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Research Paper

Pharmacokinetic Significance of Renal OAT3 (SLC22A8) for Anionic Drug Elimination in Patients with Mesangial Proliferative Glomerulonephritis

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Purpose. Our previous studies showed that the mRNA level of human organic anion transporter (hOAT) 3 in the kidney was correlated with the rate of elimination of an anionic antibiotic cefazolin. However, the correlation coefficient was not so high. In the present study, therefore, we enrolled more patients to examine whether additional factors were responsible for the correlation.

Methods. hOAT mRNA levels in renal biopsy specimens were quantified using the real-time polymerase chain reaction method. The elimination rates for the free fraction of cefazolin were determined in patients with various renal diseases.

Results. In the present study, the coefficient of correlation between the hOAT3 mRNA level and the elimination rates for the free fraction of cefazolin was not so high in the patients overall as in our previous study (r = 0.536). However, following the classification of renal diseases, a better correlation was obtained in patients with mesangial proliferative glomerulonephritis (r = 0.723). In contrast, multiple regression analyses including gender, age, and liver function did not result in any improvements in the correlation coefficients.

Conclusions. These results suggest that the hOAT3 mRNA level is a significant marker of pharmacokinetics with which to predict the rate of elimination of cefazolin in patients with mesangial proliferative glomerulonephritis.

KEY WORDS: organic anion transporter; human kidney; renal diseases; real-time PCR; renal clearance.

INTRODUCTION

In patients with renal impairment, individualized dosages are adjusted by using the plasma creatinine concentration or creatinine clearance (C_{cr}) to avoid adverse effects (1). C_{cr} is often used to estimate the glomerular filtration rate (GFR), because creatinine is mainly eliminated via glomerular filtration. However, C_{cr} would not reflect the true GFR, especially in patients with renal impairment, because the amount of tubular secretion is the negligible part of urinary excretion of creatinine (2). Recently, organic cation transporter (OCT) 2 (SLC22A2) was reported to transport creatinine suggesting that OCT2 plays an important role in the secretion of creatinine (3). Furthermore, accumulating evidence shows a poor correlation between C_{cr} and the urinary excretion of drugs (4,5), challenging the "intact nephron hypothesis" and suggesting that the renal handling of drugs may not decline in parallel. Therefore, it is necessary to identify more reliable markers to predict renal function such as the tubular secretion of drugs.

The renal handling of drugs involves three processes: glomerular filtration, tubular secretion, and reabsorption. Tubular secretion and reabsorption are mediated by various transporters expressed in the apical and basolateral membranes of the tubular epithelial cells (6,7). The organic anion transporters (OATs, SLC22A) can transport many ionic drugs and are expressed in the renal proximal tubules (8,9). However, the pharmacokinetic significance of each transporter in the clinical setting has not been fully elucidated.

Previously, we quantified the expression of hOAT1–4 mRNA and urinary excretion of the anionic antibiotic cefazolin in patients with renal diseases. The mRNA level of hOAT3, among the four anion transporters, significantly correlated with the rate of elimination of cefazolin (n = 42; r = 0.44, p < 0.01) (10). In addition, hOAT3 transported cefazolin. The renal expression level of hOAT3 possibly affects the renal handling of cefazolin. However, the coefficient of correlation between the hOAT3 mRNA level and elimination rate of cefazolin was not so high. Therefore, it is postulated that additional factors affect this coefficient.

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In our previous report, patients with various renal diseases were studied without classification. Because the cause and pathway of progression vary with the type of renal disease (11–13), proximal tubules may be exposed to different regulatory factors depending on the disease. In the present study, we enrolled more patients to examine whether the type of renal disease is a factor in the correlation between the hOAT3 mRNA level and the rate of elimination of cefazolin.

MATERIALS AND METHODS

Patients

A total of 75 patients were enrolled in the study (44 males and 31 females) aged 16–90 years (mean ± SD, 42.4 ± 18.5) with histopathologically confirmed renal disease. Patient profiles are described in Table I. Patients with mesangial proliferative GN (n = 38; 22 males and 16 females, aged 39.8 ± 18.4 years), which is recognized as the most common form of primary renal disease, were categorized as Group I. Those with other renal diseases (n = 37; 22 males and 15 females, aged 45.1 ± 18.5 years) were categorized into Group II. In Group II, eight patients had membranous nephropathy, eight had minimal change nephritic syndrome, seven had lupus nephritis, four had diabetic nephropathy, three had membranoproliferative GN, and seven had other renal diseases. Clinical tests, such as measurements of C_{cr} or 120-min values of the phenolsulfonphthalein (PSP) test (PSP120′), were routinely conducted in the hospital to evaluate renal function. 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. All patients gave written informed consent to participate in the study.

Isolation of total RNA and genomic DNA

Total RNA from renal biopsy specimens and genomic DNA from blood in a guanidinium thiocyanate solution were isolated with a MagNA Pure LC RNA isolation Kit II and DNA isolation Kit I (Roche Diagnostic, Mannheim, Germany), respectively. Total RNA was reverse-transcribed in the presence of RTmate solution (Wako Pure Chemical Industries, Osaka, Japan), and the single-stranded DNA was used for quantification of mRNA levels.

Quantification of mRNA levels

The mRNA levels of hOAT1–4 were quantified as described previously (14). Using single-stranded DNA (ssDNA), a real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in duplicate under identical conditions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also quantified as an internal control using GAPDH Control Reagent (Applied Biosystems).

Measurement of Elimination Rate Constant for Free Fraction of Cefazolin

After renal biopsy, the patients received 1 g of cefazolin by intravenous infusion for 1 h as a prophylaxis of infection. Collection of blood samples and measurement of cefazolin concentrations by high-performance liquid chromatography (HPLC) were carried out as previously described (10). The plasma unbound fraction (fu) of cefazolin was determined by ultrafiltration using a micropartition system (MRS-1; Amicon, Inc., Beverly, MA, USA) (15). Free fraction of cefazolin was expressed as the ratio of the concentration in ultrafiltrate to that in plasma. The apparent elimination rate constant of the free fraction ($Ke_{free,cez}$) was calculated using the free fraction of the plasma cefazolin concentration immediately and 1 h after cefazolin infusion.

Genotyping of the *hOAT3* gene

The genotypes of the *hOAT3* gene—C715T (Gln239Stop), T779G (Ile260Arg), C829T (Arg277Trp), A913T (Ile305Phe), C929T (Ala310Val), and G1342A (Val448Ile), which were reported in the public single nucleotide polymorphisms (SNPs) database NCBI dbSNP—were examined via the PCR restriction enzyme

Table I. Characteristics of Patients with Renal Disease

	All patients	Group I	Group II	p Value
No. of patients	75	38	37	
Sex (males/females)	44/31	22/16	22/15	
Age	42.4 ± 18.5	39.8 ± 18.4	45.1 ± 18.5	0.221
Aspartate aminotransferase (IU/L)	19.6 ± 8.9	18.3 ± 6.9	20.9 ± 10.6	0.232
Alanine aminotransferase (IU/L)	16.7 ± 10.1	14.9 ± 7.2	18.7 ± 12.2	0.113
Lactate dehydrogenase (mg/L)	180.5 ± 52.8	171.3 ± 51.6	190.3 ± 53.1	0.139
Serum creatinine (mg/dL)	1.1 ± 0.6	1.1 ± 0.7	1.0 ± 0.5	0.503
Uric acid (mg/dL)	6.3 ± 1.5	6.1 ± 1.4	6.4 ± 1.6	0.442
Blood urea nitrogen (mg/dL)	17.6 ± 11.1	16.4 ± 8.0	18.8 ± 13.5	0.341
Creatinine clearance (mL/min)	63.2 ± 28.1	64.6 ± 29.4	61.8 ± 27.0	0.672
PSP120′ (%)	64.9 ± 20.4	65.9 ± 18.7	63.4 ± 22.7	0.652
$Ke_{free\ cez}$	0.74 ± 0.25	0.73 ± 0.26	0.75 ± 0.25	0.619

Group I includes the patients with mesangial proliferative glomerulonephritis. All other patients were categorized in Group II.
Variance is indicated as the mean ± SD.
p Values are for the comparison between Group I and Group II.

Table II. Primer Sets for PCR-RFLP

Position of mutation		Primer sequences	Restriction enzyme
cDNA715	Forward	5′-CCTGGGACCTAGCAGAACACTA-3′	*Pst*I
	Reverse	5′-TGAAGAGGAGAGGGCCACAT-3′	
cDNA779	Forward	5′-CTGTTGATGTTCTTCCAGCCTCTCA-3′	*Nde*I
	Reverse	5′-TCCAGACAAGACCAACCAGCAT-3′	
cDNA829	Forward	5′-CTGTTGATGTTCTTCCAGCCTCTCA-3′	*Bsp*EI
	Reverse	5′-ATTGAAGACAGCCACCCTCC-3′	
cDNA913	Forward	5′-TAGAGGCACCACGCCTGCAT-3′	*Bgl*II
	Reverse	5′-GGTTACCAGGCCAGGGAAAGA-3′	
cDNA929	Forward	5′-TAGAGGCACCACGCCTGCAT-3′	*Stu*I
	Reverse	5′-GGAACAGGTCACTTGCGGTGTACTAG-3′	
cDNA1342	Forward	5′-CAGGTGGGACTGTGGCATTGT-3′	*Bsi*WI
	Reverse	5′-ACGCGGGTCCACAGGTTACGTA-3′	

fragment length polymorphism (RFLP) method in 67 patients. Genomic DNA could not be obtained from eight patients, who consented to the mRNA quantification and pharmacokinetic study but refused more genetic analyses. The specific primers and restriction enzymes used in this study are listed in Table II. The PCR conditions were as follows: after denaturing at 94°C for 3 min, PCR was performed with 1 μM of each primer and *Taq* DNA polymerase (Takara, Shiga, Japan), according to the following profile—94°C for 1min, 63°C for 1min, and 72°C for 1min, 35 cycles, followed by a single additional 10-min extension at 72°C. The PCR products were digested with or without apparent restriction enzymes and separated by electrophoresis on 3% agarose gel. The sequence of the *hOAT3* polymorphism (A/T913 corresponding to Ile305Phe) was confirmed by direct sequencing in heterozygotes using a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan).

Statistical Analysis

Simple and multiple regression analyses were performed using the least-squares method. Multiple regression analyses were performed to determine the impact of the patient's characteristics on the correlation between the logarithmically transformed hOAT3 mRNA data and $Ke_{free,cez}$ or the 120-min values of the PSP test (PSP120′). In multiple regression analyses, we used $Ke_{free,cez}$ or PSP120′ as the outcome variable, and the hOAT3 mRNA level and parameters of patients as predictor variables. The nonpaired Student *t* test was used to compare groups. Statistical analyses were performed with Stat View, version 5.0 (Abacus Concepts, Berkeley, CA, USA).

Materials and Methods

Cefazolin was kindly provided by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). All other chemicals used were of the highest purity available.

RESULTS

The coefficient of correlation between $Ke_{free,cez}$ and C_{cr} was 0.439 ($p < 0.01$), and between $Ke_{free,cez}$ and PSP120′ it was 0.705 ($p < 0.01$) (Fig. 1A and B). As in the previous study, it was confirmed that hOAT3 mRNA level was significantly correlated with $Ke_{free,cez}$ ($r = 0.536$; $p < 0.01$) (Fig. 1C). In addition, a significant correlation between hOAT3 mRNA level and PSP120′ ($r = 0.484$; $p < 0.01$) was found (Fig. 1D).

To investigate whether the type of renal disease affects the correlation, patients were divided into Groups I (mesangial proliferative GN) and II (other renal diseases). As shown in Table I, there were no significant differences in the population between the two groups. In addition, $Ke_{free,cez}$ was significantly correlated with PSP120′ ($r = 0.723$, $p < 0.01$; Group I, $r = 0.713$, $p < 0.01$; Group II) more than C_{cr} ($r =$

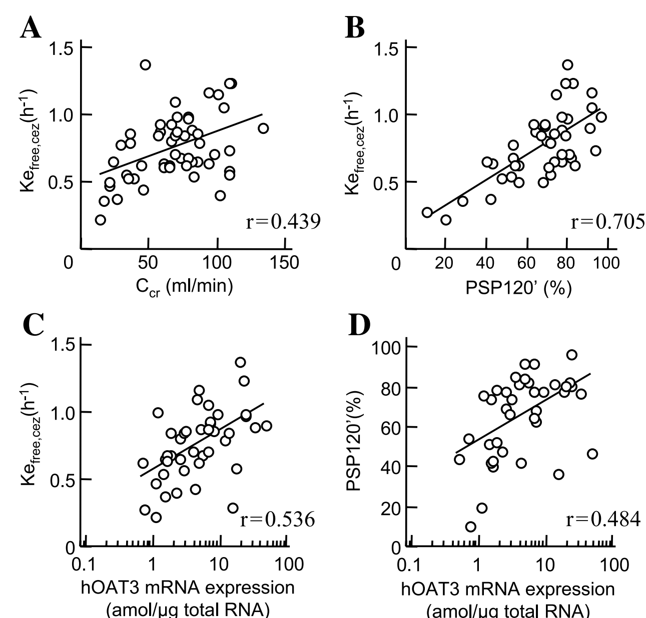

Fig. 1. The linear regression of creatinine clearance (C_{cr}) (A) or the 120-min values of the phenolsulfonphthalein test (PSP120′) (B) against the elimination rate constant for the free fraction of cefazolin ($Ke_{free,cez}$), and the linear regression between hOAT3 mRNA levels and $Ke_{free,cez}$ (C) or PSP120′ (D) in patients with renal diseases. The plasma concentration of cefazolin was measured by HPLC, and $Ke_{free,cez}$ was calculated. Total cellular RNA was extracted from the kidney biopsy specimens. The mRNA levels of hOAT3 were quantified by real-time PCR.

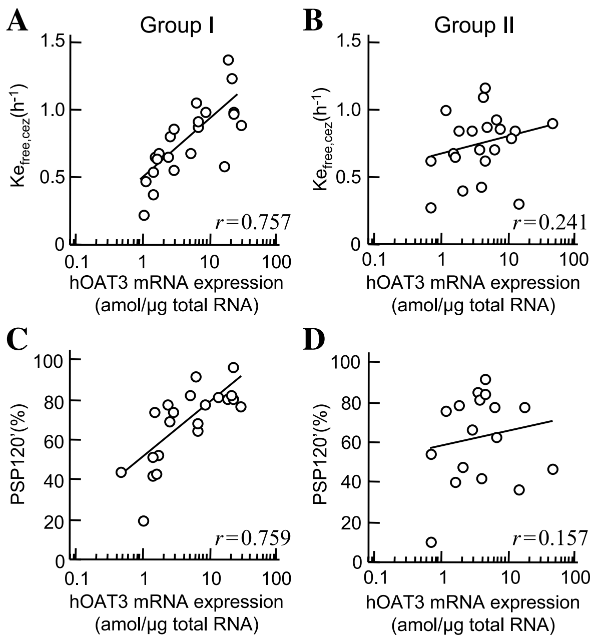

Fig. 2. The linear regression of hOAT3 mRNA levels against $Ke_{free,cez}$ (A, B) or PSP120′ (C, D) in Group I (A, C) or Group II (B, D). The plasma concentration of cefazolin was measured by HPLC, and $Ke_{free,cez}$ was calculated. Total cellular RNA was extracted from the renal biopsy specimens. The mRNA levels of hOAT3 were quantified by real-time PCR.

0.492, $p < 0.01$; Group I, $r = 0.474$, $p < 0.05$; Group II) in both groups.

Figure 2A and B shows the results of linear regression analyses with mRNA level of hOAT3 and $Ke_{free,cez}$. In Group I, the coefficient of correlation between hOAT3 mRNA level and $Ke_{free,cez}$ ($r = 0.757$; $p < 0.01$) was much higher than that in all patients. On the other hand, $Ke_{free,cez}$ was independent of hOAT3 mRNA level in Group II ($r = 0.241$; $p = 0.296$). Table III summarizes the coefficients of correlation between hOAT1–4 mRNA levels and $Ke_{free,cez}$ in each group. Although the expression level of hOAT1 mRNA was correlated with $Ke_{free,cez}$ in Group I, its coefficient was lower than the value for hOAT3. In Group II, there were no correlations between hOAT1–4 mRNA levels and $Ke_{free,cez}$. The same results were obtained between hOAT1–4 mRNA levels and PSP120′ (Fig. 2C and D, Table III).

Table III. Correlation Coefficients for $Ke_{free,cez}$, PSP120′, and the Expression Levels of hOAT mRNAs in Group I and Group II

	Group I		Group II	
mRNA expression	$Ke_{free,cez}$	PSP120′	$Ke_{free,cez}$	PSP120′
hOAT1	0.558[a]	0.556[a]	0.267	0.391
hOAT2	0.051	0.083	0.387	0.213
hOAT3	0.757[a]	0.759[a]	0.241	0.157
hOAT4	0.183	0.186	0.247	0.474

Values represent coefficients of the correlation between mRNA expression of hOATs and $Ke_{free,cez}$ or PSP120′.
[a] Each mRNA level of hOAT is significantly correlated with $Ke_{free,cez}$ or PSP120′.

Table IV. Multiple Regression Analyses for $Ke_{free,cez}$ or PSP120′

	r Value	
+ Additional parameters	$Ke_{free,cez}$	PSP120′
hOAT3 mRNA level + age	0.603	0.526
hOAT3 mRNA level + aspartate aminotransferase	0.585	0.558
hOAT3 mRNA level + alanine aminotransferase	0.543	0.534
hOAT3 mRNA level + lactate dehydrogenase	0.530	0.497

To assess whether personal profiles of patients and liver functions affect the correlation between hOAT3 mRNA level and $Ke_{free,cez}$, simple or multiple linear regression analyses were performed using gender, age, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH). Coefficients of correlation were not improved by dividing the subjects into males and females ($r = 0.450$, $p < 0.01$; males, $r = 0.600$, $p < 0.01$; females). Table IV shows the results of multiple linear regression analyses. Age and liver functions did not improve the correlation. In addition, the same results were observed for PSP120′.

Next, we investigated six SNPs in coding regions (cSNPs) with nonsynonymous changes in the *hOAT3* gene of 67 patients, because some cSNPs in the *hOAT3* gene were considered to affect the transport activity independent of the expression level. In the *hOAT3* gene, one nonsynonymous polymorphism (A913 was replaced with T) was detected via the PCR-RFLP method and confirmed by direct sequencing (Table V, Fig. 3). This polymorphism resulted in an amino acid substitution; Ile305 was changed to Phe (Ile305Phe). The allele frequency of cDNA 913 was 95.5% for allele A and 4.5% for allele T. However, there was no remarkable difference in $Ke_{free,cez}$ or PSP120′ between the two genotypic groups (Table V). Furthermore, the coefficient of correlation between hOAT3 mRNA level and $Ke_{free,cez}$ ($r = 0.551$, $p < 0.01$) or PSP120′ ($r = 0.493$, $p < 0.01$) was not changed by excluding patients with this variant.

DISCUSSION

Recent insights into the mechanisms of progressive renal dysfunction have indicated that tubulointerstitial pathology does not simply follow glomerular injury and that tubular cells may be the primary targets for various pathophysiological influences (16). Among each type of renal disease, the cause and pathway of progression are different (11–13). For each type of renal disease, renal transporters are assumed regulated in a different manner. Therefore, it may be important to consider types of renal disease when assessing the correlation between pharmacokinetics and expression levels of transporters. In the present study, we found a good relationship between the hOAT3 mRNA level and the rate of elimination of cefazolin in patients with mesangial proliferative GN (Group I), which is the most common form of primary renal disease (17). It was postulated that hOAT3 expression levels directly regulated the rate of elimination of

Table V. hOAT3 Genetic Variants in Patients with Renal Diseases and Phenotypic Indexes (n = 67)

Location	Position	Allele	Effect	Allele frequency	Genotype	Frequency (%)	$Ke_{free,cez}$ (h^{-1})	PSP120′ (%)
Exon5	cDNA715	C	239Gln	67 (100.0%)	C/C	100.0		
		T	239Stop	0 (0.0%)	C/T	0.0		
					T/T	0.0		
Exon6	cDNA779	T	260Ile	67 (100.0%)	T/T	100.0		
		G	260Arg	0 (0.0%)	T/G	0.0		
					G/G	0.0		
Exon6	cDNA829	C	277Arg	67 (100.0%)	C/C	100.0		
		T	277Trp	0 (0.0%)	C/T	0.0		
					T/T	0.0		
Exon7	cDNA913	A	305Ile	64 (100.0%)	A/A	91.0	0.75 ± 0.25	66.1 ± 20.5
		T	305Phe	3 (4.5%)	A/T	9.0	0.84 ± 0.29	64.2 ± 15.6
					T/T	0.0		
Exon7	cDNA929	C	310Ala	67 (100.0%)	C/C	100.0		
		T	310Val	0 (0.0%)	C/T	0.0		
					T/T	0.0		
Exon10	cDNA1342	G	448Val	67 (100.0%)	G/G	100.0		
		A	448Ile	0 (0.0%)	G/A	0.0		
					A/A	0.0		

Variance is indicated as the mean ± SD.

cefazolin in these patients. The level of hOAT1 mRNA was also correlated with $Ke_{free,cez}$ in Group I. However, its correlation coefficient was lower than the coefficient between hOAT3 mRNA levels and $Ke_{free,cez}$. In addition, transport of cefazolin by hOAT1 was negligible in our previous study (10,18). Therefore, it was considered that elimination rate of cefazolin was affected by the expression level of hOAT3 rather than hOAT1.

On the other hand, in Group II, there was no significant correlation between the mRNA level of hOAT3 and the rate of elimination of cefazolin. It is suggested that additional factors are affecting the rate. One possibility is that hOAT3 is regulated by a posttranslational mechanism in these patients. For example, the activation of protein kinase C (PKC) inhibited the uptake of anionic compounds by OAT3 in intact renal proximal tubules and cells stably expressing OAT3 (19). It was recently reported by Soodvilai *et al.* (20,21) that tyrosine kinase, phosphatidylinositol 3-kinase, mitogen-activated protein kinase, protein kinase A, and mitogen-activated/extracellular signal-regulated kinase kinase were involved in epidermal growth factor signaling pathways, which could affect the function of OAT3. Group II included patients with diabetic nephropathy, and the PKC activity of renal tubular cells was reported to be increased in the diabetic state (22,23). Thus, it is possible that the function of hOAT3 is modified by various kinases in some patients of Group II. However, the signaling pathways in proximal tubules specific for each renal disease, such as lupus nephritis or interstitial nephritis, are little understood in contrast to those in the glomeruli. Further studies should be performed to clarify the posttranslational regulation of tubular transporters in renal disease.

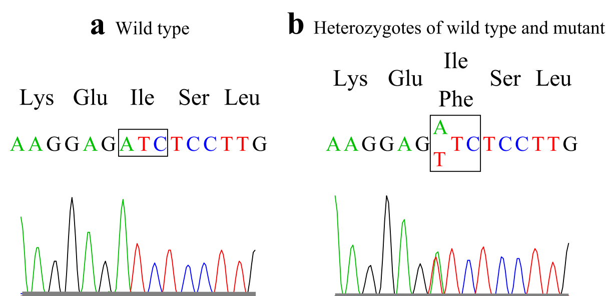

Fig. 3. Electropherograms of the SLC22A8 gene sequence in the region around the A913T mutation.

Poor correlations between C_{cr} and the renal clearance of drugs have been reported (4,5). In the present study, C_{cr} was not a good predictor of $Ke_{free,cez}$ (Fig. 1), indicating that C_{cr} can not be used to accurately assess anionic drug excretion. Alternatively, it has been indicated that tubular function should be considered when making precise adjustments of dosage. However, tubular function is not routinely evaluated in the clinical setting. In the present study, hOAT3 mRNA level was significantly correlated with PSP120′ in the patients with mesangial proliferative GN (Fig. 2). PSP is almost completely excreted into urine in the unchanged form (24,25) and in a substrate of hOAT3 (10). It is suggested that PSP tests are good predictors of the renal elimination rate of drugs, which are transported by hOAT3, with less invasive methods. Markers for the expression level of individual transporters from urine or blood will be useful to predict the rate of elimination of substrate drugs.

Genetic polymorphisms of drug transporters as well as drug-metabolizing enzymes affect the pharmacokinetics of drugs. Ishikawa *et al.* (26) suggested that cSNPs of transporter genes are responsible for the variation in responses to drugs among individuals. For example, SNPs of human organic anion transporting polypeptides (OATP-C, SLC21A6) were reported to influence the pharmacokinetics of pravastatin (27–29). In the present study, we screened for six cSNPs of the *hOAT3* gene, and found one polymorphism, Ile305Phe (allelic frequency, 4.5%). However, no pharmacokinetic significance of this cSNP was apparent. Nishizato *et al.* (28) also reported that a polymorphism in the *hOAT3* gene,

T723A (Ala389Val), was unlikely to be associated with differences in the clearance of pravastatin. Thus, it is likely that cSNPs of *hOAT3* do not account for variation in the rate of elimination in most cases, and that the expression level of hOAT3 is more important to explain the interindividual variation in the elimination rates of cefazolin and PSP than cSNPs in patients with renal diseases.

There are several differences in substrate specificity between hOAT1 and hOAT3. hOAT3, but not hOAT1, transports estrone sulfate (ES), and the cationic drug famotidine (30,31). Renal elimination rates of these substrates may also be affected by hOAT3 expression levels. While methotrexate was transported by both hOAT1 and hOAT3 (32), aciclovir and ganciclovir were transported by hOAT1 but not hOAT3 (33). It is assumed that the urinary excretion of these drugs is affected by hOAT1 expression levels.

In conclusion, the expression level of hOAT3 mRNA was suggested to be a significant pharmacokinetics marker in predicting the rate of elimination of anionic drugs in patients with mesangial proliferative GN.

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