Genetic Polymorphisms in Organic Cation Transporter 1 (OCT1) in Chinese and Japanese Populations Exhibit Altered Function[S]

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ABSTRACT

Organic cation transporter 1 (OCT1; *SLC22A1*) seems to play a role in the efficacy and disposition of the widely used antidiabetic drug metformin. Genetic variants in OCT1 have been identified largely in European populations. Metformin is increasingly being used in Asian populations where the incidence of type 2 diabetes (T2D) is on the rise. The goal of this study is to identify genetic variants of OCT1 in Chinese and Japanese populations, which may potentially modulate response to metformin. We used recent data from the 1000 Genomes Project (Chinese and Japanese) and direct sequencing of selected amplicons of OCT1 in 66 DNA samples from Japanese patients with T2D. A total of six nonsynonymous variants were identified. Three of them (Q97K, P117L, and R206C) had not been functionally characterized previously and had allele frequencies of 0.017, 0.023 and 0.008, respectively. The uptake of metformin in cells expressing Q97K, P117L, and R206C was significantly reduced relative to the OCT1 reference (62 ± 4.3, 55 ± 6.8, and 22 ± 1.5% for Q97K, P117L, and R206C, respectively). Kinetic studies indicated that P117L and R206C exhibited a reduced V_{max}, whereas Q97K showed an increased K_m. The green fluorescent protein (GFP)-tagged Q97K and P117L variants localized to the plasma membrane, whereas the GFP-tagged R206C was retained mainly in the endoplasmic reticulum. Replacement of the highly conserved R206 with different amino acids modulated the subcellular localization and function of the transporter. This study suggests that nonsynonymous variants of OCT1 in Chinese and Japanese populations may affect the differential response to metformin.

Introduction

Metformin, a biguanide, is widely used as first-line therapy for the treatment of type 2 diabetes (T2D) (Kirpichnikov et al., 2002). The molecular mechanisms underlying metformin action seem to be related to its activation (phosphorylation) of AMP-activated protein kinase (AMPK), which suppresses glucagon-stimulated glucose production and causes an increase in glucose uptake in muscle and hepatic cells (Zhou et al., 2001). Metformin has been well characterized in vitro as a substrate of organic cation transporters (OCTs), including *SLC22A1* (OCT1), which is one of the most abundantly expressed transporters in the human liver and plays a major role in the hepatic uptake of metformin (Jonker and Schinkel, 2004; Koepsell, 2004). In previous studies, we and others have shown that human OCT1 is highly polymorphic in ethnically diverse populations (Sakata et al., 2004; Shu et al., 2007). Many nonsynonymous polymorphisms of *SLC22A1*, found primarily in white populations, exhibit reduced activity in cellular assays. Genetic variants of *SLC22A1* have been shown to modulate

ABBREVIATIONS: OCT, organic cation transporter; OCT1-ref, OCT1-reference; AMPK, AMP-activate protein kinase; SNP, single-nucleotide polymorphism; nsSNP, nonsynonymous SNP; CHB, Chinese in Beijing; JPT, Japanese in Tokyo; T2D, type 2 diabetes; GFP, green fluorescent protein; ER, endoplasmic reticulum; WGA, wheat germ agglutinin; MFV, mean fluorescence value; HEK, human embryonic kidney; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; OAT, organic anion transporter.

the pharmacokinetics of metformin after oral administration and reduce the therapeutic response, presumably by decreasing the hepatic uptake of the drug (Shikata et al., 2007; Shu et al., 2007, 2008). However, genetic variants of *SLC22A1* that are related to decreased metformin uptake (i.e., S14F, R61C, S189L, G220V, G401S, 420del, and G465R) have been identified primarily in populations with European ancestries and have not been identified in Asian American, Chinese, Korean, and Japanese populations (Song et al., 2008). In one study with 33 Japanese patients with T2D, two *SLC22A1* polymorphisms (intron1 −43T>G and V408M), which do not exhibit altered function, were shown to have no significant effects on the clinical efficacy of metformin (Shikata et al., 2007).

The goal of the current study was to identify and functionally characterize nonsynonymous variants in OCT1 in Chinese and Japanese populations. To achieve this goal, we searched data from Chinese and Japanese samples in the 1000 Genomes Project (http://www.1000genomes.org/page.php) and analyzed DNA samples obtained from 66 Japanese patients with T2D. Our study demonstrates that several OCT1 nsSNPs in Chinese and Japanese samples exhibited altered function with respect to metformin uptake. One rare variant, R206C, which altered the highly conserved amino acid residue arginine, was characterized in detail to determine the molecular mechanisms by which this residue affected the function and intracellular trafficking of OCT1.

Materials and Methods

Subjects. Study subjects consisted of 66 Japanese patients with type 2 diabetes who were on metformin. Patients were recruited at the Diabetes Center of Tokyo Women's Medical University. The diagnosis of type 2 diabetes was made based on 1985 World Health Organization criteria. The subjects were 32 men and 34 women, age at the study was 58.4 ± 12.9 years (mean $\pm$ S.D.), body mass index was 25.4 ± 3.8 kg/m^2, and duration of diabetes was 13.0 ± 7.6 years. The sequencing data of the 1000 Genomes Project were obtained from http://pharmacogenetics.ucsf.edu, which combined the sequencing data from the Pharmacogenetics of Membrane Transporters Database and the 1000 Genomes Project.

Genotyping and Clinical Study. Genomic DNA samples from the 66 patients were genotyped for nine OCT1 polymorphisms (intron1 −43T>G, S14F, R61C, S189L, G220V, G401S, V408M, 420del, and G465R) by direct sequencing using an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA) in both directions. The primer design and polymerase chain reaction conditions are described in Shu et al. (2007). Other nonsynonymous variants in the sequenced amplicons were also identified. Informed consent from the patients for the DNA study was obtained. The protocol and consent were reviewed and approved by the Institutional Review Boards at

the University of California, San Francisco and Tokyo Women's Medical University.

Construction of OCT1 Variants. Variant cDNA clones of OCT1 were constructed by site-directed mutagenesis of the OCT1-reference (OCT1-ref) by using Pfu Turbo DNA polymerase (Invitrogen, Carlsbad, CA). The primers for mutagenesis are listed in Table 1. Sequences of variant cDNAs were confirmed by direct sequencing, and the full cDNA of each variant was sequenced to verify that only the intended mutation was introduced.

Green Fluorescent Protein Fusion Constructs. Variant cDNA constructs of OCT1 were subcloned in-frame with green fluorescent protein (GFP) at the C terminus of an OCT1 containing pcDNA5/FRT expression vector. The GFP fusion constructs were used to generate stable cell lines by using the Flp-In system (Invitrogen) as described above. To localize OCT1, cells were plated on poly-D-lysine-coated glass coverslips (BD Biosciences, San Jose, CA) in 24-well plates at a density of 1.5×10^5 cells per well. Twenty-four hours after seeding, cells were fixed with 4% paraformaldehyde in HBSS for 15 min and then washed three times in HBSS, followed by incubation with 5 μg/ml Alexa Fluor 594 wheat germ agglutinin (WGA) (Invitrogen) in HBSS for 10 min. To remove the stain, cells were washed three times with blank HBSS cover slips, removed from the 24-well plate, and mounted in Vectashield antifade solution (Vector Technologies, Inc., Burlingame, CA) on glass microscope slides. The nucleus was stained with 4′,6-diamidino-2-phenylindole. Endoplasmic reticulum (ER) was stained by ER-specific marker antibody (1:200) anticalnexin, which was labeled by Alexa Fluor 594 (red)-conjugated secondary antibody. A Retiga charge-coupled device cooled camera and associated QCapture Pro software (QImaging Surrey, BC, Canada) were used to visualize the cells.

Transport Studies. HEK293 Flp-in cells were routinely cultured in Dulbecco's modified Eagle's medium (DME H-21; University of California Cell Culture Facility, San Francisco, CA), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. For the reference and variants of OCT1, detailed functional studies were performed by using stably transfected HEK293 Flp-In cells generated according to the manufacturer's protocol (Invitrogen). In brief, single-copy, site-specific stable integrants were created by using the Flp-In system (Urban et al., 2006). This allows comparison among allelic variants of a gene while avoiding concerns about integration of cDNA into different sites, which may produce differences in the expression levels of the variant and reference transporters. Cells were plated at a density of 6×10^5 cells per well in six-well plates and incubated overnight in antibiotic-free media. After 24 h, cells were transfected with 0.4 μg of reference or variant OCT1 cDNA, 3.6 μg of pOG44 DNA, and 20 μg of Lipofectamine 2000 per well. Cells were split 1:4 into six-well plates after 2 days and selected for stable transfectants by using hygromycin B (75 μg/ml). After 2 weeks of exposure to hygromycin B, multiple colonies of stable cells were obtained for each variant. The colonies were pooled to obtain the reference and variant cell lines, which were used for functional analyses.

Uptake studies were performed at 37°C for 5 min as described

TABLE 1
Site-directed mutagenesis primer list
The mutated genetic codons were underlined.

Name	Sequence
OCT1-Q97K	CTATGAAGTGGACTGGAAC<u>AAG</u>AGCGCCCTCAGCTGTGTAGACC
OCT1-Q97E	CTATGAAGTGGACTGGAAC<u>GAG</u>AGCGCCCTCAGCTGTGTAGACC
OCT1-P117L	CAACAGGAGCCACCTG<u>TTG</u>CTGGGTCCCTGCCAGGATG
OCT1-P117S	CAACAGGAGCCACCTG<u>TCG</u>CTGGGTCCCTGCCAGGATG
OCT1-R206C	GTCCATGCTGCTCTTC<u>TGT</u>CTGCTGCAGGGCCTGGTCAG
OCT1-R206K	GTCCATGCTGCTCTTC<u>AAG</u>CTGCTGCAGGGCCTGGTCAG
OCT1-R206E	GTCCATGCTGCTCTTC<u>GAG</u>CTGCTGCAGGGCCTGGTCAG
OCT3-R212C	CCCTGTGTTTGTGATC<u>TTCTGC</u>TTCCTGCAAGGTGTATTTGGAAAG
OAT2-R202C	CTATGTAATGTTTGCCATCACC<u>TGC</u>ACCCTTACTGGCTCAGCCC

previously (Shu et al., 2008). Intracellular radioactivity was determined by scintillation counting and normalized to per well of protein content as measured by using the bicinchoninic acid protein assay. Results were expressed as the percentage of activity of the OCT1-ref wells. Transport of radiolabeled substrates (1-methyl-4-phenylpyridinium, tetraethylammonium, [^{14}C]metformin, and [^{3}H]serotonin) (PerkinElmer Life and Analytical Sciences, Waltham, MA) was tested in at least three separate experiments for all variants. Kinetic studies were performed as described below, with varying concentrations of unlabeled substrate (metformin) added to the uptake buffer. Rates of uptake (*V*), expressed as pmol/min/mg protein, were fit to the equation: $V = V_{max} \times [S]/K_m + [S]) + K_o \times [S]$, where K_o represents the first-order rate constant for non-OCT1-mediated uptake, [S] is the substrate concentration, and V_{max} and K_m are the Michaelis-Menten kinetic parameters. These experiments were repeated in triplicate using the OCT1-ref and its variants.

Western Blotting. For metformin treatment, HEK293 stably transfected cells were adapted with serum-free medium for 24 h (Zang et al., 2004). Then cells were treated with metformin (1 mM) for 1 h, washed with blank medium without serum, and incubated for 5 h before harvest.

Cultured cells were lysed at 4°C for 20 min in RIPA buffer (Sigma-Aldrich, St. Louis, MO) with the protease inhibitors dissolved from Complete protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN). After centrifugation for 20 min at 14,000*g* at 4°C, proteins (40 μg) from the supernatant were separated on 10% SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C with Tris-buffered saline with 0.05% Tween 20 and 5% nonfat milk. Signals from immunoblotting, performed following standard procedures, were detected by chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Primary antibodies were directed against GFP, AMPKα, AMPKα phosphorylated at Thr172, Na$^+$/K$^+$ATPase, and β-actin (Cell Signaling Technology, Danvers, MA).

Biotinylation of the Cell Surface. The biotinylation of the HEK cell surface proteins was performed with the Pierce Cell Surface Protein Isolation Kit (Pierce Chemical, Rockford, IL). In brief, the HEK cells from $2 \times 10\text{-cm}^2$ culture plates were biotinylated with 10 ml of a 490 mM sulfo-NHS-SS-biotin solution in PBS. The biotinylation reaction was terminated by adding Tris-HCl to a final concentration of 4.9 mM. The cells were harvested by scraping in 10 ml of PBS containing 490 mM oxidized glutathione. The cells were pelleted and lysed for 30 min on ice with 1 ml of RIPA lysis buffer with protease inhibitor cocktails (Sigma-Aldrich). The cell lysate was cleared by centrifugation (16,100*g*, 10 min). Clarified cell lysate (500 μl) was added to NeutrAvidin Agarose slurry. Unbound proteins were removed by washing buffers. Captured proteins were eluted from streptavidin Sepharose by incubation with 400 μl of SDS-polyacrylamide gel electrophoresis. Sample buffer with 50 mM dithiothreitol was added to the gel for 60 min at room temperature

with end-over-end mixing on a rotator. Column centrifuged for 2 min at 1,000*g*. The eluted samples were subjected to the Western blotting assay.

Flow Cytometry. Flow cytometric analysis was used to determine surface expression measured as membrane immunofluorescence using GFP chimeras of OCT1 and its variants (Chen et al., 2006). Stably transfected HEK293 cells were harvested and washed with PBS. The fluorescence density was measured with a Coulter Elite instrument (Beckman Coulter, Fullerton, CA) and analyzed with WinMDI 2.8 software provided by Duke University (Durham, NC). The expression level was evaluated as the mean fluorescence value (MFV).

Multiple Sequences Alignment. Sequences of representative transporters were downloaded from the National Center for Biotechnology Information (Bethesda, MD) database in October 2009. Multiple sequence alignments were obtained by using MUSCLE (Edgar, 2004) and visualized by using Jalview (http://www.jalview.org/). The aligned residues were colored based on their type (using the Clustlx color scheme in Jalview) and their level of conservation (the more conserved the residue in a given position, the stronger the color).

Statistical Analysis. In general, data are expressed as mean ± standard deviation. For statistical analysis, multiple comparisons were analyzed by using one-way analysis of variance followed by Dunnett's two-tailed test. The basis for comparison was OCT1-ref, unless stated otherwise. Quantitative data were analyzed with Prism 4.0 (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Six Nonsynonymous Genetic Variants in OCT1 Were Identified in Chinese and Japanese DNA Samples. To identify nsSNPs in Chinese and Japanese populations, we surveyed recent data from the 1000 Genomes Project [consisting of 30 samples each for Chinese in Beijing (CHB) and Japanese in Tokyo (JPT)] and analyzed selected exons from 66 DNA samples from Japanese patients with T2D. None of the seven previously identified reduced function-coding OCT1 variants (S14F, R61C, S189L, G220V, G401S, 420del, and G465R) in white subjects were found in the CHB/JPT sample from the 1000 Genomes Project. Rather, four nonsynonymous variants were identified in the CHB/JPT sample (Tables 2 and 3). Of these four, three (L160F, P341L, and V408M) had previously been functionally characterized and found to exhibit no changes in function (Shu et al., 2007). The fourth variant, Q97K, was found on two chromosomes at an allele frequency of 0.017. In the 66 samples from patients with T2D, OCT1 polymorphisms (intron1 −43T>G and V408M), previously identified in white populations, were

TABLE 2

Genetic variants of OCT1 identified in Chinese and Japanese from the 1000 Genomes Project (nsSNPs) (*n* = 59) and 66 Japanese patients with type 2 diabetes

Coding Position	Amino Acid Position	Ref SNP	Allele Frequency		No. of Chromosomes with Variant (a/b)
			66 Patients with T2D[a]	1000 Genome Project	
−43 T>G	Intron 1	rs4646272	0.34	0.59	45/70
289 C>A*	Q97K	N.A.	N.D	0.017	0/2
480 G>C	L160F	rs683369	N.D	0.88	0/104
350 C>T*	P117L	N.A.	0.023	N.D	3/0
616 C>T*	R206C	N.A.	0.008	N.D	1/0
1022 C>T	P341L	rs2282143	N.D	0.19	0/22
1222 G>A	V408M	rs628031	0.15	0.19	20/22

N.A., not available. N.D, not determined.

* The nsSNPs were functionally characterized in this report. Positions are relative to the ATG start site and are based on the cDNA sequence from GenBank accession no. NM_003057. a/b, the number of chromosome with variants between [a]66 patients with T2D and [b]59 CHB/JPT in the 1000 Genomes Project.

TABLE 3
Characteristics of patients carrying variants of OCT1 in 66 Japanese patients with T2D

OCT1 Variant	Age	Gender	Body Mass Index	Dose of Metformin
			kg/m^2	mg/day
P117L	73	Female	23.5	500
P117L	69	Male	22.8	750
P117L	64	Male	19.7	500
R206C	67	Male	25.5	1000

found at allele frequencies of 0.34 and 0.15 (Table 2). The seven reduced-function OCT1 polymorphisms identified in white samples were not identified in the samples either. It is noteworthy that two other nsSNPs, P117L and R206C, were identified (Table 2). The information of the patients carrying these two variants is summarized in Table 3. Previously, P117L was identified as a singleton (allele frequency of 0.004) in 116 Japanese patients with cardiac arrhythmias (Itoda et al., 2004). In the 66 samples from Japanese patients with T2D, P117L was identified in three chromosomes in three patients with an allele frequency of 0.02. R206C, identified as a singleton in the same sample set above in a male patient, has not been found in previous studies, which involved direct sequencing of OCT1 (Itoda et al., 2004; Sakata et al., 2004) (Table 3).

Genetic Variants of OCT1, Q97K, P117L, and R206C Have Reduced Function. The uptake of metformin by Q97K was significantly reduced relative to the OCT1-ref ($62 \pm 4.3\%$, $p < 0.001$) (Fig. 1A). Kinetic studies indicated that Q97K had an increased K_m (3.31 ± 0.69 mM versus 1.18 ± 0.18 mM for the reference), but the V_{max} was approximately the same as OCT1-ref (3.47 ± 0.12 nmol/mg protein/min versus 3.96 ± 0.44 nmol/mg protein/min for the reference) (Fig. 1B; Table 4). The GFP-tagged Q97K did not disrupt the subcellular localization of OCT1 and did not change the measured cellular OCT1 protein level as indicated by a lack of observable change in the overall fluorescence emission signal and Western blotting (Fig. 2, A and C)

For the nsSNPs from Japanese patients with T2D, the uptake of metformin in cells expressing P117L and R206C was significantly reduced relative to the OCT1-ref (55 ± 6.8 and $22 \pm 3.5\%$ for P117L and R206C, respectively; $p < 0.001$) (Fig. 1A). Kinetic studies indicated that both variants exhibited a reduced V_{max} (Fig. 2B). The V_{max} of P117L was approximately half of the reference (2.02 ± 0.27 versus 3.96 ± 0.74 nmol/mg protein/min), but the K_m was similar to OCT1-ref (1.51 ± 0.15 versus 1.18 ± 0.18 mM). R206C had a dramatically reduced V_{max}, which was approximately one-fifth of that for OCT1-ref (0.79 ± 0.15 versus 3.96 ± 0.74 nmol/mg protein/min). Its K_m was slightly increased (Fig. 1B; Table 4). To further characterize the function of these variants, we also tested the three variants on the uptake of the model substrates, 1-methyl-4-phenylpyridinium, tetraethylammonium, and serotonin (supplemental Fig. S1). There was no substrate selectivity among these substrates that showed a similar uptake pattern as that of metformin, implying these variants might have a universal impact on the substrate binding of OCT1.

Metformin triggers the phosphorylation of AMPK in the liver to lower blood glucose levels (Zhou et al., 2001). Genetic variants in OCT1 with impaired function can affect this process by affecting intracellular concentrations of met-
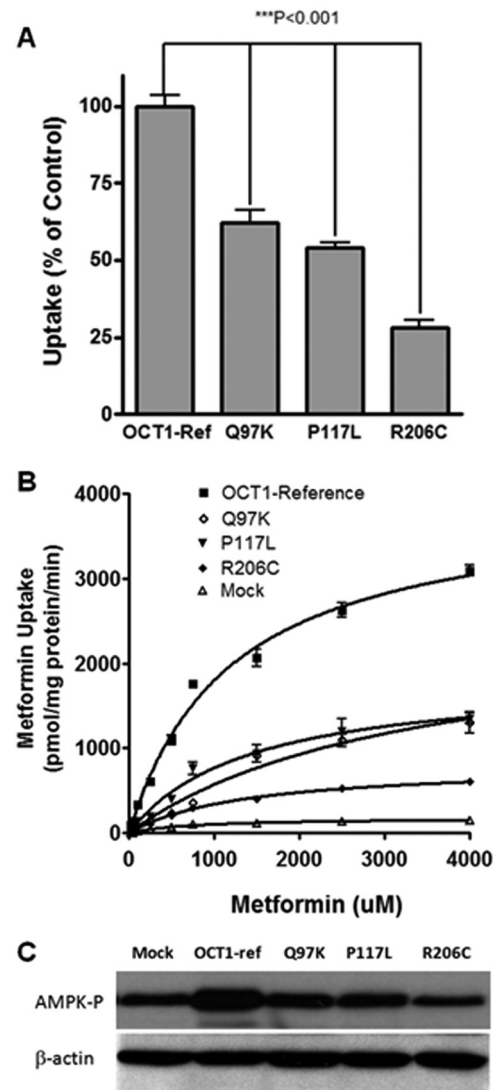

Fig. 1. Functional analyses of three nonsynonymous variants of OCT1. Variants of OCT1 were expressed in stably transfected HEK293 cells and assayed for activity by measurement of uptake of radiolabeled probe substrate (10 μM [14C]metformin) at 5 min. A, uptake activity of two variants compared with OCT1-ref (control). Uptake activity was calculated as [(OCT1or Variant-Mock)/OCT1-Mock] × 100. Values are expressed as mean ± S.D. ∗∗∗, $P < 0.001$ versus OCT1-ref. B, kinetics of metformin transport by reference OCT1 and variants P117L and R206C. HEK293 cells stably expressing either OCT1-ref, OCT1-P117L, OCT1-R206C, or Mock were incubated in the presence of 10 μM [14C]metformin and varying concentrations of unlabeled metformin for 5 min. C, metformin-stimulated AMPK phosphorylation in cell lines stably transfected mock, OCT1, and OCT1's three variants. Immunoblots were performed against phospho-AMPKα (Thr172), AMPKα, and β-actin.

formin in hepatocytes (Shu et al., 2007). To study the relationship of AMPK activation and transporter function, we treated the stably transfected HEK293-Mock, OCT1, and its three variants Q97K, P117L, and R206C with metformin. After 1 h of treatment with metformin at 1 mM, both HEK293-Q97K and HEK293-P117L showed a reduced level of phosphorylation of AMPK. The phosphorylation of AMPK was markedly reduced in HEK293-R206C and was similar to the phosphorylation of AMPK in HEK293-Mock (Fig. 1C). The ability of metformin to activate AMPK was generally proportional to the function of OCT1, which governed the availability of metformin to its target.

TABLE 4
Kinetic parameters of metformin uptake in HEK293 cells stably transfected with human OCT1 (reference) or its variants

Data were fit to a Michaelis–Menten equation. Results were expressed as nmol/mg protein/min. Data points represented the mean ± S.D. from triplicate wells in a representative experiment. The K_m and V_{max} values were determined from the fit of the data in Fig. 1B.

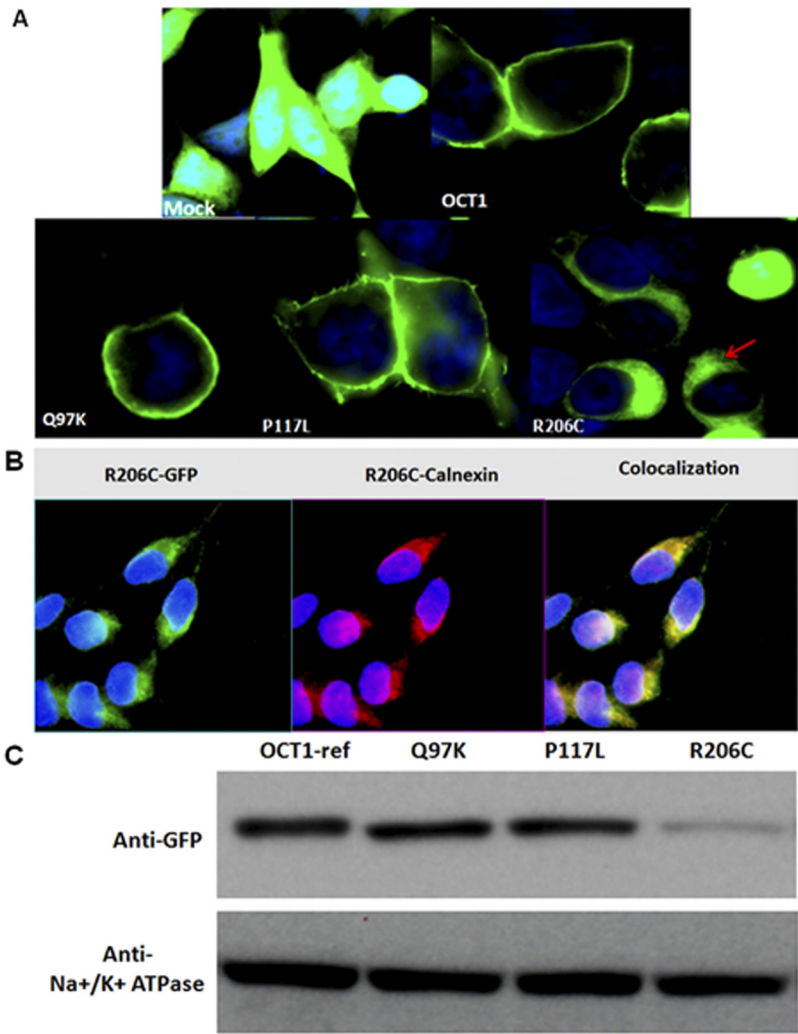| Kinetic Parameter	OCT1-ref	Q97K	P117L	R206C
V_{max} (nmol/mg protein/min)	3.96 ± 0.44	3.47 ± 0.12	2.02 ± 0.27*	0.79 ± 0.15*
K_m (mM)	1.18 ± 0.18	3.31 ± 0.69*	1.51 ± 0.15	1.31 ± 0.22

* $P < 0.05$ versus OCT1-ref.

Fig. 2. Subcellular localization and quantification of surface expression of OCT1 and three genetic variants. A, OCT1-variant GFP fusion constructs were transiently expressed in HEK293 cells and visualized by fluorescence microscopy. The nucleus, which was stained with 4′,6-diamidino-2-phenylindole, is shown in blue. GFP-OCT1 proteins are shown in green. In GFP-Mock cells, colocalization of GFP-derived signal and that from nucleus stain is shown in cyan. The red arrow in R206C indicates that this variant changed the OCT1 protein subcellular localization. B, Colocalization of GFP-R206C and ER. Orange indicates the GFP-R206C was well colocalized with ER. C, biotinylation of the plasma membrane of GFP-tagged OCT1 and its variants. Q97K and P117L had similar cell surface expression levels to the OCT1-ref. R206C showed dramatically reduced cell surface expression compared with OCT1-ref.

To further explore the underlying reason for the reduced function of the two variants of OCT1, GFP chimeras were constructed. GFP-Mock (empty vector-transfected cells) showed strong cytoplasmic and nuclear expression of GFP (Fig. 3). The GFP-tagged Q97K and P117L variants localized to the plasma membrane, similar to the GFP-OCT1-ref transporter (Fig. 3A). It is noteworthy that GFP-tagged R206C localized intracellularly and seemed to reside predominantly on the ER (Fig. 3, A and B), suggesting that there was a fundamental effect of arginine at this position influencing the localization of the protein to the plasma membrane. Consistent with the results from fluorescence microscopy, biotinylation studies showed that the surface expression was almost totally eliminated by R206C (Fig. 3C). To further characterize the genetic variants Q97K and P117L, we also created substitutions for them. For variant Q97K, we generated a mutation, Q97E, replacing the polar

uncharged glutamine with negatively charged glutamate. For variant P117L, a polar serine replaced the hydrophobic proline as P117S. Neither of the two mutations affected the subcellular localization of OCT1 and expression level. The results are shown in supplemental Fig. S2.

Various Substitutions of Arginine 206 Affected the Subcellular Localization and Function of OCT1. A positively charged residue, arginine-206, greatly affected the subcellular localization of OCT1 (Fig. 3) and showed strong expression in the membrane protein translocation apparatus. To investigate the role of charged residues in the function of OCT1 expression localization, we substituted a similarly charged amino acid, lysine, for arginine (R206K) and a negatively charged glutamate for arginine (R206E). The negatively charged substitution R206E was not only retained in the ER but also seemed to result in a significantly reduced
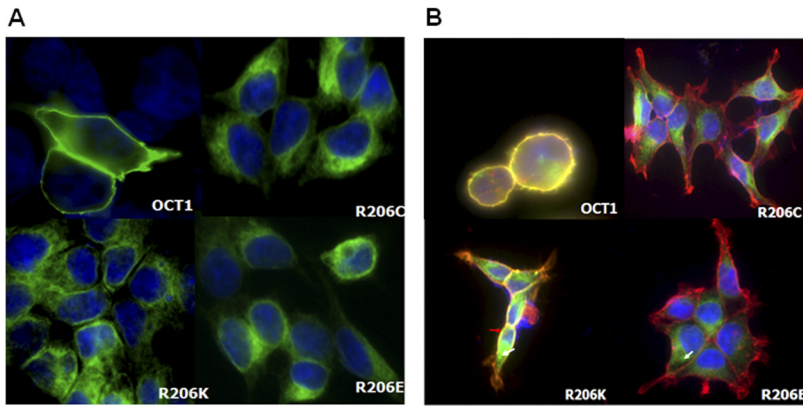
Fig. 3. Subcellular localization of different substitutions of R206. A, OCT1-variant GFP fusion constructs were transiently or stably expressed in HEK293 cells and visualized by confocal microscopy. Red arrows indicate the expression of OCT1 protein localized to the plasma membrane, and the white arrow shows the protein localized in the membrane transporting apparatus ER. B, colocalization of GFP-R206 substitutions on the plasma membrane. The plasma membrane was stained with Alexa Fluor 594-labeled WGA, shown in red. GFP-R206 substitutions are shown in green. Colocalization of GFP-derived signal and that from the plasma membrane stain is shown in orange.
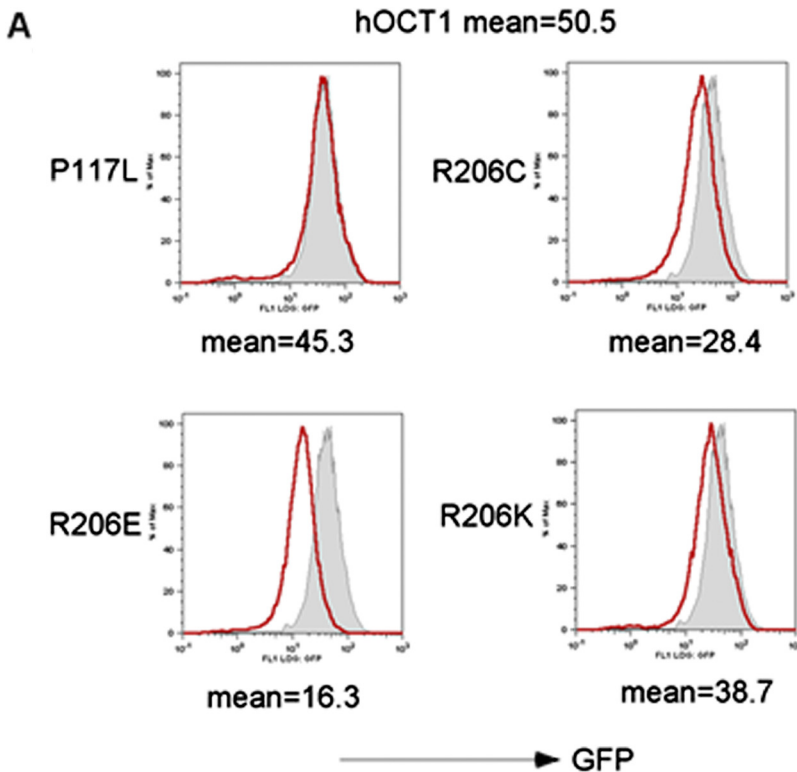

Fig. 4. Fluorescent intensity quantification and uptake assay of GFP-tagged OCT1 and its mutations. A, quantification of fluorescent intensity of GFP-tagged OCT1, its variants, or various substitutions of R206 with flow cytometry. All of the mutations (red peaks) were compared with GFP-OCT1 (gray peaks). B, uptake assays of metformin in cells expressing GFP-OCT1 and its mutations. Uptake activity was calculated as [(GFP-OCT1 or GFP-variant − Mock)/OCT1 − Mock] × 100. ∗∗∗, $P < 0.001$ versus GFP-OCT1. Data represent the mean ± S.D. from triplicate wells in a representative experiment.
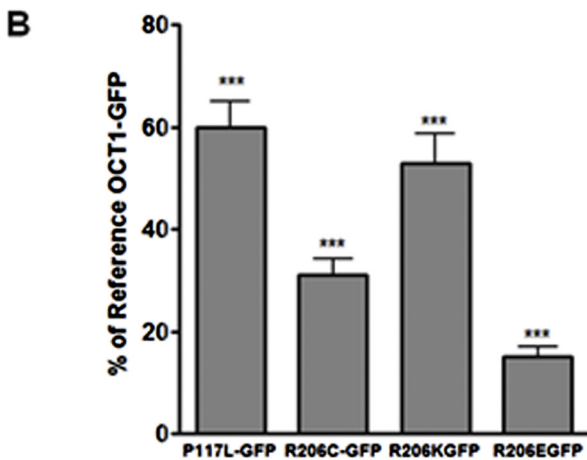
protein expression level compared with the GFP-R206C based on overall fluorescence intensity (Fig. 4A). Although the positively charged lysine residue improved expression on the plasma membrane in comparison to R206C, it still showed an appreciable retention in the ER (Fig. 4A). Costaining with the plasma membrane-specific labeling marker

WGA conjugated to the red fluorophore rhodamine indicated different patterns of colocalization with the green fluorophore-tagged OCT1 mutants (Fig. 4B). Consistent with our initial observations, cells expressing GFP-R206C had a slight overlap between the fluorescent signals of GFP and WGA in the plasma membrane, but most of the GFP signal was intracellular. Cells expressing GFP-R206E exhibited no appreciable overlap between the GFP and rhodamine signals in the plasma membrane. In contrast, cells expressing GFP-R206K had more colocalization of GFP and WGA-rhodamine than did cells expressing GFP-R206C and GFP-R206E as indicated by more yellow/orange fluorescence at the plasma membrane of the cells. These observations were further validated by both flow cytometry and metformin uptake studies (Fig. 4). The quantification of GFP expression with flow cytometry indicated that GFP-P117L (MFV = 48.1) had a slightly reduced expression level as GFP-OCT1 (MFV = 55.3). GFP-R206C reduced the total expression level by approximately 50% of the GFP-OCT1-ref (MFV = 28.3 versus 55.3) and altered its subcellular localization (Fig. 3). The lysine substitution, GFP-R206K, increased the expression level to 70% of the reference OCT1, compared with GFP-R206C. In contrast, GFP-R206E reduced the expression level even more than GFP-R206C (MFV = 16.3 for GFP-R206E compared with 28.4 for GFP-R206C). Consistent with its expression pattern, GFP-R206K demonstrated slightly increased metformin uptake compared with GFP-R206C (22% of GFP-OCT1-ref for GFP-R206C compared with 45% of GFP-OCT1-ref for GFP-R206K) (Fig. 5B). GFP-R206E reduced the function of transporter more than the cysteine substitution, retaining only approximately 15% of the OCT1-ref metformin uptake activity.

Evolutionary Conservation. The positively charged R206 regulated the localization of OCT1 to the plasma membrane. Mutations at this position changed OCT1 expression level on the plasma membrane. A positively charged residue, lysine, improved the surface expression of the transporter in comparison to a cysteine at this position, whereas a negatively charged residue, glutamate, further reduced OCT1 surface expression. A BLAST search of homologous sequences showed that R206 is not only highly conserved among species for organic cation transporters in the SLC22A family, but also some organic anion transporters (OATs) (e.g., human OAT1, OAT2, and OAT3). Representative sequences of OCT homologs in various species are listed in Fig. 5A, which shows that arginine is highly conserved not only in OCTs but also in human OATs. This evolutionary conserved arginine seems to be important in expression of the transporter on the plasma membrane. The secondary structure prediction indicated that Q97K and P117L were located in the large extracellular loop of OCT1, and R206C was in the loop of the third and fourth transmembrane domains (Fig. 5B). To test the homologous position of R206's effect on the plasma membrane expression of other transporters, we mutated the GFP-tagged OCT3 and OAT2 (Fig. 5C). R212C of OCT3 and R202C of OAT2 showed an expression pattern similar to that of R206C of OCT1. Disruption of this highly conserved arginine in other cation or anion transporters clearly resulted in a disruption of transporter subcellular localization.

Discussion

Organic cation transporters are critical for the absorption, distribution, and elimination of many small molecular weight basic compounds including endogenous amines and a wide array of drugs and environmental toxins (Jonker and Schinkel, 2004). Genetic polymorphisms in drug transporters have been increasingly recognized as possible sources of variation in drug disposition and response (Giacomini et al., 2007). As one of the most abundantly expressed transporters in the liver, OCT1 is important for metformin therapeutic action, and genetic variation in OCT1 may contribute to variation in response to the drug (Shu et al., 2007), although this remains controversial (Zhou et al., 2009). In particular, several studies (Shu et al., 2007; Becker et al., 2009) have demonstrated an association with response to metformin, whereas other studies have shown no association with response to the drug (Shu et al., 2007; Becker et al., 2009; Zhou et al., 2009).

To date, most of the studies of genetic variation in OCT1 have focused on European or European-American populations. In this study, combining the sequencing data from the 1000 Genomes Project with 66 Japanese patients with T2D, none of the seven previously identified reduced function OCT1 nonsynonymous variants (S14F, R61C, S189L, G220V, G401S, 420del, and G465R) were identified, consistent with previous observations in Japanese populations (Itoda et al., 2004; Sakata et al., 2004; Shikata et al., 2007; Shu et al., 2007). Only V408M has been found in both white and Japanese populations; however, this OCT1 variant seems to have normal function. In the current study, we identified three reduced function variants in OCT1 (Q97K, P117L, and R206C). To determine the frequencies of these variants in the Japanese population, we genotyped two nsSNPs (P117L and R206C) in the *SLC22A1* gene by using 3000 T2D and 3392 controls from samples stored in the Biobank Japan Project (RIKEN, Yokohama, Japan). For P117L (c.350C>T), we found 24 cases and 18 controls that were heterozygous for the minor allele. Minor allele frequencies were 0.4% in cases and 0.27% in controls. These data suggest that this nsSNP is not associated with type 2 diabetes. R206C (c.616C>T) was not found in either the cases or controls, suggesting that it is a very rare variant.

Although the in vitro studies demonstrated that metformin uptake and metformin-stimulated phosphorylation was reduced in cells expressing the two variants, because only four individuals carried them, it was not possible to determine whether the variants were associated with altered response to metformin. It is noteworthy that metformin dose, a known determinant of response to metformin, differed among the four. A large study will be needed to determine the clinical effect of rare variants of OCT1 on response to metformin. However, with new advances in next-generation sequencing technologies such studies are now possible. Other transporters such as MATE1/2 and OCT2/3, which also transport metformin, may modulate response to the drug, and genetic variants in these transporters and in other genes in the metformin response pathway may associate with the antidiabetic effect of the drug.

Examination of the functional effects of the variants on metformin transport has implications on our understanding of the amino acids that determine OCT1 activity and traf-
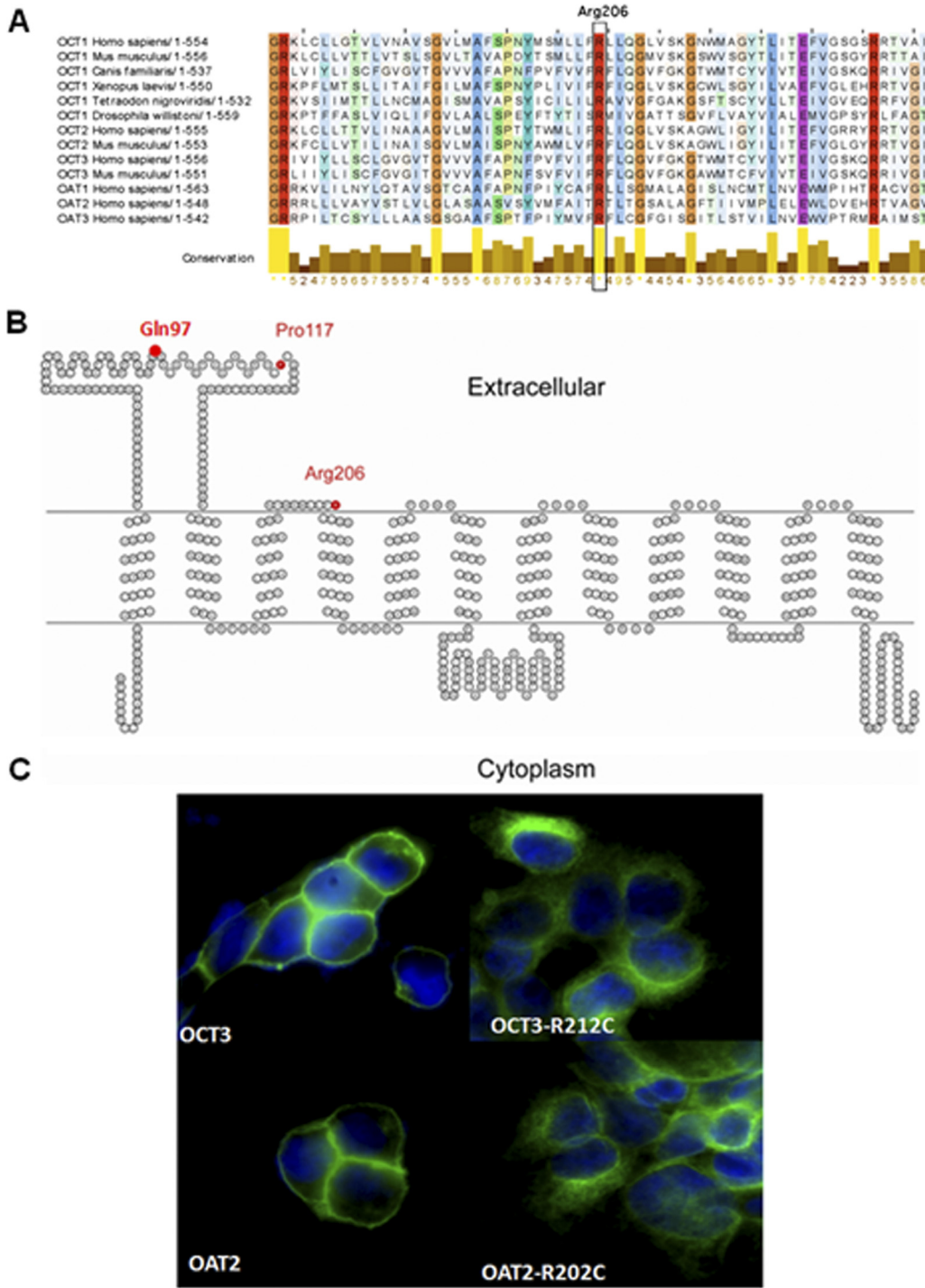
Fig. 5. Sequence alignment of the proximal region of R206 of OCT1 with its homologs. A, species are listed as representative species from invertebrate (*Drosophila*) to primates (*Homo sapiens*). The genes are identified or predicted cation transporters. Arginine 206 is not only highly conserved in all of the cation transporters of all of the species but it is also highly conserved in human organic anion transporters, OAT1–OAT3. B, topology of OCT1 revealed that Q97K and P117 were located at the extra-large loop and R206 was between the third and fourth transmembrane domains. Three variants are highlighted in the topology figure as red. C, cysteine mutation of highly conserved homologous arginine in OCT3 and OAT2 showed similar intracellular retention to the R206C in OCT1.

ficking. For example, in comparison to the reference OCT1, P117L exhibited a reduced uptake activity and V_{max} for metformin with a similar localization to the plasma membrane. A replacement of a rigid proline with a leucine, which has relatively flexible side chain, could potentially change the local structure of OCT1. Thus, the reduced V_{max} of metformin in cells expressing P117L could be caused by a structural change in OCT1. The K_m change of Q97K might suggest that this residue involved the substrate binding affinity. The replacement of the polar glutamate with a stronger positively charged lysine at 97 could increase the repulsion of cation substrates. Topology prediction indicates that these two residues are located at the large extracellular domain, which is generally considered as containing the transporter regulatory and substrate recognition domains (Jonker and Schinkel, 2004; Koepsell, 2004).

In contrast, the reduced activity of the R206C seemed to be explained by a much reduced surface expression level of the transporter. The reduced total GFP-tagged signal in cells expressing GFP-R206C or GFP-R206E may be caused by accelerated protein degradation in the ER with the misfolded immature protein. R206C reduced the export of OCT1 from the endoplasmic reticulum to the plasma membrane. Topology prediction indicated that R206 is located in the interlinking loop

between transmembranes 3 and 4 and is highly conserved in all OCTs and human OATs. The residue may be an evolutionarily conserved arginine-based ER-sorting motif of OCT1 and its homologs. Mutation of the homologous position of OCT3 and OAT2 strongly supported the idea that this highly conserved arginine is critical to the plasma membrane expression of both human cation and anion transporters that may share similar mechanisms for intracellular trafficking. Arginine-based ER localization signals are sorting motifs that are involved in the biosynthetic transport of membrane proteins (Kalandadze et al., 2004). Often, di-leucine endocytic signals occur close to arginine-based signals (Kalandadze et al., 2004). A previous study indicates that arginine-427 plays a critical role in the trafficking of the sodium-dependent glucose cotransporter (SGLT1) to the plasma membrane (Lostao et al., 1995), suggesting that the charged residue is essential in membrane protein trafficking. The disruption of R206 by the genetic variant cysteine and other substitutions might interfere with the conversed arginine-based motif (-LLFRLL-) (Fig. 5A) in OCT1 and result in a dysfunction of trafficking to the plasma membrane (Michelsen et al., 2005).

In summary, our study demonstrates that in marked contrast to the common reduced-function coding region variants identified in white populations, reduced-function coding region variants of OCT1 in Asian populations are rare. This study is the first to demonstrate reduced-function variants of OCT1 in Asian populations and suggests that, although rare, these variants may contribute to altered response to metformin in individuals who carry them. Moreover, examination of the function of these naturally occurring variants provides new insights into the role of particular amino acids in OCT1 function and subcellular localization. In particular, the newly identified R206C resulted in a significantly reduced expression and function of OCT1. On the basis of these data, we suggest that an evolutionarily conserved arginine functions as an ER-sorting motif of transporters.

Acknowledgments

References

Becker ML, Visser LE, van Schaik RH, Hofman A, Uitterlinden AG, and Stricker BH (2009) Genetic variation in the organic cation transporter 1 is associated with metformin response in patients with diabetes mellitus. *Pharmacogenomics J* **9:**242–247.

Chen L, Durkin KA, and Casida JE (2006) Spontaneous mobility of GABA$_A$ receptor M2 extracellular half relative to noncompetitive antagonist action. *J Biol Chem* **281:**38871–38878.

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5:**113.

Giacomini KM, Brett CM, Altman RB, Benowitz NL, Dolan ME, Flockhart DA, Johnson JA, Hayes DF, Klein T, Krauss RM, et al. (2007) The Pharmacogenetics Research Network: from SNP discovery to clinical drug response. *Clin Pharmacol Ther* **81:**328–345.

Itoda M, Saito Y, Maekawa K, Hichiya H, Komamura K, Kamakura S, Kitakaze M, Tomoike H, Ueno K, Ozawa S, et al. (2004) Seven novel single nucleotide polymorphisms in the human SLC22A1 gene encoding organic cation transporter 1 (OCT1). *Drug Metab Pharmacokinet* **19:**308–312.

Jonker JW and Schinkel AH (2004) Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1–3). *J Pharmacol Exp Ther* **308:**2–9.

Kalandadze A, Wu Y, Fournier K, and Robinson MB (2004) Identification of motifs involved in endoplasmic reticulum retention-forward trafficking of the GLT-1 subtype of glutamate transporter. *J Neurosci* **24:**5183–5192.

Kirpichnikov D, McFarlane SI, and Sowers JR (2002) Metformin: an update. *Ann Intern Med* **137:**25–33.

Koepsell H (2004) Polyspecific organic cation transporters: their functions and interactions with drugs. *Trends Pharmacol Sci* **25:**375–381.

Lostao MP, Hirayama BA, Panayotova-Heiermann M, Sampogna SL, Bok D, and Wright EM (1995) Arginine-427 in the Na$^+$/glucose cotransporter (SGLT1) is involved in trafficking to the plasma membrane. *FEBS Lett* **377:**181–184.

Michelsen K, Yuan H, and Schwappach B (2005) Hide and run. Arginine-based endoplasmic-reticulum-sorting motifs in the assembly of heteromultimeric membrane proteins. *Embo Rep* **6:**717–722.

Sakata T, Anzai N, Shin HJ, Noshiro R, Hirata T, Yokoyama H, Kanai Y, and Endou H (2004) Novel single nucleotide polymorphisms of organic cation transporter 1 (SLC22A1) affecting transport functions. *Biochem Biophys Res Commun* **313:**789–793.

Shikata E, Yamamoto R, Takane H, Shigemasa C, Ikeda T, Otsubo K, and Ieiri I (2007) Human organic cation transporter (OCT1 and OCT2) gene polymorphisms and therapeutic effects of metformin. *J Hum Genet* **52:**117–122.

Shu Y, Brown C, Castro RA, Shi RJ, Lin ET, Owen RP, Sheardown SA, Yue L, Burchard EG, Brett CM, et al. (2008) Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther* **83:**273–280.

Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, Ianculescu AG, Yue L, Lo JC, Burchard EG, et al. (2007) Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest* **117:**1422–1431.

Song IS, Shin HJ, Shim EJ, Jung IS, Kim WY, Shon JH, and Shin JG (2008) Genetic variants of the organic cation transporter 2 influence the disposition of metformin. *Clin Pharmacol Ther* **84:**559–562.

Urban TJ, Gallagher RC, Brown C, Castro RA, Lagpacan LL, Brett CM, Taylor TR, Carlson EJ, Ferrin TE, Burchard EG, et al. (2006) Functional genetic diversity in the high-affinity carnitine transporter OCTN2 (SLC22A5). *Mol Pharmacol* **70:**1602–1611.

Zang M, Zuccollo A, Hou X, Nagata D, Walsh K, Herscovitz H, Brecher P, Ruderman NB, and Cohen RA (2004) AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. *J Biol Chem* **279:**47898–47905.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, et al. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108:**1167–1174.

Zhou K, Donnelly LA, Kimber CH, Donnan PT, Doney AS, Leese G, Hattersley AT, McCarthy MI, Morris AD, Palmer CN, et al. (2009) Reduced-function SLC22A1 polymorphisms encoding organic cation transporter 1 and glycemic response to metformin: a GoDARTS study. *Diabetes* **58:**1434–1439.

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