPs in the *MATE1* and two non-synonymous SNPs in the *MATE2-K* genes were identified in Japanese subjects for the first time. All of the mutated proteins except for MATE1 V10L showed a significant decrease in transport activity, and especially those of MATE1 G64D and MATE2-K G211V were completely abolished by the impairment of cell surface expression. These polymorphisms may affect the renal handling of various cationic drugs and cause drug-induced nephrotoxicity.

ACKNOWLEDGEMENTS

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臨床編 3
免疫抑制薬の動態と薬効・毒性

増田智先　乾 賢一

ますだ さとひろ，いぬい けんいち：京都大学医学部附属病院 薬剤部

「免疫」は，ウイルスなどの体内への侵入に対する生体の防御機構として，重要な役割を担っている。一方，自己と非自己を区別する仕組みが破綻すると，「自己免疫疾患」として理解される数多くの病状を示す。また，近年の目覚ましい発展をみせている臓器移植医療の領域では，非自己であるドナー由来の臓器を移植することから，拒絶反応や移植片対宿主病の抑制は治療の正否を決定する重要な要素と考えられる。これらの疾病に対する治療薬として，また臓器移植術後の拒絶反応抑制薬として，免疫抑制薬は使用される。

免疫抑制薬は，その作用機序に基づいていくつかのグループに分類される[1]。リンパ球の増殖を直接的に阻害するステロイド薬，リンパ球において盛んな *de novo* 核酸合成に拮抗する代謝拮抗薬（アザチオプリン，ミコフェノール酸モフェチル，メトトレキサート），リンパ球の増殖シグナルであるインターロイキン2（IL-2）産生の抑制を示すカルシニューリン阻害薬（シクロスポリン，タクロリムス），リンパ球の種々表面抗原を標的とした抗体などがあげられる。まず，これらの免疫抑制薬についてそれぞれの特徴を述べ，京都大学において経験を重ねてきたタクロリムスに関する知見を紹介したい。

ステロイド薬

経口投与されたステロイド薬はほぼ完全に消化管から吸収され，血漿中における蛋白結合率も高い[2,3]。リンパ球におけるステロイド薬の受容体はグルココルチコイド受容体（GR）であり，nuclear factor kappa B（NFκB）の活性化抑制などを介してリンパ球の活性化抑制，増殖抑制を示す。また，ステロイド薬は，肝臓において核内受容体（constitutive androstane receptor：CAR）と結合し，シトクロムP450（CYP）系の発現誘導を媒介することから，薬物の代謝速度の亢進につながる。すなわち，高用量のステロイド投与による併用薬物の動態変化，特に全身クリアランスの増大に注意を要する。

代謝拮抗薬

アザチオプリン（AZP）は，生体内で6-メルカプトプリン（6-MP）に代謝され，プリンヌクレオチドの生合成を阻害する。経口投与されたAZPの血漿中蛋白結合は30%程度，6-MPでは20%程度といわれている。肝臓において代謝を受け，その代謝物はほとんどが尿中へと排泄される[4]。

ミコフェノール酸モフェチルは，速やかに吸収された後加水分解されミコフェノール酸となり，TおよびB細胞における *de novo* プリン合成を阻害し，それに基づくリンパ球選択的な増

殖抑制効果を発揮する[5]。ミコフェノール酸は肝臓において，おもにUGT1A9によるグルクロン酸抱合反応を受け，胆管側膜のMRP2を介して胆汁中へと排泄される。したがって，MRP2に対する阻害能を有するシクロスポリンの併用例では，胆汁中ミコフェノール酸のグルクロン酸抱合体排泄量が低下する。腎臓移植後の拒絶反応発症との対応を評価した検討から，ミコフェノール酸は血中濃度-時間曲線下面積(AUC)と最も良い相関を示すこと，その薬効は血中イノシンモノリン酸デヒドロゲナーゼ（IMPDH）活性で推定可能なことが示されているが，ミコフェノール酸の血中濃度モニタリング（TDM）に対する保険算定はわが国においていまだ認められていない。

葉酸の誘導体であるメトトレキサートは，葉酸代謝系におけるジヒドロ葉酸還元酵素を特異的に阻害することによって，テトラヒドロ葉酸の生成を抑制し，DNA合成のみならずRNA，蛋白質合成の抑制を示す。ヒトでは，生体に投与されたメトトレキサートの約70%が未変化体として尿中へと排泄される。特に，近位尿細管側底膜に発現する有機アニオントランスポーターOAT1やOAT3によって上皮細胞内に取り込まれ，刷子縁膜側のトランスポーターを介して尿中へと排泄される。現在，メトトレキサートはMRP2やMRP4を介して尿細管分泌を受けると考えられているが，未同定トランスポーターとして注目される膜電位依存性有機アニオントランスポーターならびにアニオン交換輸送系の寄与も無視できない[6]。このようにメトトレキサートは，尿細管上皮細胞に濃縮的に取り込まれるため，その脂溶性の高さから尿細管管腔中での析出による尿管閉塞が重要な副作用としてあげられる。したがって，メトトレキサートは，血中濃度をモニターしながら用量調節することが推奨されている。

● カルシニューリン阻害薬

T細胞の脱リン酸化酵素であるカルシニューリンに対する特異的な阻害薬として，シクロスポリンとタクロリムスが使用される。シクロスポリンは，細胞内受容体であるシクロフィリンと結合し，カルシニューリン阻害活性を示す。一方，タクロリムスは白血球内FK506 binding protein 12 kd（FKBP1A）と結合し，シクロスポリンと同様にカルシニューリン阻害活性を示す。いずれも化学構造上まったく異なるにもかかわらず，薬理効果の作用機序や薬物動態の制御因子は類似すると考えられてきた。また，いずれの薬物も副作用として腎毒性や中枢毒性を示す。

シクロスポリンとタクロリムスは，上市当初よりTDMに基づく用量調節が推奨され，移植領域においてTDM保険算定が認められている数少ない薬物である。また，シクロスポリンは近年マイクロエマルジョン製剤が発売され，そのバイオアベイラビリティが改善された。それに伴い，シクロスポリンのAUCと最も相関するポイントが精査され，従来のトラフ値（次回投与直前）に比して投与2時間目の血中濃度(C2)を免疫抑制効果の指標とする傾向にある。一方，従来のトラフモニタリングは，腎毒性などの副作用モニターの指標として利用されている。タクロリムスについては，トラフ値がAUCと良好な相関関係を示すことが小児肝移植患者を対象にして明確になって以来，トラフモニタリングが主流である。

タクロリムスやシクロスポリンは，体循環に移行の後に主として肝臓に発現するCYP3A4またはCYP3A5によって代謝されること，代謝物および未変化体薬物は引き続きP-糖蛋白質(Pgp, MDR1遺伝子にコードされる)を介して，胆汁中に排泄されることが知られている。また，CYP3A4, 5やPgpは小腸上皮細胞にも発現しており，経口投与されたこれら薬物の吸収過程における代謝・排泄を媒介することによって，薬物の吸収障壁として協働的に機能している（**図1**）。したがって，これら代謝酵素やトランスポーターは，経口投与された薬物の血中濃度を支配する重要な生体因子として位置付けられている。すなわち，小腸のPgpやCYP3A4, 5の同一患者における発現変動や個人差に関する情報は，免疫抑制薬の個別投与設計を行ううえで有

用な指標になる[7]。

われわれは，生体肝移植患者を対象に検討した結果，術時の小腸 MDR1 mRNA 発現レベルが，タクロリムスの血中濃度/投与量（C/D）と良好な負の相関関係を示すことを見いだした。一方，CYP3A4 発現レベルとの相関関係は認められなかった。さらに，一定のタクロリムス血中濃度を得るために MDR1 高発現群では，MDR1 低発現群と比較して約 2 倍のタクロリムス投与量を必要とすること，MDR1 高発現群における平均 C/D 比は MDR1 低発現群の約 50%であることが明らかとなり，小腸 Pgp 発現量はタクロリムス体内動態に大きく影響することが判明した。

CYP3A4 のアイソフォームである CYP3A5 の多型性について，その発現は，*CYP3A5* ゲノム上のイントロン 3 における一塩基多型（SNP）が引き起こす，CYP3A5 mRNA のスプライシング異常とそれに引き続く機能蛋白の欠失に左右される。この遺伝子変異はコーカサス人においては高い頻度で認められ，その結果，60〜90%のコーカサス人は CYP3A5 機能を欠失する。一方，少なくとも 50%以上のアフリカ系人種は野生型である *CYP3A5*1* 遺伝子型を有し，機能蛋白を発現するとされている。そして日本人では，*CYP3A5*1* 遺伝子多型の頻度は 27.6%であり，コーカサス人とアフリカ系人種の中間と理解できる[8]。

生体肝移植術を受けた患者については，小腸は患者自身の遺伝子型を反映するものの肝臓はドナー由来であり，両者の遺伝子型が異なる場合が想定される。したがって，肝臓移植患者においては CYP3A5 活性が肝臓のみ，あるいは小腸のみに認められる症例が存在する。そこで，移植術後の経過日数に伴うタクロリムス体内動態の個体内変動と *CYP3A5* 多型との関係について調べた結果，術直後では小腸における多型が，術後 2 週以降では移植肝の多型が強く影響を及ぼすことが明らかになっている。これらの検討により，移植肝/患者体重比（%），術時の小腸 MDR1 mRNA 発現量，患者小腸および移植肝 *CYP3A5* 多型情報をタクロリムス動態影響因子として日常診療への応用を進めている（**図 2, 3**）[9〜11]。

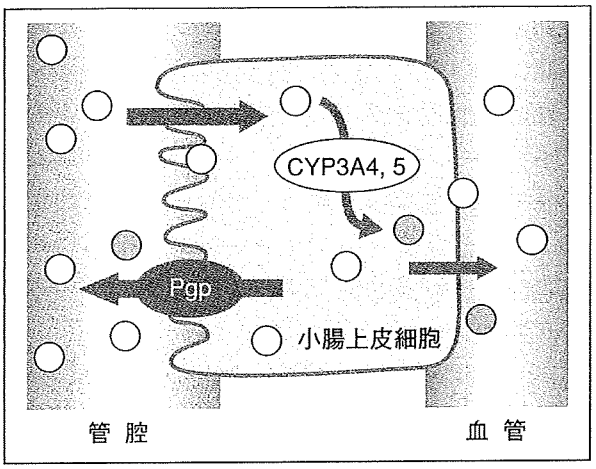

図 1　小腸上皮細胞における薬物の吸収機構
○：未変化体，●：代謝物

● 抗　体

種々白血球に特徴的な細胞表面の抗原を認識する抗体が，医薬品として利用されており，大きくマウス抗体，キメラ抗体，ヒト化抗体，ヒト抗体などのように，分類されている[12〜14]（**表 1**）。移植治療において免疫反応の抑制を目的に使用されている抗体医薬品は，ムロモナブ-CD3（白血球表面の CD3 に対するマウス抗体），バシリキシマブ〔活性化 T リンパ球インターロイキン 2（IL-2）受容体 α 鎖（CD25）に対するキメラ抗体〕が腎移植後の急性拒絶反応抑制という適応を有している。同様に，CD25 に対するヒト化抗体として daclizumab が米国では認可されているが，わが国では承認されていない。また，キメラ抗体であるリツキシマブは CD20 陽性の B 細胞性非ホジキンリンパ腫に対して適応とされているが，ABO 血液型不適合のドナー由来の生体肝移植ならびに腎移植症例において，抗 A，抗 B 抗体を産生する B リンパ球の除去を目的に使用される場合がある。

マウス抗体であるムロモナブ-CD3 の半減期は 8〜9 時間であるが，ヒト-マウスのキメラ抗体であるバシリキシマブやリツキシマブの半減期はそれぞれ 7.7 日，18.5 日と長い。さらに，ヒト化抗体である daclizumab の消失半減期は

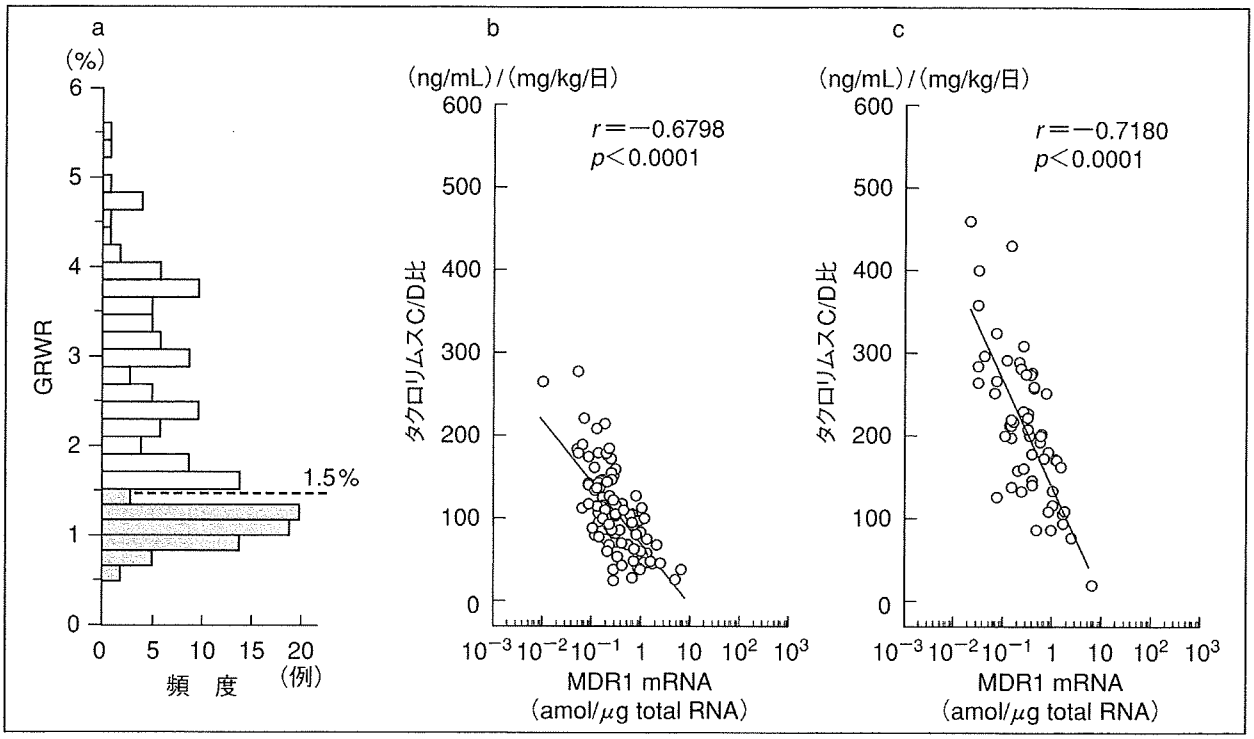

図 2　生体肝移植術時の小腸 MDR1 mRNA 発現量と術後 4 日間のタクロリムス血中濃度/投与量（C/D）比との関係

a：移植肝重量/患者体重比（GRWR）の分布。白は 1.5%以上，ブルーは 1.5%未満を示す。小腸 MDR1 mRNA 発現量とタクロリムス C/D 比との関係を GRWR が 1.5%以上（**b**）と 1.5%未満（**c**）に分けて解析。

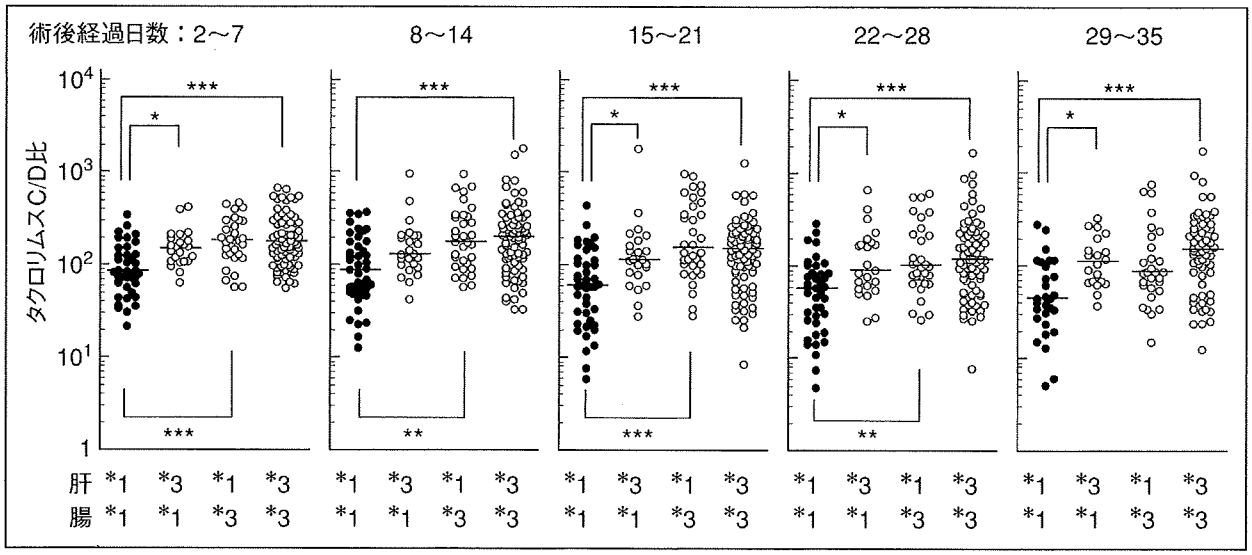

図 3　タクロリムス C/D 比に及ぼす移植肝および小腸 *CYP3A5*3* 遺伝子型の影響

*1：*CYP3A5*1/*1* and *CYP3A5*1/*3*, *3：*CYP3A5*3/*3*, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

20 日とされる。

● まとめ

免疫抑制を期待して使用される薬物の体内動態や作用機序は薬物によってさまざまであり，これらを組み合わせて用いられる場合も多い。最近では，mTOR 阻害薬（mammalian target of rapamycin）であるエベロリムスがわが国で承認され，chronic allograft nephropathy（慢性移植腎症）に対してシクロスポリンと併用されてい

表 1　モノクローナル抗体の分類

	マウス由来	ヒト由来の割合
キメラ抗体	可変部	70%
ヒト化抗体	超可変部*	90%
ヒト抗体**	なし	100%

* 超可変部：抗原と直接相互作用する部位，** ヒト抗体：ヒト免疫グロブリンのみを産生するマウスを用いて作製した抗体

る[15]。一方，海外では sirolimus が mTOR 阻害薬として以前から用いられており，日本では未承認ながら膵島移植治療の主要な免疫抑制薬として位置付けられている。これらはタクロリムスと同様にマクロライド系の化合物であり，その動態制御因子として CYP3A4, 5 や Pgp の関与が示唆されているが，わが国では使用経験が浅く今後の情報収集を待ちたい。

以上，臓器移植後の免疫抑制療法としてまとめられてきた時代から，多様化する時代へとますます個別化免疫抑制療法の需要は高まりつつある。それぞれの原疾患，移植臓器・細胞，患者背景，術後経過，薬物感受性および動態特性に応じた個別化の確立と実用化が期待される。

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ORIGINAL ARTICLE

Risk Factors for Recurrence of Primary Sclerosing Cholangitis After Living Donor Liver Transplantation: A Single Center Experience

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Abstract We retrospectively reviewed our 10-year experience with living donor liver transplantation (LDLT) in 30 consecutive patients with end-stage primary sclerosing cholangitis (PSC) to determine long-term patient and graft survival and risk factors for recurrence of PSC. For strict diagnosis of recurrence, patients with hepatic artery thrombosis ($n = 2$), ABO blood type incompatible transplantation ($n = 3$), and postoperative survival shorter than 1 year ($n = 5$) were excluded from the study, leaving 20 patients for analysis. Recurrence was diagnosed in 11 patients 26–71 months after transplantation. Multivariate analysis showed that cytomegalovirus diseases within 3 months after transplantation and related donors were independent risk factors for recurrence. When the effects on recurrence were compared among donor-recipient relationships, there were significant differences, especially between nonrelated donors and parents. Multivariate analysis showed that age was an independent risk factor for time to graft loss. Cytomegalovirus prophylaxis and avoidance of related donors are important in reducing PSC recurrence, although this is a preliminary report with limitations due to the small number of patients. LDLT for young patients with PSC using grafts from their parents might have to be avoided where deceased donor liver transplantation is available.

Keywords Primary sclerosing cholangitis · Living donor liver transplantation · Cytomegalovirus · Recurrence · Risk factor

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Abbreviations

PSC	Primary sclerosing cholangitis
LDLT	Living donor liver transplantation
CMV	Cytomegalovirus
ALP	Alkaline phosphatase
γ-GTP	Gamma-glutamyl transferase
MELD	Model for end-stage liver disease
HLA	Human leukocyte antigen
HR	Hazard ratio
CI	Confidence interval

Introduction

Primary sclerosing cholangitis (PSC) recurs in 20–30% of patients within 5 years after liver transplantation, and the

incidence of recurrence appears to increase with the time following transplantation [1]. Recurrent PSC is usually first apparent more than 1 year after transplantation with selective elevation of alkaline phosphatase (ALP) and gamma-glutamyl transferase (γ-GTP) [1]. Symptoms of ascending cholangitis and biliary cirrhosis can develop, but patient and allograft survival are not adversely influenced up to 5 years after deceased donor liver transplantation [1]. In a review by Gordon, eight of 13 peer-reviewed publications from 1995 through 2005 found no clinical variables to be associated with an increased risk of PSC recurrence [2]. The remaining studies showed cytomegalovirus (CMV) infection, intact colon, male sex, OKT3 treatment, gender mismatch, and corticosteroid-resistant rejection to be risk factors for recurrent PSC [2]. Gautam et al. failed to analyze risk factors for recurrence in their meta-analysis [3]. Although both Demetris and Gordon mentioned in their reviews that living donor liver transplantation (LDLT) could be a risk for PSC recurrence, there have been no large-scale studies of recurrent PSC after LDLT.

In this report, we reviewed our 10-year experience with LDLT in 30 patients with end-stage PSC, such as intractable pruritus, bacterial cholangitis, gastrointestinal bleeding, ascites, jaundice, and hepatic encephalopathy. An emphasis was placed on examining long-term patient and graft survival, and the incidence and outcome of recurrent PSC. Risk factors for recurrence and graft loss were also evaluated. Based on this experience, we propose a strategy to improve the outcome of LDLT for PSC.

Patients and Methods

Patients

Thirty patients with PSC (14 male and 16 female) underwent primary LDLT, including three domino transplantations, from July 1996 through August 2005. Ages ranged from 5 to 58 years, with a median age of 29 years. Seventeen patients also had ulcerative colitis, and one patient had early cholangiocarcinoma. Preoperative status was "at home" in 11 patients and "hospitalized" in 19 patients. The model for end-stage liver disease (MELD) score ranged from 8 to 36, with a median of 22 in the 28 adult patients. The New Mayo score for PSC ranged from 1.557 to 35.403, with a median of 8.916. The donors were parents in 16 cases, siblings in seven cases, sons in two cases, husbands in two cases, and domino donors of familial amyloid polyneuropathy in three cases. The graft type was the whole liver in two cases, the right lobe in 21 cases, the left lobe in five cases, and the left lateral segment in two cases. The blood type combination was identical in 21 cases, compatible in six cases, and incompatible in three cases. The follow-up period ranged from 1 to 133 months, with a median length of 63 months.

Written informed consent was obtained from each patient and donor, and the study was approved by the Ethics Committee of Kyoto University Hospital according to the Declaration of Helsinki of 1975 as revised in 1996.

Donor Selection

Donors were selected from parents, grandparents, siblings, offspring, and spouses of recipients for standard LDLT. In domino transplantation, the donors were recipients of LDLT for familial amyloid polyneuropathy. Donors were fully informed of the risks and benefits of LDLT and confirmed their voluntary decision to become a live donor, and consent was obtained from each. Our institutional donor age limit is 65 years. Preoperative evaluations for estimating graft and remnant liver volume in the donor were performed using three-dimensional reconstructed images of the hepatic vascular anatomy.

Definition of Recurrence

Recurrence of PSC was strictly defined using both positive and negative criteria according to Graziadei et al. [4]. Criteria included a confirmed diagnosis of PSC before transplantation and intrahepatic multiple biliary strictures confirmed by cholangiography occurring more than 90 days after transplantation, or biopsy findings showing fibrous cholangitis and/or fibro-obliterative lesions with or without ductopenia, biliary fibrosis, or biliary cirrhosis. All of the above findings occurred in the absence of hepatic artery thrombosis/stenosis, established chronic (ductopenic) rejection, anastomotic strictures alone, nonanastomotic strictures occurring before post-transplantation day 90, and ABO blood group incompatibility between donor and recipient. When clinical signs (fever, abdominal pain, jaundice, pruritus) or laboratory signs (hepatic enzyme levels or serum bilirubin levels at least 1.5 times greater than the normal upper range) indicated cholangitis, a liver biopsy was performed percutaneously. Pathological diagnosis was made by a pathologist (H·H.) [5]. Cholangiography was performed by occasional percutaneous transhepatic cholangiography, or endoscopic retrograde cholangiography, or recently magnetic resonance cholangiography.

In our 1,350-patient experience with LDLT, none of the patients developed multiple intrahepatic biliary strictures without gallstones or biliary casts, other than after ABO incompatible transplantation, after hepatic artery thrombosis/stenosis, or with the original disease of PSC.

Surgery and Immunosuppression Regimen

Twenty-seven patients underwent standard LDLT, and three underwent domino LDLT. The standard LDLT

procedures for both donors and recipients were performed according to our previously reported methods [6, 7], and we have also reported our method of domino transplantation [8].

Briefly, all grafts for standard LDLT were flushed and preserved with a histidine-tryptophan-ketoglutarate solution (Dr. Franz Köhler Chemie, Alsbach-Hähnlein, Germany), and three grafts from domino donors were flushed with University of Wisconsin solution. The grafts were revascularized by reconstruction first of the portal vein and then of the hepatic artery. Regarding biliary reconstruction, the biliary system was reconstructed with a Roux-en-Y choledochojejunostomy if inflammation was noted in routine frozen section biopsy specimens of the common bile duct. If atypia or inflammation was present in the frozen section biopsy specimen, the entire common bile duct was resected to the level of the pancreas.

Methylprednisolone (10 mg/kg) was administered just before the start of graft reperfusion, followed by 1 mg/kg of intravenous methylprednisolone for 3 days and 0.5 mg/kg of intravenous methylprednisolone for a further 3 days. Oral prednisolone (0.3 mg/kg) was continued. Tacrolimus was administered on the postoperative day 1 according to our standard procedure [9]. Antimetabolites (azathioprine or mycophenolate mophetil) were added in patients with refractory rejection or by decision of the attending physicians during long-term follow-up. If acute cellular rejection was confirmed with Banff criteria [10], patients received a 3- to 5-day course of intravenous bolus therapy (methylprednisolone, 10 mg/kg).

Tissue typing was performed in patients and donors for human leukocyte antigen (HLA)-A, HLA-B (Bw), HLA-C, HLA-DR, and HLA-DQ for class I and II loci according to Terasaki methods. We found that HLA-DR 15 was most frequent (15/30; 50%) in 30 patients of PSC and that the reported incidence of DR 15 was 14.8% in Japanese population [11].

Prophylaxis for Viral Infection

Oral acyclovir (10 mg/kg/day) was administered on postoperative day 7 and continued for 3 months as prophylaxis generally for herpes virus family. Preemptive treatment for CMV was performed instead of prophylaxis. Gancyclovir was administered when weekly CMV antigenemia was detected over two cells in 20,000 cells or CMV diseases were diagnosed. CMV diseases were diagnosed when clinical manifestations concomitant with positive CMV antigenemia developed or when positive findings, such as microabscess in the liver and inclusion body in the intestinal wall, were observed on pathological examination of biopsy specimens.

Statistical Analysis

Overall survival curves were calculated with the Kaplan–Meier method. The univariate log-rank test was used to evaluate the effects of characteristics on recurrence and graft loss. Multivariate Cox regression analysis was used to evaluate the association between time to recurrence or time to graft loss and patient characteristics and to estimate hazard ratios (HRs). To assess the effect of PSC recurrence on graft loss, we performed Cox regression analysis with recurrence as the time-dependent covariate. Graft loss was defined as death or retransplantation. The software program SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. All statistic analysis was done by S.T.

Results

Analysis of Risk Factors for Recurrence and Graft Loss

Three patients with ABO incompatible transplantation and two patients with hepatic artery thrombosis were excluded from analysis for recurrence according to Graziadei et al. [4]. To evaluate long-term effects of characteristics on recurrence and graft loss, five patients who died within 1 year after transplantation were excluded. These five patients did not have recurrence of PSC until death. The remaining 20 patients were enrolled in the statistic analysis.

The age of the remaining 20 patients analyzed in this study ranged from 5 to 58 years, and the median age was 32 years. The characteristics of donors and recipients are shown in Table 1. The 5- and 10-year graft survival was 68.6 and 39.7%, respectively, and the 5- and 10-year patient survival was 81.4 and 81.4%, respectively (Fig. 1). The patients were divided into three groups: under 18 years, 18–29 years, and 30 years or older. Recurrence was diagnosed in 11 patients at 26–71 months after transplantation. In all 11 patients, the recurrence was diagnosed by liver biopsy specimens and five patients by PTC and six patients by MRCP. Recurrence developed in six patients within 36 months after transplantation, and in three patients between 36 month and 48 month, and two patients thereafter. Recurrence developed in a patient receiving a graft from her husband 71 months after transplantation. Two patients became pregnant before recurrence.

Each clinical course of 11 patients with recurrence is shown in Fig. 2. The interval from recurrence to retransplantation ranged from 9 to 93 months. Four of the patients received second grafts from the other parent, two from siblings, one from her husband, and one from a nonrelated

Table 1 Characteristics and outcome according to patient characteristics ($n = 20$)

Characteristics	Number (%)	Recurrence		Graft loss	
		Incidence (%)	*P*-value	Incidence (%)	*P*-value
Age					
Under 18 years old	3 (10)	67	0.016	100	0.001
18–29 years old	5 (25)	80		80	
30 years old -	12 (60)	42		17	
MELD score					
−21	10 (56)	40	0.042	30	0.257
22−	8 (44)	75		50	
Unknown	2				
New Mayo					
<9.0	10 (50)	60	0.422	50	0.498
9.0≤	10 (50)	50		40	
Ulcerative colitis					
Without	7 (35)	43	0.642	29	0.835
With	13 (65)	62		54	
Number of immunosuppressants at 1 year after transplant					
1	3 (15)	100	0.728	33	0.376
2	12 (60)	50		58	
3	5 (25)	40		20	
Biliary reconstruction					
Choledocho–choledochostomy	4 (20)	75	0.852	50	0.779
Choledocho–jejunostomy	16 (80)	50		44	
Number of biliary anastomosis					
1	13 (65)	69	0.142	54	0.596
2 or more	7 (35)	29		29	
Biliary anastomotic complications within 1 year					
Without	10 (50)	40	0.464	40	0.454
With	10 (50)	70		50	
Corticosteroid pulse for ACR within 3 months					
No	9 (45)	56	0.160	44	0.923
1 time	7 (35)	86		57	
2 times	4 (20)	0		25	
CMV diseases within 3 months					
Without	14 (70)	43	0.007	36	0.085
With	6 (30)	83		67	
Donor					
Nonrelated	5 (25)	20	0.008	20	0.152
Related	15 (75)	67		53	
Sex (recipient)					
Male	8 (40)	25	0.080	50	0.912
Female	12 (60)	75		42	
Sex (donor)					
Male	16 (80)	44	0.106	44	0.945
Female	4 (20)	100		50	
Gender mismatch					
Match	10 (50)	40	0.419	40	0.745
Mismatch	10 (50)	70		50	
Graft type					
Left lobe	5 (25)	80	0.083	80	0.696

Table 1 continued

Characteristics	Number (%)	Recurrence		Graft loss	
		Incidence (%)	*P*-value	Incidence (%)	*P*-value
Right lobe	13 (65)	54		31	
Whole liver	2 (10)	0		50	
HLA-DR15 (recipient)					
Without	11 (55)	55	0.861	55	0.293
With	9 (45)	56		33	
HLA-DR15 (donor)					
Without	13 (65)	31	0.025	38	0.504
With	7 (35)	100		57	
HLA-DR15 (recipient and donor)					
Without	15 (75)	40	0.068	40	0.633
With	5 (25)	100		60	
HLA-DR15 (either donor or recipient)					
Without	9 (45)	44	0.456	56	0.188
With	11 (55)	64		36	
HLA-DR1501/1502 (recipient and donor)					
Without	16 (80)	44	0.252	44	0.420
With	4 (20)	100		50	

P-value: log rank test

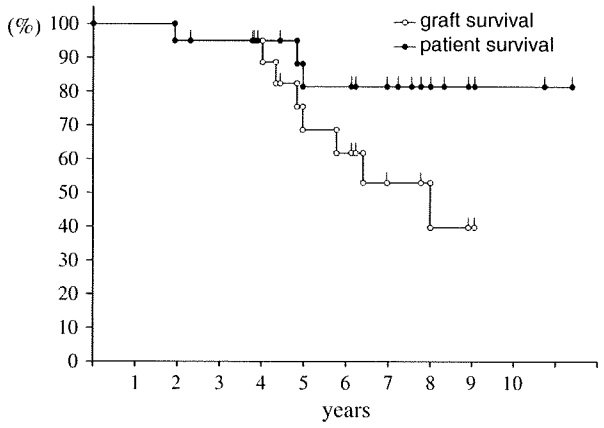

Fig. 1 Patient and graft survival after LDLT for PSC of 20 patients

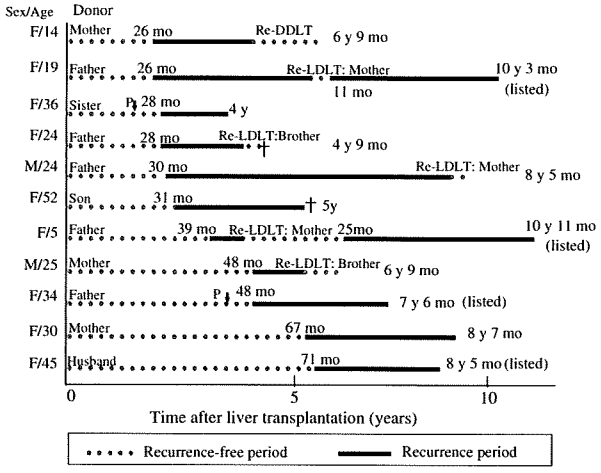

Fig. 2 Clinical courses of 11 patients with PSC recurrence. Sex/age: sex and age (years) of recipients, *Re-DDLT* retransplantation with deceased donor liver transplantation, *Re-LDLT* retransplantation with LDLT, *listed* on waiting list for retransplantation, *P* pregnancy, *y* years, and *mo* months

deceased donor. One patient died of surgical complications 1 month after retransplantation. In two patients who received the second grafts from the other parent, PSC recurred at 11 and 25 months after retransplantation, respectively. The interval between a second recurrence and retransplantation was shorter than the interval between the first transplantation and the first recurrence (the second recurrence interval versus the first recurrence interval in each patient: 11 months versus 28 months, and 25 months versus 39 months). Four patients are on waiting lists to undergo deceased donor liver transplantation because of graft failure due to PSC recurrence. Two patients are waiting for second grafts, and two patients are waiting for third grafts.

Five nonrelated donors were two husbands and three domino-donors. Fifteen related donors were eight fathers, two mothers, two brothers, one sister and two sons. Recurrence of PSC developed in eight of ten patients (80%) receiving grafts from parents, including one of two (50%) from children, one of three (33%) from siblings, and one of five (20%) from non-related donors.

The hepatic chemistries at the time of recurrence were shown in Fig. 3. The levels of ALP and γ-GTP were