Short Communication

A Null Allele Impairs Function of CYP2C76 Gene in Cynomolgus Monkeys: A Possible Genetic Tool for Generation of a Better Animal Model in Drug Metabolism

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ABSTRACT:
The monkey CYP2C76 gene does not correspond to any of the human CYP2C genes, and its enzyme is at least partly responsible for the species difference occasionally seen in drug metabolism between monkeys and humans. To establish a line and/or lines of monkeys that are expected to show metabolic patterns highly similar to humans, we set out to find monkeys that lacked CYP2C76 activity. By genetic screening of 73 monkeys and a database search of expressed sequence tags, we found a total of 10 nonsynonymous genetic variants in the coding region of CYP2C76, including a null genotype (c.449TG>A). Some of the variants were differently distributed between two animal groups originating from different geographical regions (Indochina and Indonesia). After screening 170 additional genomic samples, we identified a total of eight animals (six males and two females) that were heterozygous for c.449TG>A, which could be used for establishing a homozygous line. If the homozygotes show drug-metabolizing properties more similar to humans than wild-type monkeys, the homozygotes may serve as a better animal model for drug metabolism. The data presented in this article provide the essential genetic information to perform a successful study by using cynomolgus monkeys and present a possible tool to generate a better animal model for drug metabolism.

Monkeys, especially macaques, which include the cynomolgus monkeys (*Macaca fascicularis*), are frequently used to predict the metabolic fate of drugs in humans due to their similar pharmacokinetics to humans. However, differences are occasionally seen between the two species for some drugs (Stevens et al., 1993; Sharer et al., 1995; Weaver et al., 1999). To predict the metabolism of drugs and the influence of drug-drug interactions in humans, species differences in drug-metabolizing enzymes including cytochrome P450s (CYPs) should be clarified and taken into account when extrapolating monkey data to humans.

CYP is one of the most important drug-metabolizing enzymes, and it is known to form a superfamily consisting of a large number of subfamilies (Nelson et al., 2004). One of the subfamilies, the CYP2C subfamily, including CYP2C8, CYP2C9, and CYP2C19, in humans is important for drug metabolism because the CYP2Cs metabolize approximately 20% of all prescribed drugs, such as tolbutamide, phenytoin, warfarin, and ibuprofen (Goldstein, 2001). For macaques, other groups as well as ours have identified cDNAs for CYP2C20, CYP2C43, CYP2C75, and CYP2C76 from cynomolgus or rhesus monkeys (Komori et al., 1992; Matsunaga et al., 2002; Mitsuda et al., 2006; Uno et al., 2006). Among these, the failure to identify a human gene homologous to CYP2C76 at the corresponding region of the human genome as well as the absence of protein in the human liver strongly suggest that none of the human genes are orthologous to CYP2C76 (Uno et al., 2006). CYP2C76 was at least partly responsible for the species difference in the metabolism of pitavastatin between monkeys and humans (Uno et al., 2007a). Moreover, investigation of 11 other CYPs that were isolated from a liver cDNA library revealed that these CYPs were, unlike CYP2C76, not apparent by species-specific, raising the possibility that CYP2C76 might be one of a few species-specific CYPs that is substantially expressed in the cynomolgus monkey liver (Uno et al., 2007b). These results suggest the importance of CYP2C76 for species difference in drug metabolism between monkeys and humans.

One way to improve accuracy in extrapolating animal data to humans is to establish better animal models. Eliminating the function of the species-specific genes, such as CYP2C76, in monkeys might lead to drug-metabolizing properties that are more similar to humans. Inactivation of the gene itself can be accomplished by gene targeting, but the technique has not been successfully applied to nonhuman primate species (Norgren, 2004). As in humans, monkeys have a diverse genetic background with a variety of genetic polymorphisms. Functionally null or defective alleles have been detected in the human CYP genes such as CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 (see http://www.imm.ki.se/CYPalleles/). Therefore, in this study, we made efforts to comprehensively identify genetic variants to identify null and/or defective alleles that are present in CYP2C76.

Materials and Methods
Animals and Genomic DNA Extraction. Genomic DNA was prepared from whole blood samples by using the PUREGENE DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer’s instructions. The blood samples used in this study were collected from 243

ABBREVIATIONS: CYP, cytochrome P450; PCR, polymerase chain reaction; EST, expressed sequence tag; SRS, substrate recognition site.
was approved by the local ethics committee. Cynomolgus monkeys (39 from Indochina and 204 from Indonesia). The study used the genome samples of 73 cynomolgus monkeys (39 from Indochina and 204 from Indonesia). The study was approved by the local ethics committee. Genetic variants and to determine allele frequencies of the identified variants, we used the genome samples of 73 cynomolgus monkeys (39 from Indochina and 204 from Indonesia). Genetic variants from animals of Indonesian origin were analyzed. Each exon as well as flanking DNA was amplified by polymerase chain reaction (PCR) followed by direct sequencing of the PCR products. PCR was carried out in 20-μl reactions containing 1 ng of genomic DNA, 5 pmol of forward and reverse primers, and 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The amplification was performed on the MJ Research thermal cycler (Bio-Rad, Hercules, CA) with an initial denaturation at 95°C for 10 min and 30 cycles of 20 s at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. Sequencing was conducted by using ABI PRISM BigDye Terminator version 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) followed by electrophoresis with the ABI PRISM 3730 DNA Analyzer (Applied Biosystems). The primer pairs that were used for PCR and sequencing are listed in Table 1. Sequence data were analyzed using DNAStat Pro (Hitachi Software, Tokyo, Japan), which was also used for the identification of genetic variants from an in-house expressed sequence tag (EST) database.

Preparation of Expression Plasmids and Protein Expression. For the functional characterization of genetic variants, the construction of expression plasmids, protein expression, and measurement of CYP proteins were performed as previously known (Uno et al., 2006). A mutation was introduced into the CYP2C76 expression plasmid by using the QuikChange XL II kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The primer pairs that were used are listed in Table 1. The entire sequence of each insert was confirmed by sequencing.

Measurement of Tolbutamide Methyl-Hydroxylation. To measure the drug-metabolizing activity of CYP2C76 variants, tolbutamide methyl-hydroxylation was measured, toward which CYP2C76 shows the drug-metabolizing activity (Uno et al., 2006, 2007a). To prepare the reaction mixture, tolbutamide was preincubated in 250 mM potassium phosphate buffer solution (pH 7.4) with the partially purified recombinant CYP proteins at 37°C for 5 min. The reaction was initiated by the addition of the NADPH-regenerating system (BD Biosciences, San Jose, CA) in a total volume of 0.5 ml. The incubations were carried out at 37°C in triplicate at linear conditions for 30 min using tolbutamide at 5 concentrations between 0.25 and 3.0 mM. Ice-cold 70% perchloric acid (50 μl) was added to stop the reaction. An internal standard solution and ultrapure water were added to the reaction mixture, and the resulting mixture was centrifuged (7500g, 4°C, 10 min). The metabolites in the supernatant were analyzed by liquid chromatography/tandem mass spectrometry (Shimadzu, Kyoto, Japan). The Michaelis-Menten parameters of the apparent Km and Vmax were determined using WinNonlin (version 4.0; Pharsight, Mountain View, CA).

Results and Discussion

Our initial survey of 73 genome samples (39 from Indochina and 34 from Indonesia) and our in-house EST database identified a number of variants over all exons and their vicinities of CYP2C76 in cynomolgus monkeys. To describe genetic variants, the CYP2C76 cDNA sequence (DQ074807) originally identified (Uno et al., 2006) was regarded as a reference sequence in this study due to the unavailability of a consensus wild-type sequence for CYP2C76. Because we were interested in the null or defective alleles to generate an animal model, only nonsynonymous variants were listed (Table 2). A total of 10 nonsynonymous variants were identified, 3 of which, c.707A>T, c.883A>G, and c.1292A>G, were located in the regions that are important for protein function, such as the substrate recognition site (SRS) (Gotoh, 1992) and the heme binding region. Because of the potential relevance of these variants, their influence on protein function was investigated by using recombinant proteins that are heterologously expressed in Escherichia coli. The drug-metabolizing capacity of the recombinant proteins using tolbutamide as a substrate indicated that these 3 variants showed an appreciable reduction in metabolic activity, compared with the CYP2C76 protein of the reference sequence (Table 2).

Importantly, we found a null allele c.449TG>A in exon 3 (Table 2), causing a frameshift and a premature termination codon in the same exon. The sequencing chromatogram for this allele is described in Fig. 1A. With this allele, approximately three fourths of the entire protein is predicted to be truncated including the heme binding region and all of the SRs, with the exception of SRS1, indicating that the protein, even if translated, would not be functional. Indeed, the CYP-specific peaks with a maximum at 450 nm observed for the protein of reference sequence were not found for the protein with this
A for establishing a better animal model, importance of c.449TG/H11022 strongly indicating the c.449TG genotype by reduced carbon monoxide-difference spectra (Fig. 1B), for the CYP2C76 of the reference sequence were 704 and 1570, respectively. The genetic variants identified in cynomolgus CYP2C76

<table>
<thead>
<tr>
<th>Variant</th>
<th>Exon</th>
<th>Nucleotide Changea</th>
<th>Amino Acid Change</th>
<th>Location on cDNA</th>
<th>Allele Frequency</th>
<th>Site</th>
<th>Kinetic Parametersb</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.178G&gt;T</td>
<td>2</td>
<td>caaa(G&gt;T)attat</td>
<td>D60Y</td>
<td>178</td>
<td>0.513</td>
<td>0.500</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>c.193C&gt;T</td>
<td>2</td>
<td>ctgtg(C&gt;T)tctct</td>
<td>L65F</td>
<td>193</td>
<td>0.513</td>
<td>0.500</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>c.241G&gt;A</td>
<td>2</td>
<td>gatac(G&gt;A)aagca</td>
<td>E81K</td>
<td>241</td>
<td>0.513</td>
<td>0.500</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>c.449TG&gt;A</td>
<td>3</td>
<td>agctc(TG&gt;A)tctct</td>
<td>Frameshift</td>
<td>449</td>
<td>0.020</td>
<td></td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>c.707A&gt;T</td>
<td>5</td>
<td>aattt(A&gt;T)tgctc</td>
<td>Y236F</td>
<td>707</td>
<td>0.036</td>
<td>1.000</td>
<td>N.D. 710 412</td>
</tr>
<tr>
<td>c.883G&gt;A</td>
<td>6</td>
<td>ttgtt(A&gt;G)tctca</td>
<td>S295G</td>
<td>883</td>
<td>0.013</td>
<td>0</td>
<td>N.D. 824 927</td>
</tr>
<tr>
<td>c.928A&gt;T</td>
<td>6</td>
<td>gactg(A&gt;G)gtgca</td>
<td>M310L</td>
<td>928</td>
<td>0.731</td>
<td>1.000</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>c.968G&gt;A</td>
<td>7</td>
<td>aatgc(G&gt;T)ggaag</td>
<td>R323Q</td>
<td>968</td>
<td>0.231</td>
<td>0</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>c.1292A&gt;Gc</td>
<td>9</td>
<td>aggaag(A&gt;G)gaaga</td>
<td>K431R</td>
<td>1292</td>
<td>0</td>
<td>0</td>
<td>Heme binding 836 1393</td>
</tr>
<tr>
<td>c.1436C&gt;T</td>
<td>9</td>
<td>tatac(C&gt;T)acctc</td>
<td>P479L</td>
<td>1436</td>
<td>0.205</td>
<td>0.074</td>
<td>N.D. N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

a Nucleotide changes were detected by comparing with the CYP2C76 cDNA sequence (GenBank accession no. DQ074807) as a reference sequence.

b $K_m$ (μM) and $V_{max}$ (pmol/min/nmol CYP) of each recombinant protein was determined with tolbutamide methyl-hydroxylation, for which the detection limit was 40 pmol/ml. The $K_m$ and $V_{max}$ for the CYP2C76 of the reference sequence were 704 and 1570, respectively.

The allele frequency of the other identified variants was determined with the genomic samples of the 73 animals that were used for the analysis, probably reflecting the genetic difference of the animals used to identify CYP2C76 cDNA and those used in this study. Some of the alleles were identified by searching the EST database, and was not found in any genome samples that were analyzed in this study.

The frequency was estimated to be 0.020 for the animals of Indonesian origin that were analyzed in this study. One allele, c.1292A>G, which was detected by searching the EST database, was not found in any genome samples that were analyzed in this study.

The genetic variants identified in cynomolgus CYP2C76 were distributed differently between the two animal groups. One reason for this distinction could be the regional difference in the allele frequency, because the two animal groups originated from different geographic regions, Indochina and Indonesia, and are separated by an ocean. Likewise, the regional difference of allele frequency has been revealed for the rhesus monkeys of Chinese and Indian origin as well (Ferguson et al., 2007; Rhesus Macaque Genome Sequencing and Analysis Consortium, 2007). Alternatively, the difference might simply be attributable to the different colonies from which the animals used in this study were derived. One allele, c.1292A>G, which was detected by searching the EST database, was not found in the 73 animals analyzed in this study (Table 2). The reason for this discrepancy may be accounted for by assuming that the animals used in this study and those that were used for EST sequencing were derived from different colonies. Further screening of animals from various colonies in different regions should give a clearer answer to this question. Consid-
ering the possibility that genetic variants such as c.449TG>A could substantially affect drug metabolism in monkeys, the origin of the animals that were used is one of the most important factors for a successful study on drug metabolism.

Inactivation of species-specific genes such as CYP2C76 could lead to a better animal model in monkeys. However, the techniques of gene knockout or knockdown in vivo have not been available in this species (Norgren, 2004). Our results present an alternative way to produce animals lacking the function of a specific protein, by using genetic polymorphisms such as null or defective alleles in monkeys. It should be noted that, in our case, this is only possible if CYP2C76 does not play a crucial role in embryonic or fetal development. This might be one of the reasons for failing to identify homozygotes after screening 243 animals. For example, CYP26A1 protects tissues from overexposure to all-trans retinoic acid by metabolizing (degrading) it; otherwise, embryos cannot survive, as evidenced by prenatal death of the CYP26A1 null mouse (Niederreither et al., 2002). CYP2C76 might play a similar developmental role if CYP2C76 is expressed in embryos or fetuses. Nevertheless, this approach is not only limited to CYP2C76, but it is also applicable for many other genes, and it should be useful in generating better animal models for humans in a variety of physiological investigations.

In this study, our initial screening for genetic variants in cynomolgus CYP2C76 has successfully identified a total of eight nonsynonymous variants. These include the null allele (c.449TG>A), which resulted in a frameshift and premature termination codon introduced in exon 3. So far, six males and two females have been found to be heterozygous for this allele. These animals can now be used to generate homozygotes through mating. If the homozygotes show drug-metabolizing properties more similar to humans, they may serve as a better animal model for drug metabolism.

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