Membrane transporters play an important role in the metabolism of drugs and endogenous substrates. Recently, it was reported that certain single nucleotide polymorphisms (SNPs) in transporters including ABC (ATP-binding cassette) transporters and SLC (solute carrier) transporters are related to their altered transport activities, which may have clinical implications (1). Thus, functional analysis of genetic polymorphism of transporters is clinically important.

Organic cation transporters (OCTs) mediate sodium-independent electrogenic transport of small organic cations with different molecular structures (2). These organic cations include clinically used drugs (e.g., metformin), endogenous compounds (e.g., catecholamine), as well as toxic substances (e.g., MPP+). Recently, several functionally relevant genetic variations in human OCT1 (hOCT1, SLC22A1) (3, 4) and hOCT2 (SLC22A2) (5) were reported. In contrast, there is no report concerning the functional characterization of genetic variations of hOCT3, although the coding region polymorphisms were already reported (6).

hOCT3 (SLC22A3), also designated as extraneuronal monoamine transporter (EMT), expressed in the kidney, liver, and placenta, participates in the cellular uptake and elimination of various cationic substrates including therapeutically important agents as well as in the inactivation of biogenic amines such as catecholamines and histamine (7). In the public SNP database (NCBI dbSNP, http://www.ncbi.nlm.nih.gov/SNP/), five nonsynonymous SNPs of SLC22A3 are reported, but functional analysis of these genetic variations has not been performed yet. In this report, we characterized the functional properties of the hOCT3 variants in transiently transfected cells.

Full-length cDNA of hOCT3 was obtained as described previously (8). The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to introduce point mutations into hOCT3 cDNA in the expression vector according to the manufacturer’s instructions. Complementary oligonucleotides used for mutagenesis are described in Table 1. All the final sequences were confirmed by DNA sequencing.

HEK293 cells and COS cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO2. Transient transfection with Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer’s instructions. After transfection, the cells were grown 36 – 48 h before the experiments.

Cellular uptake of [3H]histamine (2.05 Bq/mmol) and [3H]MPP+ (2.96 TBq/mmol), purchased from American...
Radiolabeled Chemicals (St. Louis, MO, USA), were measured in hOCT3 (or its mutants)–transfected HEK293 cells grown on poly-D-lysine-coated 24-well plates. The cells were incubated in serum-free Hanks’ balanced salt solution (HBSS) containing the following: 125 mM Na gluconate, 4.8 mM K gluconate, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 1.3 mM Ca gluconate, 5.6 mM glucose, and 25 mM HEPES (pH 7.4) for 10 min. The uptake study was started by adding HBSS containing [$^3$H]histamine (200 nM) or [$^3$H]MPP$^+$ (30 nM) to the plate. After 2 min, the cells were washed twice in ice-cold HBSS and then lysed in 0.1 N NaOH for 20 min, followed by measurement of radioactivity by scintillation counting.

COS cells transfected with vectors containing wild-type hOCT3 and mutants were washed with PBS containing 1 mM MgCl$_2$ and 0.1 mM CaCl$_2$ (PBS$^{++}$), fixed in cold methanol for 7 min, and washed 3 times with PBS$^{++}$. Fixed cells were permeabilized in permeabilization buffer (0.1% BSA and 1% Triton X-100 in PBS$^{++}$) for 15 min and blocked in goat serum dilution buffer (GSDB) (10% goat serum, 1% Triton X-100, and 10 mM glycine in PBS$^{++}$) for 30 min. Cells were incubated with primary antibodies diluted in GSDB buffer overnight at 4°C. An anti-Na,K-ATPase monoclonal antibody directed against an epitope between residues 338 to 724 of the chicken Na,K-ATPase (9) and anti-OCT3 polyclonal antibody (Transgenic Inc., Kumamoto) were used for primary antibodies. Cells were washed 3 times with permeabilization buffer and then incubated with Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 546 anti-mouse IgG (Invitrogen), and 1 μg/ml DAPI (Roche, Mannheim, Germany) diluted in GSDB buffer for 1 h, after which they were washed 3 times in PBS$^{++}$ and once in water. Cells were mounted in Vectashield (Vector laboratories, Burlingame, CA, USA). Fluorescence was visualized with a laser confocal microscope. Contrast and brightness settings were chosen so that all pixels were within the linear range.

In the public SNP database (NCBI dbSNP), we found five nonsynonymous nucleotide polymorphisms of hOCT3 (SLC22A3): T44M, A116S, T400I, A439V, and G475S. As shown in Fig. 1A, T44 is located near the 3′-end of the first transmembrane domain (TMD); A116 is in the middle of the long extracellular loop between the first and second TMDs; T400 is located near the 3′-end of the eighth TMD; A439 is located near the 5′-end of the tenth TMD; G475 is located near the 5′-end of the eleventh TMD. Among these, T44 is conserved from hOCT1 to hOCT3 as well as hOCTN1 and hOCTN2, and T400 is conserved from OCT1 to OCT3, but A116, A439, and G475 are found only for OCT3 (2).

To examine whether hOCT3 polymorphisms found in the SNP database affect functional activities, we constructed site-directed mutants and expressed them in HEK293 cells. As shown in Fig. 1, B and C, the uptakes of representative OCT3 substrates, [$^3$H]histamine and [$^3$H]MPP$^+$, were largely reduced for A116S and A439V and moderately for T400I. The uptake of [$^3$H]histamine for G475S was slightly decreased. In contrast, the T44M mutant showed no significant changes in the uptake. Plasma membrane localizations of hOCT3 variants were examined by immunofluorescent analysis. Figure 2 shows the staining of hOCT3 proteins in COS cells transfected with cDNAs of hOCT3 variants. Wild-type and mutant hOCT3 proteins were localized in the plasma membrane, detected by anti-Na,K-ATPase monoclonal antibody, as well as in the cytoplasm, confirming that altered transport activities are not likely to be induced by

<table>
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<th>refSNP ID</th>
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Transport Activities of SLC22A3 SNPs

Since membrane transporters and metabolic enzymes are both involved in renal and hepatic clearance of drugs, alteration of drug transport activities in the tissues could have an important influence on pharmacokinetics of its substrate drugs. For example, Zwart et al. reported that Oct3/Slc22a3 (mouse homologue of hOCT3)-deficient mice exhibit decreased accumulation of the neurotoxin MPP⁺ in the heart (10). In the same way, aforementioned functionally relevant hOCT3 variations are likely to explain some of the inter-individual variations in cationic drug disposition. Thus, it would be interesting to know if there are changes in the MPP⁺ susceptibility of individuals with altered hOCT3 function.

To date, there is one report on phenotypic differences suspected to be due to hOCT3 polymorphism. Aoyama et al. found that some SNPs in hOCT3/SLC22A3 are related to the development of polysubstance abuse in Japanese individuals with dependence on the amphetamine derivative methamphetamine (MAP) (11). It may be that altered disposition of MAP affects one’s behavior to seek other addictive substances. Interestingly, it was reported that OCT3 is involved in the disposition of MAP and behavioral changes induced by MAP in an animal model (12). Obviously, it is necessary to determine whether MAP is a transport substrate of hOCT3 and whether the SNPs reported in this study affect transport activity of hOCT3.

Furthermore, removal of endogenous cations such as catecholamine and histamine may be affected by the transport function of OCT3 (13). For example, Schneider et al. reported that pharmacologic modulation of histamine transport via OCT3 might become important in the control of basophil functions during allergic diseases (14) and Vialou et al. demonstrated that the presence of OCT3 is critical for salt-intake regulation that is closely related to blood pressure (15). Thus, it would be interesting to see the effect of functionally relevant SNPs in hOCT3 on the disease process of allergy and hypertension.

From the point of the structure–function relationship, the reduction of transport function in T400I of hOCT3 seems interesting because residue T400 is conserved from OCT1/Oct1 to OCT3/Oct3 (2). Functional analysis of the mutants that have the corresponding amino acid replacement in hOCT1 and hOCT2 may provide information about the importance of this residue in the function of organic cation transport in hOCTs. Further study is necessary to clarify this structure–function relationship.

In summary, we characterized the functional properties of the hOCT3 variants and found, for the first time, that three variants are associated with reduced transport activity. This study suggests that the hOCT3 variants will contribute to inter-individual variations in cationic drug disposition as well as certain disease processes such as hypertension and allergic and neuropsychiatric diseases.
Acknowledgments

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References


