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Systematic Screening of Human *ABCC3* Polymorphisms and Their Effects on MRP3 Expression and Function

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Summary: The present study was undertaken to identify genetic polymorphisms of multidrug resistance-associated protein 3 (MRP3, gene name *ABCC3*), an ATP-binding cassette transporter that mediates the transport of substrates across the basolateral membrane into the blood, and to investigate their effects on *ABCC3* expression and MRP3 function. We identified genetic polymorphisms of *ABCC3* and evaluated the effects by (1) a luciferase reporter gene assay, (2) measuring mRNA levels, and (3) a human pharmacogenomics study with 4-methylumbelliferone glucuronide (4-MUG). Overall, 61 genetic variants were identified in three ethnic populations; of these variants 17 were novel (7 were non-synonymous: 61Arg>Cys, 132Gln>Stop, 221Trp>Stop, 270His>Gln, 548Leu>Gln, 600Lys>Arg, and 1324Arg>His). However, these mutations occurred at very low frequencies (max. 4.7%). The observed allele frequencies showed considerable inter-ethnic differences. The reporter gene assay indicated a significant reduction of transcriptional activity with the −1767G>A allele compared to the wild-type allele; however, a decreased expression of ABCC3 mRNA was not detected in human liver samples. A human pharmacokinetic study showed that the *ABCC3* genotype in the promoter region was not associated with changes in the pharmacokinetics of 4-MUG, a substrate of MRP3. This is the first study to assess the effects of *ABCC3* polymorphisms on human pharmacokinetics; however, further investigations are needed to complete the picture.

Keywords: ***ABCC3*; drug transporter; human; MRP3; pharmacogenomics; pharmacokinetics; polymorphism**

Introduction

Multidrug resistance-associated protein 3 (MRP3, gene *ABCC3*) is an ATP-binding cassette (ABC) transporter expressed in adrenal gland, liver, small intestine, colon, and gall bladder in humans.[1)] Typical substrates of MRP3 are glutathione, glucuronide, sulfate conjugates, and bile salts.[2)] The expression of MRP3 is up-regulated in patients with Dubin-Johnson syndrome in response to elevated bile salt levels and appears to compensate for the impaired function of MRP2 in the liver.[3,4)]

Recent studies suggest that MRP3 has a significant role in the pharmacokinetics (PK) of some compounds in mice. MRP3 is highly expressed on the basolateral membrane of enterocytes and hepatocytes, where it transports substrates into the bloodstream. Kitamura *et al.* revealed that the permeability of the serosal membrane to 4-methylumbelliferone glucuronide (4-MUG), suggested as a substrate of MRP3 by *in vitro* experiments,[5)] was significantly reduced in *Abcc3*$^{-/-}$ mice (to 33% of that in wild-type mice), suggesting that MRP3 plays a major role in the transport of substrates from enterocytes into the portal vein.[6)] Also in mice, MRP3 was demonstrated to significantly contribute to the hepatic basolateral excretion of 4-MUG.[7)] Systemic exposure to 4-MUG (AUC in plasma) after the oral administration of 4-MU in *Abcc3*$^{-/-}$ mice was decreased to 25% of that in wild-

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type mice.[8] Similarly, for clinically important drugs such as methotrexate and fexofenadine, significant reduction of the AUC in plasma and basolateral excretion were observed in *Abcc3*$^{-/-}$ mice.[9,10]

mRNA and protein levels of MRP3 in human liver vary extensively among individuals by 86- and 84-fold, respectively.[11] These findings imply that variability in the expression and transport activity of MRP3 is responsible for the inter-individual variability in the pharmacokinetics of MRP3 substrates. However, the mechanism responsible for the variability has not yet been elucidated; furthermore, few human studies have tested MRP3 function. One recent clinical study showed that a single nucleotide polymorphism (SNP) in the promoter region of *ABCC3* (−211C>T) altered the response to anti-tumor drugs. It found that the (−211C/T+T/T) group had significantly lower response rates to chemotherapy (most patients treated with etoposide and platinum-based drugs) than the −211C/C group.[12] Although the effect of the −211C>T genotype on the pharmacokinetics of MRP3 substrates is not clear, these findings suggest some clinical implications of MRP3 function.

In this study, to assess the mechanism responsible for the variability in MRP3 expression and the effect of the −211C>T variant on MRP3 function, we first analyzed genetic variations of the *ABCC3* gene in three ethnic groups (Japanese, Caucasians, and African-Americans). We then assessed their functional impact by (i) luciferase reporter gene assay, (ii) quantification of mRNA expression in human liver samples, and (iii) a human pharmacogenomics (PGx) study using 4-MUG, one of the substrates of MRP3, conducted in 50 healthy volunteers.

Materials and Methods

Blood and liver samples: Blood samples were obtained from unrelated Japanese, Caucasian, and African-American subjects (96 of each; Tennessee Blood Services, Memphis, TN). Liver tissue samples from 51 Caucasian donors (National Disease Research Interchange, Philadelphia, PA) were used for the quantification of ABCC3 mRNA expression. Histological records revealed that these samples were non-cirrhotic, non-tumorous, and showed no signs of local cholestasis.

Genomic DNA isolation and cDNA synthesis: Genomic DNA was isolated from blood samples with the use of the Toyobo blood kit (Toyobo, Osaka, Japan) on a Toyobo HMX-2000 robot (Toyobo). Total RNA extraction and reverse-transcription (RT)-polymerase chain reaction (PCR) procedures for liver samples were previously described.[13] Briefly, total RNA extracted with an RNAeasy Kit (Qiagen, Hilden, Germany) was treated with RNase-free DNase I. The samples were then treated with *Hae*III and *Mbo*II to digest the potential DNA template, prior to RT. The RNA samples were reverse transcribed into first-strand cDNA with 1 μg of total RNA, 4 μl of 5 × first strand buffer, 4 μl of 0.1 mM DTT, 1 μl of 500 μg/ml random primer (Promega, Madison, WI), 4 μl of 10 mM dNTP mixture, and 200 units of SuperScript II RNase H$^-$ reverse transcriptase (Life Technologies, Rockville, MD). The reaction was run at 42°C for 60 min.

Genotyping and haplotype analysis of *ABCC3* in three ethnic populations: Genetic variations of *ABCC3* sequences, including all 31 exons, their surrounding introns, and approximately 2000 bp of the 5′-flanking region, were examined using DNA samples from the three ethnic populations. *ABCC3*-specific primers for PCR were designed on the basis of reference sequences of the *ABCC3* gene (GenBank accession No. AC004591 and AC005921) to avoid the amplification of sequences from homologous genes (**Table 1**). The PCR was run for 35–40 cycles of 95°C for 40 s, 50–65°C for 45 s, and 72°C for 1 min. The products were analyzed with the single-strand conformation polymorphism (SSCP) method for screening of genetic variants followed by sequencing. Sequencing was performed directly or after sub-cloning on an ABI 3100 automatic sequencer (Applied Biosystems, Foster City, CA) using a Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Some variants listed in **Supplemental Table S1** were identified by the PCR-restriction fragment length polymorphism (RFLP) method. The haplotype for approximately 2000 bp of the 5′-flanking region was determined with ARLEQUIN Ver. 3.1 software.

Luciferase reporter gene assay: Six major haplotypes were amplified in length for approximately 2000 bp (from −2047 to −11 upstream of the ATG codon). For amplification, a *Kpn*I restriction site was attached to the forward primer, whereas the reverse primer was flanked by a *Sma*I site: 5′-CGGGGTACCCTCAAGCCCAGAGCAATCTG-3′ (forward) and 5′-TCCCCCGGGCGCGGCTGCAAGGAAGGCGAGC-3′ (reverse). The PCR product was ligated into the *Kpn*I and *Sma*I sites of the pGL3-basic vector (Promega). HepG2 cells were transiently transfected by the LipofectAMINE method and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum. Briefly, a mixture of 1.0 μl of LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) and 1 μg of reporter plasmid was transfected into the cells and kept there for 48 h. Then, 0.25 μg of the plasmid pRL-CMV (Promega) containing *Renilla* luciferase driven by the CMV (cytomegalovirus) promoter was co-transfected as a control. Luciferase activity was measured using the Dual Luciferase assay system (Promega). Promoter activity is given as the mean ± S.D. of triplicate determinations.

Quantitative real-time PCR: The mRNA levels were measured by real-time PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems). Amplification mixtures contained 10 μl of SYBR Premix Ex Taq (Takara, Kyoto, Japan), 0.4 μl of Rox reference dye, 1 μl of cDNA synthesis mixture, 4 pmol each of the