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Identification of novel functional Organic Anion-transporting Polypeptide 1B3 (OATP1B3) polymorphisms and assessment of substrate specificity

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Abstract

Objective—The uptake carrier Organic Anion-transporting Polypeptide 1B3 (OATP1B3, gene *SLCO1B3*) is involved in the hepatic clearance of xenobiotics including statins, taxanes and mycophenolic acid. We thought to assess the *SLCO1B3* coding region for yet unidentified polymorphisms, and to analyze their functional relevance.

Methods—We used DNA of ethnically diverse subjects for polymerase chain reaction (PCR), and determined polymorphisms by sequencing or temperature-dependent capillary electrophoresis. We then created variant OATP1B3 expression plasmids by site-directed mutagenesis, which were transiently expressed and functionally characterized in HeLa cells using radiolabeled substrates.

Results—We identified six non-synonymous polymorphisms including novel variants 439A>G (Thr147Ala), 767G>C (Gly256Ala), 1559A>C (His520Pro) and 1679T>C (Val560Ala). Allelic frequencies occurred ethnicity-dependent, with the latter observed only in African Americans (3.6%). After expression in HeLa cells, variants His520Pro, Val560Ala, and Met233Ile or Met233Ile_Ser112Ala haplotype demonstrated decreased uptake activity compared to wildtype for cholecystokinin-8 (CCK8) and rosuvastatin, but not atorvastatin. Kinetic CCK8 analysis revealed reduced V_{max} without altering K_m. His520Pro and Val560Ala exhibited decreased total and plasma membrane protein expression. Val560 mapped onto a structural model of OATP1B3 revealed this is a key region for substrate–transporter interaction. His520 resides in a predicted extracellular region thought to be critical to the pH-dependent component of OATP1B3 activity. Loss of activity at pH 7.4 and 8.0 relative to pH 6.5 was significantly greater for the Pro520 variant.

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Conclusion—OATP1B3 polymorphisms that result in altered expression, substrate specificity, and pH-dependent activity may be of potential relevance to hepatic clearance of substrate drugs *in vivo*.

Keywords

OATP1B3 polymorphisms; drug transporter; drug disposition

Introduction

The human organic anion-transporting polypeptide (OATP) 1B3 (also OATP-8 or LST-2, gene name *SLCO1B3*) belongs to a liver-enriched OATP subfamily that is predominantly expressed on the basolateral membrane domain of hepatocytes. OATP1B3 shares 80% sequence identity with the other member of this subfamily, human OATP1B1 (also OATP-C or LST-1, gene name *SLCO1B1*) [1]. In contrast to OATP1B1, OATP1B3 has also shown to be expressed in cancer tissues [2,3]. Both transporters share a broad spectrum of drug substrates including the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) rosuvastatin and atorvastatin [4,5], the anticancer agent methotrexate [3], the angiotensin II receptor antagonist olmesartan [6], and the antibiotic rifampicin [7]. However, OATP1B3 but not OATP1B1 has been shown capable of mediating the cellular uptake for drugs such as the angiotensin II receptor antagonist telmisartan [8] and the anticancer agents paclitaxel and docetaxel [9,10].

Sequence variation in genes encoding OATP family members has been reported to contribute significantly to interindividual differences in the disposition of many clinically relevant drugs [11–13]. Previously, our group identified and characterized naturally occurring polymorphisms in the *SLCO1B1* gene associated with significantly impaired transport activity [14]. Among those, the extensively studied common *SLCO1B1* variant 521C>T (Val174Ala) was shown to be associated with increased plasma levels of various statins, including rosuvastatin, atorvastatin, and pravastatin [15,16]. Moreover, this variant has been recently reported in a genome wide association study as the most important predictor of myopathy, a common side effect of statin therapy, which in rare cases can be life-threatening [17].

The presence of *SLCO1B3* coding region variants had been previously noted using public databases and genotyping of 182 Caucasian Europeans, where three non-synonymous polymorphisms were identified [18]. Two common variants, *SLCO1B3* 334T>G (Ser112Ala) and 699G>A (Met233Ile), were found to be associated with altered transport function *in vitro* compared to the expressed wildtype protein. Functional implications and clinical relevance of these two variants have been the subject of several *in vivo* studies. Importantly, recent studies suggest *SLCO1B3* 334T>G and 699G>A influences the pharmacokinetic profile of a newly identified substrate, the glucuronide metabolite (MPAG) of the immunosuppressant mycophenolic acid (MPA) [19,20]. Renal transplant patients carrying two variant *SLCO1B3* 334GG alleles exhibited lower exposure to MPA but higher plasma levels of MPAG in comparison to 334TG and TT carriers, resulting in a significantly reduced metabolic ratio for MPA/MPAG among the GG carriers.

In the present study we report the identification and functional assessment of novel, ethnicity-dependent non-synonymous variants in the *SLCO1B3* gene associated with impaired transport activity for the OATP1B3 specific substrate cholecystokinin (CCK8) as well as the drug substrate rosuvastatin related to altered expression. We also mapped the transport-deficient variants onto a recently predicted structural model of OATP1B3 to further illustrate potential structure-function relationships.

Methods

Materials

Radiolabeled [^{3}H]-CCK8 (97.0 Ci/mmol, > 96.9% purity) was purchased from Amersham GE Healthcare UK Limited (Buckinghamshire, England), [^{3}H]-atorvastatin calcium (10.5 Ci/mmol, > 99% purity) from American Radiolabeled Chemicals, Inc. (Saint Luis, US), and unlabeled atorvastatin from Toronto Research Chemicals (Toronto, Canada). [^{3}H]-rosuvastatin (79 Ci/mmol, 97.1% radiochemical purity) and unlabeled rosuvastatin were provided by Dr. Yi Wang (AstraZeneca, Wilmington, DE). Additional [^{3}H]-rosuvastatin (5 Ci/mmol, 99% radiochemical purity) was sourced from American Radiolabeled Chemicals (St. Louis, MO). The pEF6/V5-His-TOPO® expression vector was purchased from Invitrogen. Genomic DNA isolated from peripheral blood lymphocytes of healthy Caucasian-, African-, Chinese- and Hispanic-American volunteers was purchased from Coriell Cell Repositories (Camden, NJ). All other chemicals and reagents, unless stated otherwise, were obtained from Sigma-Aldrich Research (St. Louis, MO) and were of the highest grade available.

Identification of SLCO1B3 polymorphisms and determination of allelic frequencies

Variations in the *SLCO1B3* gene were assessed in ethnically defined genomic DNA samples from healthy subjects (Caucasian-, African-, Chinese- and Hispanic-American descent). After amplification of the 14 exons using polymerase chain reaction (PCR), single nucleotide polymorphisms were determined by either direct sequencing (n = 92 for each ethnicity, exons 8–11; ABI 3700 DNA Analyzer, Applied Biosystems Inc., Foster City, CA) or temperature-dependent capillary electrophoresis (n = 46 for each ethnicity, exons 1 – 7 and 12 – 14, Reveal®, SpectruMedix, State College, PA) of PCR products with subsequent sequencing of the identified variant samples. Primer sequences are summarized in Table 1.

Variant SLCO1B3 plasmid construction

SLCO1B3 variant expression plasmids were created using site-directed mutagenesis as described previously [14]. Reference sequence (also referred to as *SLCO1B3* wildtype; NM_019844) and presence of polymorphisms was verified by sequencing.

Transient transfection and uptake transport assays

Transient transfection assays were performed using the recombinant vaccinia virus (vTF-7) expression method detailed previously [21]. Briefly, human cervical carcinoma cells (HeLa) (American Type Culture Collection, Manassas, VA) were seeded into 12-well plates, infected with vaccinia virus, and then transfected with expression plasmid or vector control lacking any insert using Lipofectin reagent (Invitrogen). Sixteen hours thereafter, cells were washed with transport media (OPTIMEM, Invitrogen) and treated with radiolabeled drug. At various time intervals, cells were washed three times with ice-cold Dulbecco's Phosphate Buffered Saline (PBS) buffer and then lysed with 1% sodium dodecyl sulfate (SDS). Retained cellular radioactivity was quantified by liquid scintillation spectrometry. Transport experiments evaluating pH sensitivity were carried out as described using Krebs-Henseleit buffer (KHB; 118 mM NaCl, 23.8 mM $NaHCO_3$, 4.8 mM KCl, 0.96 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$) adjusted to pH 6.5, 7.4, or 8 instead of OPTIMEM. Parameters for saturation kinetics (*V*max and *K*m) were estimated by nonlinear curve fitting using Prism (GraphPad Software, Inc., San Diego, CA). All experiments were carried out in duplicate on 2 to 3 separate days.

OATP1B3 cell surface and total protein expression and structural mapping

Expression analysis of OATP1B3 at the cell surface and in total cell lysates was performed as described previously [14,22]. Total cell lysates and biotinylated fractions were subjected to Western blot analysis for detection of OATP1B3 with a rabbit polyclonal antibody (1:4000) [4], and for the intracellular protein calnexin (1:4000; StressGen, Victoria, BC) [14]. Bands were visualized using enhanced chemiluminescence (ECL plus, Amersham, Piscataway, NJ). Mapping of the OATP1B3 variants His520Pro and Val560Ala on a recently predicted structural model of OATP1B3 was carried out as previously described using PyMOL (www.pymol.org) [23].

Statistical analysis

Determination of statistical differences between various group parameters was determined using either Student *t*-test, Mann Whitney U-test or ANOVA, as appropriate. A p-value <0.05 was considered to reflect statistical significance.

Results

Polymorphisms in *SLCO1B3* and allelic frequency

Using genomic DNA from ethnic diverse populations, we identified 14 coding region *SLCO1B3* variants (Table 2). Among six non-synonymous polymorphisms identified, *SLCO1B3* 439A>G, 767G>C, 1559A>C and 1679T>C were new and previously unreported [24]. Their location is depicted on a putative OATP1B3 secondary structure (Fig. 1). The previously described rare 1564G>T (Gly522Cys) [18] was not detected in our population. The prevalence of *SLCO1B3* 334T>G, 699G>A, 767G>C, and 1679T>C was ethnicity-dependent (Tables 3 and 4). Complete linkage of 334T>G and 699G>A as previously described by some authors was not observed in our study [10,25,26]. *SLCO1B3* 439A>G and 1559A>C were rare in our study population.

OATP1B3 is the main CCK8 uptake transporter in liver

The peptide hormone CCK8 has been shown to be substrate of OATP1B3 [27], but not OATP1B1 [28]. In order to evaluate CCK8 transport activities among other members of the solute carrier (SLC) superfamily, we transiently expressed an array of human and rat OATPs, organic cation transporters (OCTs), organic anion transporters (OATs), as well as the hepatic bile acid transporter sodium taurocholate cotransporting polypeptide (NTCP). The capacity for CCK8 uptake by OATP1B3 exhibited nearly 150-fold greater transport activity (Fig. 2A) compared to cells transfected with the plasmid lacking OATP1B3 (vector control). The closely related OATP1B1 showed minimal, although statistically significant transport of CCK8, but approximately 100-fold less than that mediated by OATP1B3. Other OATPs, OCT and OAT transporters failed to exhibit any uptake activity for CCK8. The initial rate of CCK8 uptake by OATP1B3 was demonstrated to be linear over the first 5 minutes (Fig. 2B).

Functional analysis of OATP1B3 variants

An array of expression plasmids comprising *SLCO1B3* wildtype, six non-synonymous variants, and the haplotype combination of 334T>G and 699G>A were created. CCK8 transport activities were significantly lower for Met233Ile, His520Pro, and Val560Ala ($p < 0.05$) variants relative to wildtype (Fig. 2C). His520Pro and Val560Ala exhibited approximately 20% CCK8 uptake relative to wildtype, and Met233Ile about 65%. Expression of the Ser112Ala_Met233Ile haplotype revealed a slightly more pronounced decrease to 57% ($p < 0.05$) than Met233Ile alone.

The transport kinetics of CCK8 was examined for those allelic variants that exhibited a significant decrease in transport activity (Fig. 3). The maximum uptake rate (V_{max} ± SD, pmol.mg protein^{-1}.min^{-1}) of CCK8 was decreased for His520Pro, Val560Ala, and Met233Ile compared to wildtype, whereas Ser112Ala did not differ. The concentration at which half the maximal uptake occurs (K_m ± SD, μM) was slightly altered with an about 2-fold increase for Met233Ile and Val560Ala. Expression of Ser112Ala_Met233Ile haplotype resulted in values similar to those observed for Met233Ile when expressed alone.

We also show that rosuvastatin and atorvastatin are substrates of OATP1B3 (Fig. 4A). Rosuvastatin transport activity was significantly lower for Met233Ile, His520Pro, and Val560Ala ($p < 0.01$) variants relative to wildtype, exhibiting 56%, 45% and 54% uptake, respectively, whereas the linked Ser112Ala_Met233Ile haplotype yielded activity that is 87% of wildtype (Fig. 4B). Interestingly, no significant differences were observed for atorvastatin (Fig. 4C).

Total and cell surface expression of OATP1B3 variants

Immunoblot analysis (Fig. 5) suggested a reduction in total OATP1B3 protein expression for Gly256Ala, His520Pro and Val560Ala, whereas Met233Ile was not different from wildtype. Interestingly, Ser112Ala and Thr147Ala bands appear slightly stronger than wildtype. Cell-surface biotinylation with the membrane-impermeant biotinylation reagent, sulfo-NHS-SS-biotin, was performed to quantify the cell surface expression of each variant. Only His520Pro and Val560Ala revealed lower cell surface expression levels compared to wildtype. Cell surface-expressed OATP1B3 proteins from transfected HeLa cells were highly glycosylated proteins with an apparent molecular mass of ~100-kDa, which is similar to that observed in human liver and total protein lysates. An additional major band of ~70 to 75-kDa was detected in cell lysates in comparison to human liver, consistent with the calculated molecular mass of OATP1B3. The two forms of OATP1B3 in HeLa cells may suggest that a significant fraction of the transfected OATP1B3 is not glycosylated. The enrichment of cell surface proteins within the biotinylated fractions was evidenced by the lack of the intracellular protein calnexin.

Amino acid conservation and structural model

The two functionally most relevant variants His520Pro and Val560Ala were mapped onto a recently proposed structural model of OATP1B3 (Fig. 6A, B) [23]. Accordingly, the amino acid residue His520 (Fig. 6A) is located to the large extracellular loop 5 (ECL) between the transmembrane (TM) regions 9 and 10, and Val560 (Fig. 6A, B) in the C-terminal region of TM10, thereby facing the putative central pore of the transport protein that is speculated to be crucial to substrate translocation.

The amino acid alignment of OATP1B3 within various orthologs representing the conserved residues corresponding to the identified non-synonymous variants is shown in Fig. 6C. Only Gly256 appears to be conserved among the five assessed species, whereas substantial variability is observed for the other amino acid residues.

Effect of extracellular pH on CCK8 transport by OATP1B3 His520Pro variant

A conserved histidine residue located on the extracellular portion of TM3 was recently shown to mediate the pH-sensitive OATP1B3 transport activity [29]. Based on the predicted extracellular location of the His520Pro variant (ECL5), we evaluated the effect of extracellular pH on His520 mediated CCK8-transport compared to Pro520 (Fig. 7). Interestingly, CCK8 transport demonstrated pH dependence for His520 as well as Pro520 with maximum stimulation at pH 6.5 with a substantial 3.4-, 4- and 5-fold decrease in CCK8 uptake by Pro520 compared to His520 at pH 6.5, 7.4, and 8, respectively (Fig. 7A).

Compared to the maximum stimulation at pH 6.5, CCK8 uptake was significantly reduced at pH 8 to 67.7% for His520, and to 46% for Pro520 (Fig. 7B). Interestingly, in comparison to His520, the magnitude in reduction of transporter function was greater for the Pro520 variant when the extracellular pH was increased from 6.5 to 7.4 or 8.

Discussion

OATP transporters, particularly those in the OATP1B subfamily, have shown to be important to the liver-specific drug uptake. For OATP1B1, commonly occurring functional variants, first reported by our group [14], have now been shown to be a key predictor of altered substrate drug levels and response [15–17]. *In vitro* evidence further suggests that OATP1B3 is involved in the hepatic uptake of clinically relevant drugs such as the anticancer agents methotrexate, docetaxel and paclitaxel [3,9], and the angiotensin II receptor antagonists olmesartan and telmisartan [6,8]. We had previously shown that in liver, expression of OATP1B1 and 1B3 are similar, at least when mRNA levels were quantified [4]. Given that many drug substrates of OATP1B1 are also shared substrates of OATP1B3, genetic variation in *SLCO1B3* is likely one of the determinants of their variable disposition *in vivo*. Presence of genetic variation in *SLCO1B3* has been reported, and in some cases, clinical relevance noted [19,20].

In this study, we conducted a comprehensive analysis for OATP1B3 function in terms of genetic variation and substrate specificity. First, we performed a large genotypic analysis to identify *SLCO1B3* variants in ethnically diverse subjects. Within the *SLCO1B3* coding region, we identified a total of six non-synonymous polymorphisms, including four novel variants. We further detected the previously described common variants 334T>G and 699G>A [18] that were not observed to be in complete linkage. This was also noted by some authors [18] but not others [10,25,26]. Overall our results indicate that the coding region of *SLCO1B3* is less polymorphic in comparison to *SLCO1B1,* encoding the closely related OATP1B1 protein of the same subfamily [14]. Interestingly, the novel impaired-function polymorphism 1679T>C (Val560Ala) was only observed in African Americans with an allelic frequency of 3.6%. On the other hand *SLCO1B3* 334T>G and 699G>A were less frequent in African Americans compared to other ethnicities (Table 4).

Two novel variants, OATP1B3 His520Pro and Val560Ala, exhibited markedly lower uptake activity for CCK8 and rosuvastatin compared to wildtype when studied in HeLa cells. Among previously reported variants, only Met233Ile but not Ser112Ala revealed decreased uptake of CCK8 and rosuvastatin, and the Ser112Ala_Met233Ile haplotype did not exhibit any additional reduction in transport activity. Previous studies have reported conflicting results regarding the effect of these two variants, either alone or co-expressed. Letschert et al. observed increased CCK8 transport in MDCKII cells stably expressing Ser112Ala and Met233Ile whereas their expression in HEK239 cells did not result in a difference compared to wildtype [18]. On the other hand, expression of the Ser112Ala_Met233Ile haplotype showed decreased uptake activity for two recently reported OATP1B3 substrates, testosterone and MPAG [20,30], after expression in Cos-7 and HEK cells, respectively, but no difference for the chemotherapeutic drug substrate paclitaxel when assessed in Xenopus laevis oocytes [9,10]. The divergent findings may suggest substrate-specific effects but also be in part explained by the use of different cell systems with varying levels of uncharacterized endogenous transporters with overlapping substrate specificity.

Kinetic analysis of OATP1B3-mediated CCK8 transport in HeLa cells revealed that Met233Ile, His520Pro and Val560Ala as well as the Ser112Ala_Met233Ile combination result in reduced V_{max} without altering the apparent affinity (K_m). The K_m value of 4.2μmol/L for the wildtype protein observed in this study was in close agreement with CCK8 uptake

results measured in *X. leavis* oocytes expressing human OATP1B3 (K_m 11.1μmol/L) as well as in primary cultured rat hepatocytes (K_m 6.7 μmol/L) [27].

In addition to altered substrate recognition, changes in expression of functional proteins or failure of the variant protein to reach the cell surface are known to influence the magnitude of overall transport function. In our study, the total expressed level of the two variant proteins His520Pro and Val560Ala was found to be reduced compared to the wildtype. Furthermore, these two variant proteins revealed a reduced cell surface expression. This is consistent with previously reported findings [14,18]. Two other OATP1B3 variants, namely Gly522Cys and Gly583Glu, demonstrated decreased intrinsic clearance using bromosulfophthalein as a result of impaired V_{max} values but comparable K_m [18], and variant proteins were shown to be mainly retained intracellularly in comparison to wildtype. Similarly, the OATP1B1 Val174Ala (*5) variant was found to decrease V_{max} with no significant effect on K_m using various substrates, and cell surface trafficking was shown to be the major determinant for its functional effect *in vitro* [14]. Subsequently, numerous clinical studies were able to demonstrate the functional relevance of OATP1B1*5 *in vivo* [15,16]. In summary, our results suggest that the observed alterations in transport activity, in part, may result from decreased total protein and, in some cases, cell surface targeting of the variant OATP1B3. Decreased total protein expression was also observed for Gly256Ala, however there was no significant impairment of CCK8 transport activity.

The impaired-function His520Pro substitution is predicted to localize to the putative large ECL5 of OATP1B3 between TM9 and TM10. The large ECL – common to all OATPs – has been proposed to play an important role in the substrate-transporter interaction and surface expression [14,23,31]. Ten conserved cysteine residues within this loop were shown to affect transport function as well as cell surface trafficking in OATP2B1, another OATP family member [31]. Interestingly, naturally occurring polymorphisms resulting in amino acid substitutions within ECL5 of OATP1B3 (His520Pro, Gly522Cys, Gly583Glu) [18,24] and OATP1B1 (Gly488Ala) [14] do not directly involve any cysteine residues but were observed to be functionally impaired, partially due to decreased total protein expression and/or reduced cell surface trafficking. This suggests that non-cysteine amino acid variations present in the large ECL may be indirectly associated with the disulfide bond formation between cysteine residues. The possibility of altered substrate interaction with the transporter is also substantiated by the fact that the variant amino acid substitutions alter transport activity only for some but not all OATP1B3 substrates, as observed in this study for OATP1B3 His520Pro and reported for the OATP1B3 Gly522Cys variant [18].

The loss-of-function Val560Ala substitution is localized to TM10 of OATP1B3. A recent report utilizing a transmembrane domain swapping strategy indicated that TM10 is important for CCK8 transport by OATP1B3 [28]. Replacing TM10 of OATP1B3 with TM10 of OATP1B1 nearly abolished its ability to transport the OATP1B3 specific substrate CCK8. Three key amino acids within this region, Tyr537, Ser545, and Thr550, were shown to cause the observed effect. Interestingly, replacement of Thr559 in OATP1B3 to the respective residue in OATP1B1 (Ile559) also caused a significant decrease in CCK8 uptake (70% of OATP1B3), thus the profound loss-of-function noted in this study for the variant that results in the exchange of Val560 to Ala560 is consistent with this region being critical for substrate specificity. Notably when mapped onto a recently suggested model of OATP1B3 [23], the side chain of the Val560 residue at the C-terminal region of TM10 faces the putative central pore (Fig. 6). Thus, our findings provide new and important evidence that the central pore, particularly the C-terminal region of TM10, is most likely of functional relevance for OATP1B3.

Recent studies also suggest that transport activity for OATPs is modulated by pH, and that higher transport activity is observed at a lower (acidic) extracellular pH, resulting in increased substrate affinity as evidenced by lower K_m values [29]. The His130 residue (TM3) likely facing the extracellular milieu had been identified to be crucial for the observed pH sensitivity in OATPs including OATP1B3. The replacement of the basic histidine residue with a neutral glutamine abolished pH dependence of transport of a rat Oatp family member, Oatp1a1, and exchange of glutamine with histidine in OATP1C1 created pH dependence of a formerly pH independent transporter. Our study identified a non-synonymous variant that results in a histidine residue in ECL5 of OATP1B3 that is replaced by the less polar and weekly basic proline. Comparing CCK8 transport activity of the wildtype protein containing His520 with the variant Pro520 at extracellular pH of 6.5, 7.4, and 8.5 revealed pH dependence for His520 as well as the Pro520 variant with maximum activation of uptake under acidic conditions (pH 6.5 > pH 7.4 > pH 8), although the magnitude in reduction of transporter function appeared to be greater for the Pro520 variant when the extracellular pH was increased from 6.5 to 7.4 or 8.

Recently, the clinical relevance of *SLCO1B3* polymorphisms has been evaluated for various drug substrates of OATP1B3. Drug exposure of two widely used chemotherapeutics, paclitaxel and docetaxel, was assessed with respect to *SLCO1B3* gene variants in cancer patients [10,32]. No association was observed between *SLCO1B3* variants with pharmacokinetics of docetaxel and paclitaxel. This negative finding may be explained in part by the small patient numbers (<100) in case of the more rare variants, 1559A>C and 1679T>C, and the potential involvement of other transporter proteins. More recently, *SLCO1B3* 334T>G and 699G>A were reported to determine the pharmacokinetics of the immunosuppressant mycophenolic acid in renal transplant patients, with higher mycophenolic acid glucuronide exposure in variant carriers compared to wildtype suggesting lower systemic clearance due to decreased hepatic uptake [20,33].

In conclusion, we have identified and functionally characterized novel non-synonymous polymorphisms in the *SLCO1B3* gene associated with impaired transport *in vitro*. Notably, *SLCO1B3* 1679T>C (Val560Ala) with an allelic frequency of 3.6% in African Americans is a heretofore unrecognized loss-of-function polymorphism with the potential to affect substrate drug disposition *in vivo*. Importantly, detailed kinetic analysis, total and cell surface expression data, variant-associated pH dependence, and computational mapping of OATP1B3 variants further outline the complexity and potential functional relevance of these polymorphisms.

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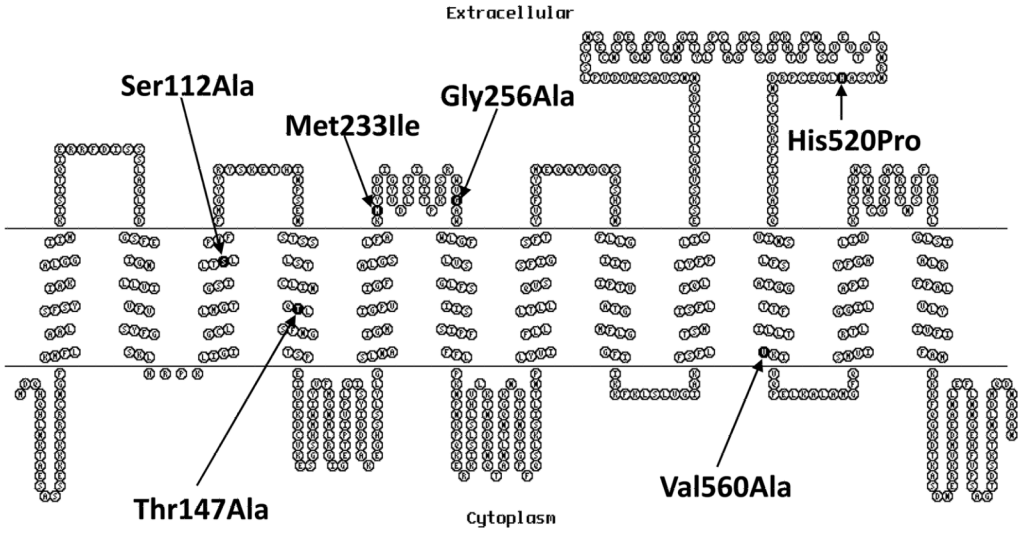

Fig. 1.

Schematic representation of OATP1B3. Transmembrane topology is based on predictions using TopPred2 (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) and is in agreement with the recently published OATP1B3 model.[23] Non-synonymous nucleotide substitutions are indicated by *arrows*. The transmembrane topology schematic was rendered using TOPO2 (S.J. Johns and R.C. Speth, transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO/topo.html, unpublished).

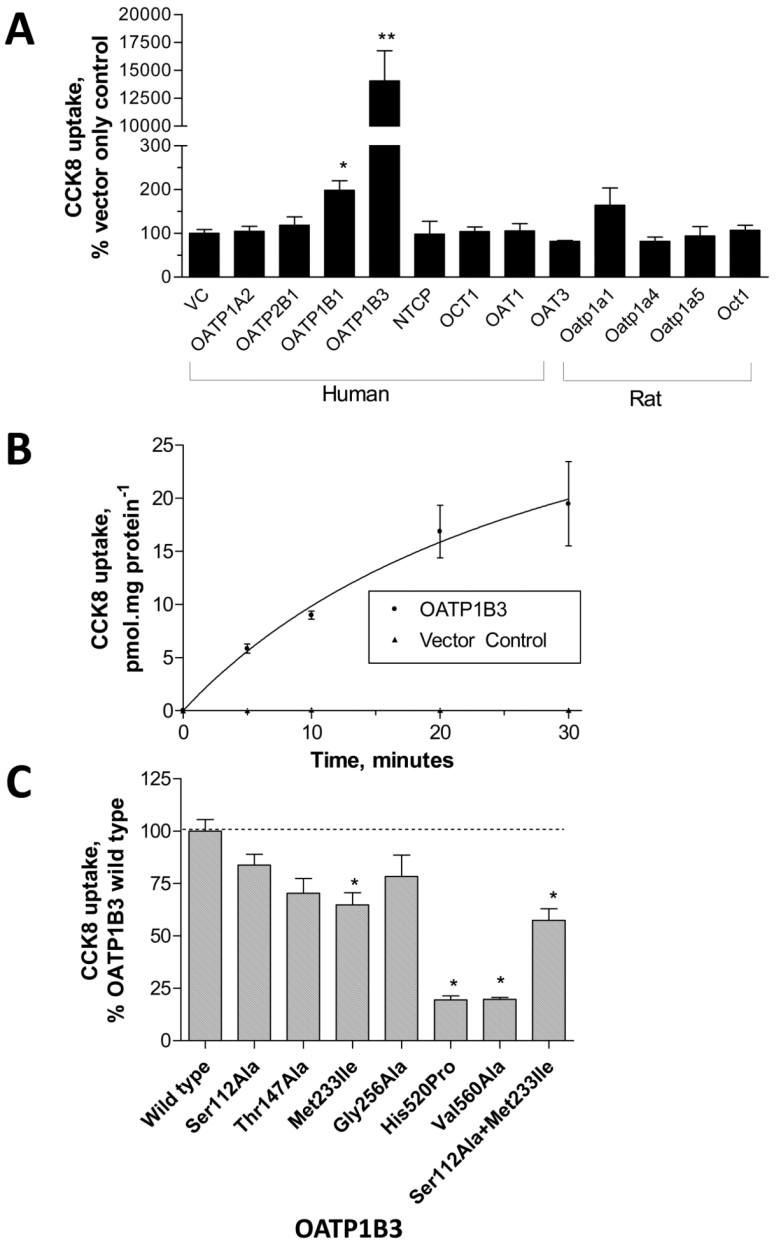

Fig. 2.

[^{3}H]-CCK8 uptake transport activity (0.1 μM) following transient heterologous expression in HeLa cells. **(A)** Uptake of [^{3}H]-CCK8 by cells transfected with various human OATPs including OATP1B3, OATP2B1, OATP1B1, and OATP1A2, and the uptake transporters NTCP (Na^+-Taurocholate Cotransporting Polypeptide), OCT1 (Organic Cation Transporter 1), and OAT (Organic Anion Transporter) 1 and 3 as well as several rat orthologs. Values are expressed as percent of cellular uptake by vector control. **(B)** Time course of [^{3}H]-CCK8 uptake [pmol/mg protein] in OATP1B3 over-expressing cells compared to transporter-lacking vector control (VC). **(C)** Uptake of [^{3}H]-CCK8 by cells transfected with *SLCO1B3* variants relative to wildtype at 10 minutes. Values are expressed as percent of cellular uptake by OATP1B3 wildtype. All values are given as mean ± S.E. or mean ± SD (Fig 2B only) of at least two to three independent experiments with a total of n = 5 – 6. **p < 0.01, *p<0.05.

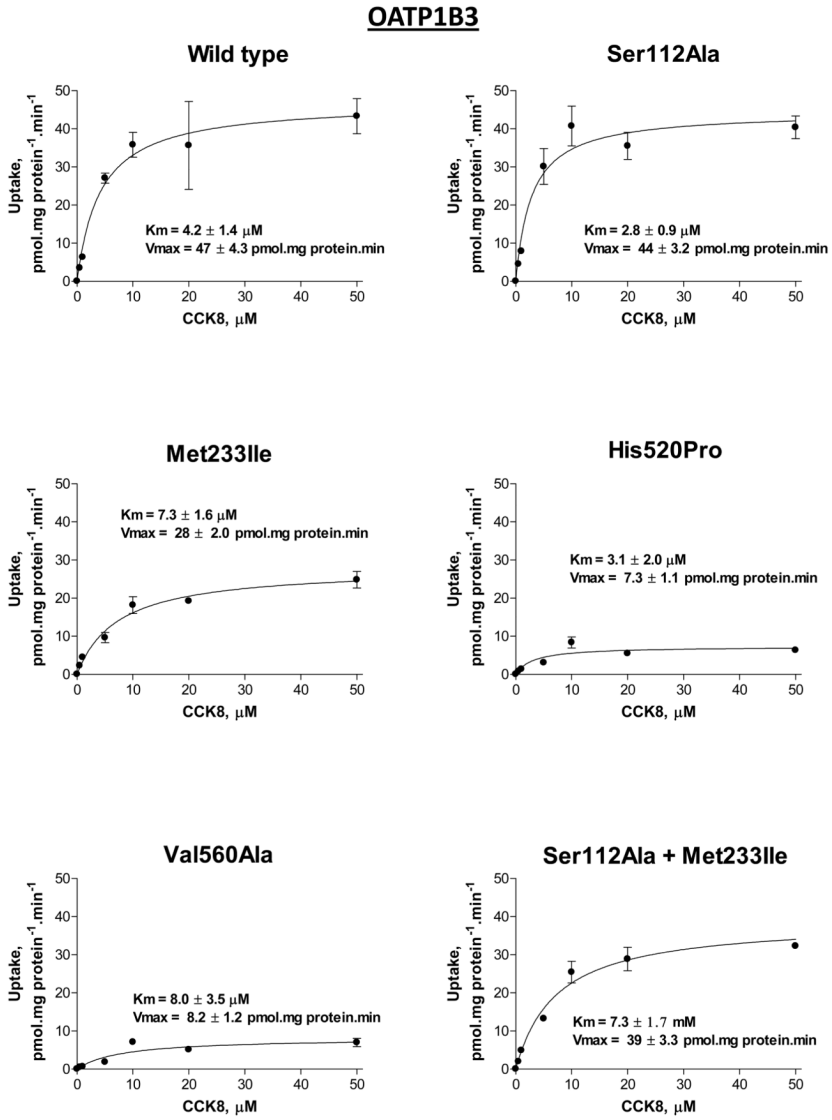

Fig. 3.

Transport kinetics of OATP1B3 following transient heterologous expression in HeLa cells conducted at 5 min at varying concentrations of CCK8 (1–50 μM). Parameters for saturation kinetics (V_{max} and K_m) were estimated by nonlinear curve fitting using Prism (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean ± S.E. (n = 4 – 6).

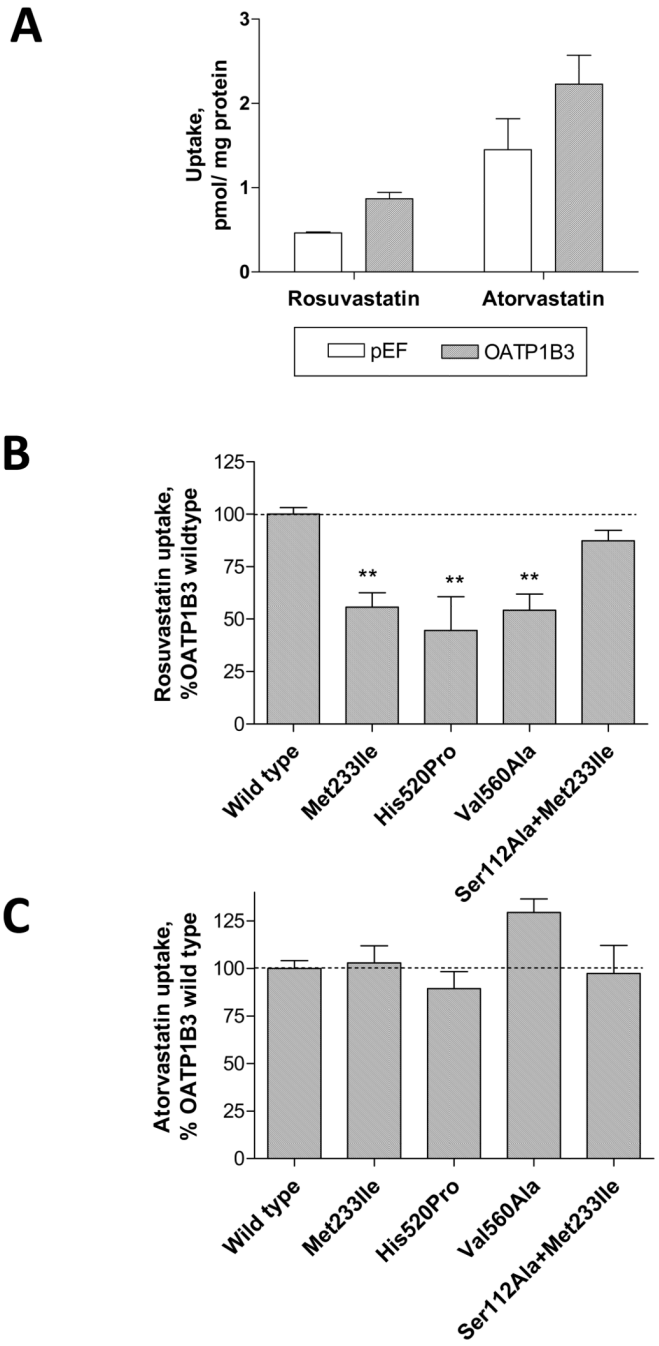

Fig. 4.

Rosuvastatin and atorvastatin uptake transport activity (0.1μM) following transient heterologous expression of OATP1B3 in HeLa cells. **(A)** Uptake of [^{3}H]-rosuvastatin and [^{3}H]-atorvastatin by OATP1B3 over-expressing cells at 5 minutes compared to transporter lacking vector control [pmol/mg protein]. **(B)** Uptake of [^{3}H]-rosuvastatin and **(C)** [^{3}H]-atorvastatin by cells transfected with *SLCO1B3* variants relative to wildtype at 5 min. Values are expressed as percent of cellular uptake by OATP1B3 wildtype and given as mean ± S.E. of three independent experiments with a total n ≥ 5 (two for atorvastatin with n = 4). **p < 0.001

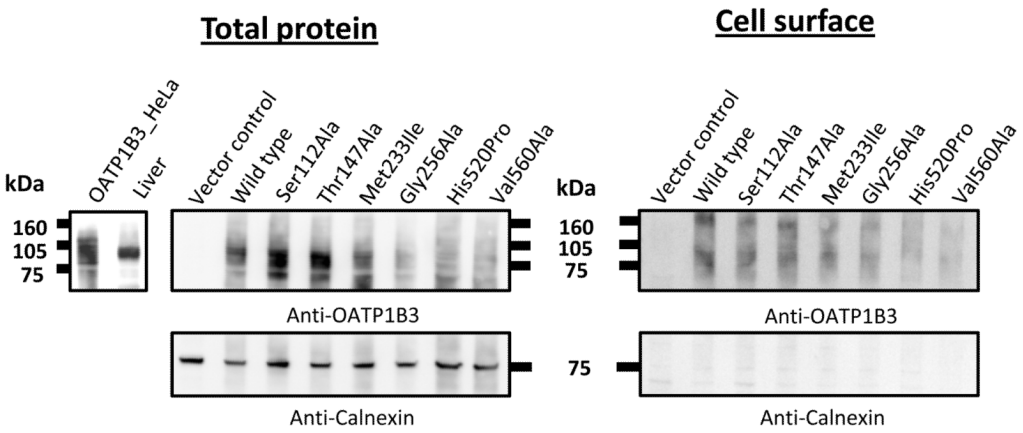

Fig. 5.

Total (*left panel*) and cell surface protein expression (*right panel*) of OATP1B3 variants in comparison to wildtype and vector control (VC) lacking any insert. Total cell lysate containing biotinylated and non-biotinylated proteins, and cell surface-expressed biotinylated proteins (captured on streptavidin beads) were subjected to SDS-PAGE, and transferred onto nitrocellulose membranes. Immunoblots were probed with anti-OATP1B3 antibody (*top panels*) and then stripped and probed with anti-calnexin antibody (*bottom panels)*. For comparison, immunoblot of human liver protein lysate (*most left panel*) probed with anti-OATP1B3 antibody.

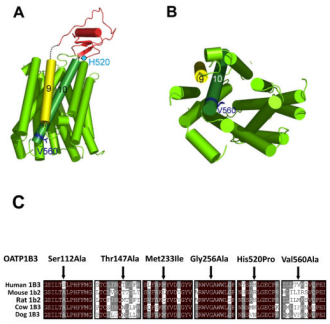

Fig. 6.

(A) and **(B)** OATP1B3 variants V560A and H520P mapped on to the previously described structural model of OATP1B3[23] viewed from (**A**) the lateral side and (**B**) the intracellular side. Helices are shown as cylinders and beta sheets as sheets. The model consists of two main regions: the membrane-spanning helices (TM, green) that form a pore and the extracellular Kazal-type serine protease inhibitor-like domain (red) which links TM9 and TM10 (labeled and colored yellow and dark green, respectively). The side chain of V560 at the C-terminal region of TM10 is shown to point towards the pore. The position of H520 is mapped roughly on to the un-modeled loop which connects the Kazal-type domain and the N-terminus of TM10. The Figure was generated with PyMOL (www.pymol.org). **(C)** Amino acid alignment of human OATP1B3 with various orthologs by ClustalW algorithm. Arrows indicate positions of non-synonymous variants.

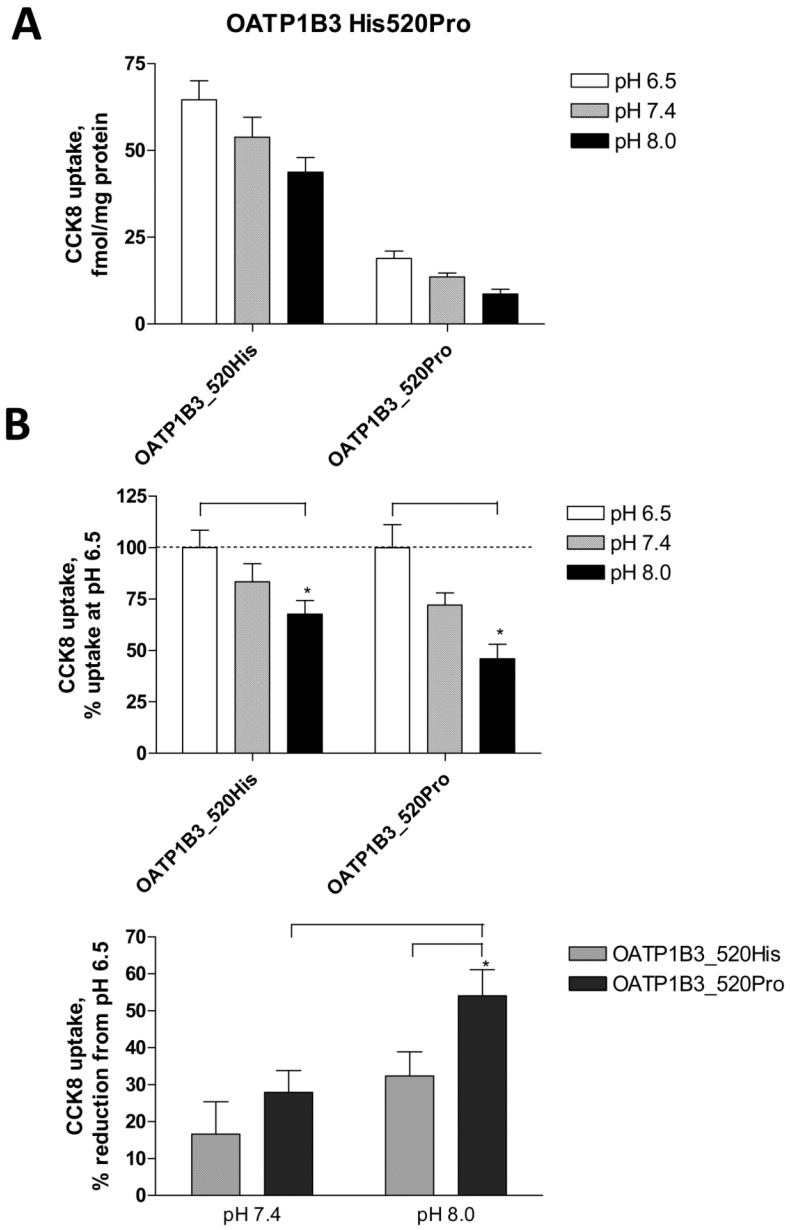

Fig. 7.

Effect of OATP1B3 His520 in comparison to the variant OATP1B3 Pro520 on pH-sensitivity. [^{3}H]-CCK8 uptake in transiently transfected HeLa cells at 3 nM was measured for 5 min at 37°C at pH 6.5, 7.4, and 8.0. Results are expressed as mean ± S.E. of duplicate determinations from 3 independently performed transport studies. **(A)** Absolute CCK8 uptake [fmol/mg protein], OATP1B3 520His: pH6.5: 64.6 ± 5.5, pH7.4: 53.9 ± 5.6, pH8: 43.7 ± 4.2; and for OATP1B3 520Pro: pH6.5: 18.9 ± 2.1, pH7.4: 13.6 ± 1.1, pH8: 8.7 ± 1.3. **(B)** [^{3}H]-CCK8 uptake relative to pH6.5 with uptake values corrected for unspecific transport in HeLa cells and normalized to uptake at pH 6.5 (= 100%). (**C**) Reduction of transporter function [%] from pH 6.5 to 7.4 or 8. *$p < 0.05$

Table 1

Oligonucleotide primer sequences to carry out PCR of the *SLCO1B3* coding region

Exon	Forward primer	Reverse primer
1	5′-catttcaaaccaagcatcagc-3′	5′-taataaatggctcagagctg-3′
2	5′-cgagtaaagaagaaaaactg-3′	5′-acttatgcaagtatggttatca-3′
3	5′-gggcattcagttctactaga-3′	5′-taataaatggctcagagctg-3′
4	5′-atttctctgtatttctggga-3′	5′-tcagaaactttatagacgtg-3′
5	5′-taaaacactctcttgtctcg-3′	5′-gtagatccagggaatgtaat-3′
6	5′-ccaagtatttgtgacatctga-3′	5′-aatggtgtcctgcacttaaaa-3′
7	5′-aacgatttttgactggcttctt-3′	5′-aatcctcttccccttttatgta-3′
8	5′-gcctcacaaatcatttgtaac-3′	5′-gcagtgtttcatttatcaagc-3′
9	5′-gacatatcagaaaaaccata-3′	5′-gatggttaacatattacaca-3′
10	5′-tcttctgctctttctctact-3′	5′-taaggagaggagaaaaagtg-3′
11	5′-ggcaaatgtatttgttaatatttcaa-3′	5′-tgttttacaggatcatactta-3′
12	5′-gaatgatgctgataaatgtt-3′	5′-tgcattcagtctttagtatt-3′
13	5′-cgctcagttacatttgaagc-3′	5′-gaaatgtgtttacgacaact-3′
14	5′-ctggggagaaaaaaatgtaag-3′	5′-caggaacacctcaaaaataac-3′

Table 2

SLCO1B3 gene coding region variants

Nucleotide change	AA change	Exon	Reference sequence	Variant sequence	rs number
69C>T	Arg23Arg	1	GACG**C**TGCA	GACG**T**TGCA	
334T>G	Ser112Ala	3	GACA**T**CTTT	GACA**G**CTTT	rs4149117
439A>G	Thr147Ala	4	TCAA**A**CCTT	TCAA**G**CCTT	rs57585902
699G>A	Met233Ile	6	AAAT**G**TACG	AAAT**A**TACG	rs7311358
759T>A	Arg253Arg	7	CTCG**T**TGGG	CTCG**A**TGGG	rs61736830
767G>C	Gly256Ala	7	GTTG**G**AGCT	GTTG**C**AGCT	rs60140950
924A>T	Thr308Thr	7	AAAC**A**GCTA	AAAC**T**GCTA	
1557A>G	Ala519Ala	11	CAGC**A**CACT	CAGC**G**CACT	rs2053098
1559A>C	His520Pro	11	GCAC**A**CTTG	GCAC**C**CTTG	
1593A>G	Thr531Thr	11	GTAC**A**AGGA	GTAC**G**AGGA	
1614T>C	Val538Val	11	ATGT**T**GCAA	ATGT**C**GCAA	rs77851390
1679T>C	Val560Ala	11	ACTG**T**GAAg	ACTG**C**GAAg	rs12299012
1833G>A	Gly611Gly	13	AAGG**G**GCTT	AAGG**A**GCTT	rs3764006
1997G>A	Ser659Ser	14	CATC**G**GACA	CATC**A**GACA	rs60571683

Table 3

Allelic and genotype frequencies of non-synonymous *SLCO1B3* gene variants among different ethnic populations

	African Americans		Caucasians		Chinese		Hispanics		All	
SLCO1B3	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
334T>G										
T/T	19	43.2	17	38.6	12	26.1	8	17.8	56	31.3
T/G	16	36.4	8	18.2	11	23.9	10	22.2	45	25.1
G/G	9	20.5	19	43.2	23	50	27	60	78	43.6
Total	44	100	44	100	46	100	45	100	179	100
q	0.386		0.523		0.620		0.711		0.561	
439A>G										
A/A	45	97.8	45	100	46	100	44	100	180	99.4
A/G	1	2.2	0	0	0	0	0	0	1	0.6
G/G	0	0	0	0	0	0	0	0	0	0
Total	46	100	45	100	46	100	44	100	181	100
q	0.011		0		0		0		0.003	
699G>A										
G/G	10	21.7	6	14.0	1	2.3	2	4.4	19	10.7
G/A	28	60.9	7	16.3	16	36.4	11	24.4	62	34.8
A/A	8	17.4	30	69.8	27	61.4	32	71.1	97	54.5
Total	46	100	43	100	44	100	45	100	178	100
q	0.478		0.779		0.795		0.833		0.719	
767G>C										
G/G	43	93.5	30	66.7	46	100	40	88.9	159	87.4
G/C	3	6.5	14	31.1	0	0	5	11.1	22	12.1
C/C	0	0	1	2.2	0	0	0	0.0	1	0.5
Total	46	100	45	100	46	100	45	100	182	100
q	0.033		0.178		0		0.056		0.066	

	African Americans		Caucasians		Chinese		Hispanics		All	
SLCO1B3	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
1559A>C										
A/A	69	100	87	100	81	98.8	76	100	313	99.7
A/C	0	0	0	0	1	1.2	0	0	1	0.3
C/C	0	0	0	0	0	0	0	0	0	0
Total	69	100	87	100	82	100	76	100	314	100
q	0		0		0.006		0		0.002	
1679T>C										
T/T	64	92.8	87	100	82	100	76	100	309	98.4
T/C	5	7.2	0	0	0	0	0	0	5	1.6
C/C	0	0	0	0	0	0	0	0	0	0
Total	69	100	87	100	82	100	76	100	314	100
q	0.036		0		0		0		0.008	

N, number of individuals; *q*, frequency of variant allele

Table 4

Minor Allelic Frequency (%) of functional *SLCO1B3* polymorphisms. Results from this study were compared with previous entries in pharmacogenomic databases and publications among different ethnicities.

	Minor Allelic Frequency (%)				
	rs number	**rs4149117**	**rs7311358**	**-**	**rs12299012**
	SLCO1B3	**334T>G**	**699G>A**	**1559A>C**	**1679T>C**
	Effect	**Ser112Ala**	**Met233Ile**	**His520Pro**	**Val560Ala**
Reference	**Ethnicity**				
Our study	European American	52	78	0	0
Our study	African American	39	48	0	3.6
Our study	Asian (Chinese)	62	80	0.6	0
Our study	Hispanic	71	83	0	0
Perlegen	European	89	90	nd	nd
Perlegen	African American	35	35	nd	nd
Perlegen	Asian (Chinese)	83	84	nd	nd
HapMap	European	87	88	nd	0
HapMap	Asian (Chinese)	71	74	nd	0
HapMap	Asian (Japanese)	67	71	nd	0
HapMap	Sub-Saharan African	35	34	nd	1.7
Letschert et al. 2004	European	78	71	nd	nd
Franke et al. 2008	not specified	82	80	0	3
Smith et al. 2007	European Caucasian	81	83	nd	nd
Smith et al. 2007	American Caucasian	88	87	nd	nd
Smith et al. 2007	African American	41	41	nd	nd
Smith et al. 2007	Mexican	78	79	nd	nd
Smith et al. 2007	Han Chinese	80	77	nd	nd
Smith et al. 2007	Ghanaian	38	38	nd	nd
Baker et al. 2008	not specified	85	84	0	1.6
Tsujimoto et al. 2006	Japanese	73	73	nd	nd

Perlegen (AFD) and HapMap data were accessed on March 3rd 2009 via http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp;

nd, not determined