Short Communication

Genetic Variants of CYP3A4 and CYP3A5 in Cynomolgus and Rhesus Macaques

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ABSTRACT:

Cynomolgus and rhesus macaques are frequently used in preclinical trials due to their close evolutionary relationships to humans. We conducted an initial screening for genetic variants in cynomolgus and rhesus macaque genes orthologous to human CYP3A4 and CYP3A5. Genetic screening of 78 Indochinese and Indonesian cynomolgus macaques and 34 Chinese rhesus macaques revealed a combined total of 42 CYP3A4 genetic variants, including 12 nonsynonymous variants, and 34 CYP3A5 genetic variants, including nine nonsynonymous variants. Four of these nonsynonymous variants were located at substrate recognition sites or the heme-binding region, domains essential for protein function, including c.886G>A (V296M) and c.1310G>A (T437S) in CYP3A4 and c.1437C>G (N479K) and c.1310G>C (T437S) in CYP3A5. The mutant proteins of these genetic variants were expressed in Escherichia coli and purified. Metabolic activity of these proteins measured using midazolam and nifedipine as substrates showed that none of these protein variants substantially influences the drug-metabolizing capacity of CYP3A4 or CYP3A5 protein. In Indonesian cynomolgus macaques, we also found IVS3+1delG in CYP3A4 and c.625A>T in CYP3A5, with which an intact protein cannot be produced due to a frameshift generated. Screening additional genomes revealed that two of 239 animals and three of 258 animals were heterozygous for IVS3+1delG of CYP3A4 and c.625A>T of CYP3A5, respectively. Some genetic variants were unevenly distributed between Indochinese and Indonesian cynomolgus macaques and between cynomolgus and rhesus macaques. Information on genetic diversity of macaque CYP3A4 and CYP3A5 presented here could be useful for successful drug metabolism studies conducted in macaques.

Cynomolgus (Macaca fascicularis) and rhesus (Macaca mulatta) macaques have been used to predict the metabolic fate of drugs in humans due to their evolutionary closeness to humans. As with humans, macaques have a diverse genetic background as evidenced by numerous genetic polymorphisms that have been reported (Ferguson et al., 2007; Hernandez et al., 2007; Street et al., 2007). In cynomolgus macaques, interanimal differences have been noted in drug metabolism by in vivo analysis using dextromethorphan and S-mephentoin as probe substrates (Jacqz et al., 1988), which could be, in some part, attributable to genetic variability of drug-metabolizing enzymes, because genetic variants of cytochromes P450 (P450s) such as CYP2C76 have been identified (Uno et al., 2009).

Human CYP3As are considered the major drug-metabolizing cytochrome P450 (P450) subfamily, comprised of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (Gellner et al., 2001). In humans, CYP3As account for more than half of the total P450 content in human liver (Thummel and Wilkinson, 1998) and metabolize more than half of all prescription drugs, such as nifedipine, midazolam, and testosterone (Thummel and Wilkinson, 1998; Evans and Relling, 1999). Numerous interindividual differences in drug-metabolizing capability mediated by CYP3A4 and CYP3A5 have been reported in humans, some of which are partially caused by genetic polymorphisms (see http://www.imm.ki.se/CYPalleles/). For example, hepatic CYP3A5 protein is present at detectable levels in 10 to 30% of whites and 60% of African Americans (Hustert et al., 2001; Kuehl et al., 2001; Lin et al., 2002). This interindividual variability in CYP3A5 protein expression is highly correlated with CYP3A5*3, a defective allele, because the aberrant splicing imposed by the mutant allele gives rise to a non-functional protein (Kuehl et al., 2001). Identification and characterization of such genetic variants are essential for understanding drug-metabolizing properties of CYP3A4 and CYP3A5 enzymes.

Several groups, including ours, have identified cDNAs highly homologous to human CYP3A4 or CYP3A5 cDNA in cynomolgus and rhesus macaques (Komori et al., 1992; Carr et al., 2006; Uno et al., 2007). Between these macaque species and humans, sequence identity of CYP3A4 and CYP3A5 is approximately 95 and 94% in cDNA and 93 and 91% in amino acid sequence, respectively. After consulting with the P450 Nomenclature Committee (http://drnelson.utmem.edu/cytochromeP450), in this article, we designate cynomolgus CYP3A8 and rhesus CYP3A64, both orthologous to human CYP3A4, as CYP3A4, and rhesus CYP3A66, orthologous to human CYP3A5, as CYP3A5. Rhesus CYP3A4 possesses metabolic capabilities and induction properties similar to human CYP3A4 (Carr et al., 2006). Cynomolgus CYP3A4 and CYP3A5 exhibited testosterone 6β-hydroxylation (Uno et al., 2007), similar to their human P450 counterparts, indicating similar drug-metabolizing properties of macaque and human CYP3A.

ABBREVIATIONS: P450, cytochrome P450; PCR, polymerase chain reaction; UTR, untranslated region; RT, reverse transcription; SRS; substrate recognition site.
In this study, we attempted to comprehensively identify and characterize genetic variants for CYP3A4 and CYP3A5 in cynomolgus and rhesus macaques. Genome samples from Indochinese and Indonesian cynomolgus macaques and from Chinese rhesus macaques were used to elucidate the regional and lineage differences in allele frequency of the identified variants. Metabolic activities were measured by using the human CYP3A substrates, midazolam and nifedipine, to characterize potentially important variants.

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, Minneapolis, MN) according to the manufacturer’s instructions. The blood samples used in this study were collected from 296 cynomolgus macaques (38 from Indochina and 258 from Indonesia) and 34 rhesus macaques (from China). This study was reviewed and approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd.

DNA Sequencing. Genetic variants were identified by polymerase chain reaction (PCR) amplification and sequencing of all CYP3A4 and CYP3A5 exons, including the 5' untranslated region (UTR), the coding region, and the 3'UTR, using genome samples from 78 cynomolgus macaques (38 from Indochina, 40 from Indonesia) and 34 rhesus macaques. For IVS3+1delG of CYP3A4 and c.625A>T of CYP3A5, 199 and 218 cynomolgus macaques were also genotyped, respectively. A 20-μl PCR reaction contained 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 in a thermal cycler (Applied Biosystems) 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 performed by using an ABI PRISM BigDye Terminator version 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems), followed by electrophoresis on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems). The primers used for PCR and sequencing are listed in Supplemental Material Table 1 (supplemental tables are available at http://dmd.aspetjournals.org). Sequence data were analyzed by using DNASIS Pro (Hitachi Software, Tokyo, Japan). Genetic variants of CYP3A4 were identified by comparison with CYP3A4 cDNA sequences of cynomolgus macaque (GenBank accession number S53047) and rhesus macaque (GenBank accession number NM_017460), whereas CYP3A5 variants were identified by comparison with CYP3A5 cDNA sequences of cynomolgus macaque (GenBank accession number DQ074795) and rhesus macaque (GenBank accession number NM_001040219).

Preparation of Expression Plasmids and Protein Expression. For functional characterization of the genetic variants, expression plasmids were prepared as described previously (Iwata et al., 1998; Uno et al., 2006). The mutation was introduced into the expression plasmid containing cynomolgus CYP3A4 cDNA (GenBank accession number S53047) by using the QuiqChang XL II kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The primer pairs used were as follows: 5'-ATGAAAAAGTGTGGCGCTTTTATGATGTC-3' and 5'-GACCATCTATATAAGCCCAGATTTTCTTATC-3' for IVS3+1delG; 5'-GTCTGATGCAGCTGCTGACCAATTACACATTT-3' and 5'-AA-GATAATGTGGCGGCGATGCTCCAGATGAC-3' for c.625A>T; 5'-CCCTTACTATATACGCTGTCGAAAATGCTCTCTGAG-3' and 5'-GAGTGTGTCGCTCTGATCTCCAAAAAGGCTGTAAG-3' for c.1310G>A. Likewise, the mutation was introduced into the expression plasmid containing cynomolgus CYP3A5 cDNA (GenBank accession number DQ074795) by using the following primers: 5'-GGGAAAAGTTTAAGTCTTCTAATTATTTG-3' and 5'-CAATTTTATACGTTGCTGACCTCAGAATCCATTG-3' for c.625A>T; 5'-CATATAACACCCTCTGGAAGCTGACGCAAAGTCTG-3' and 5'-CCAATGCTGCTGCTGACCTCAGAATCCATTG-3' for c.1310G>A; and 5'-CTGAGATGAGTTAAGCTGACGCAAAGTCTG-3' and 5'-CTGAGATGAGTTAAGCTGACGCAAAGTCTG-3' for c.1310G>C. The entire sequence of the cDNA insert was confirmed by sequencing. The PCR products were cloned into a pcDNA vector containing human NADPH-P450 reductase cDNA. The plasmids were used for protein expression in Escherichia coli performed according to the method of Iwata et al. (1998). Membrane fractions were prepared from bacterial cells as described previously (Sandhu et al., 1994). Measurement of P450 protein content in the membrane preparations was determined spectrally, according to the method reported by Omura and Sato (1964). The yield of NADPH-P450 reductase was estimated as described previously (Phillips and Langdon, 1962).

Measurement of Enzyme Activity. Midazolam hydroxylation and nifedipine oxidation were determined as described previously (Yamazaki et al., 1999). In brief, a typical incubation mixture (0.25 ml) contained recombinant CYP3A4 or CYP3A5 proteins (5 pmol), an NADPH-generating system (0.25 mM NADP+, 2.5 mM glucose 6-phosphate, and 0.25 unit/ml glucose 6-phosphate dehydrogenase), and substrate (100 μM midazolam or 100 μM nifedipine) in 0.10 M potassium phosphate buffer (pH 7.4). Midazolam reactions were incubated at 37°C for 10 min and terminated by addition of 0.25 ml of ice-cold acetonitrile. After centrifugation at 2000g for 10 min, the supernatant was analyzed by high-performance liquid chromatography with an ultraviolet detector. Nifedipine reactions were incubated at 37°C for 5 min and terminated by addition of 1.5 ml of CH3Cl2, 0.2 M NaCl, and 0.1 M Na2CO3. Organic phases were evaporated under a stream of nitrogen gas, and product formation was determined by high-performance liquid chromatography with an ultraviolet detector.

RNA Preparation and Reverse Transcription-PCR. To examine transcripts of macaque CYP3A4 and CYP3A5, total RNA was extracted from livers of seven cynomolgus macaques and reverse transcription (RT)-PCR was carried out as described previously (Uno et al., 2006). In brief, the first-strand cDNA was generated in a mixture containing 1 μg of total RNA, oligo(dT), and SuperScript II RT reverse transcriptase (Invitrogen, Carlsbad, CA) at 37°C for 1 h. One twenty-fifth of this reaction was used for the subsequent PCR that was carried out by using AccuPrime Taq DNA polymerase (Invitrogen) according to the manufacturer’s protocol. PCR conditions include an initial denaturation at 94°C for 2 min and 35 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 2 min, followed by a final extension at 68°C for 5 min. The primers used were as follows: 5'-CACACACAGCCAGCAACC-3' and 5'-CCGCTTCATTTTCAGGGTCTC-3' for CYP3A4; and 5'-CGATGGAC-CTATCCCAAAAT-3' and 5'-CTCTTTCTCCATGTTGTTT-3' for CYP3A5. The amplified cDNAs were cloned into vectors, and the inserts were sequenced as described earlier.

Results and Discussion

To identify genetic variants, all CYP3A4 and CYP3A5 exons including the 5'UTR, the coding region, and the 3'UTR, were amplified and sequenced by using genome samples from 78 cynomolgus macaques (38 from Indochina and 40 from Indonesia) and 34 rhesus macaques. A total of 42 variants was identified in CYP3A4 exons, including five in the 5'UTR, 27 in the coding region, and 10 in the 3'UTR (Table 1). c.886G>A in substrate recognition site (SRS) 4 and c.1310G>A in the heme-binding region were among 12 nonsynonymous variants found. A total of 34 variants was found in CYP3A5 exons, including five in the 5' UTR and 29 in the coding region (Table 2). No variants were found in the 3' UTR. Nine of the variants found were nonsynonymous including c.1310G>C in the heme-binding region and c.1437C>G in SRS6, c.387T>A and c.1310G>C in CYP3A5 were found to be major alleles in the animals analyzed, probably reflecting genetic differences between the animals used to identify CYP3A5 cDNA and those used in this study. The frequency of all genotypes was in Hardy-Weinberg equilibrium within each population (χ² test).

A comparison of allele frequency between cynomolgus and rhesus macaques revealed that 10 (23.8%) of total 42 variants for CYP3A4 and 6 (17.6%) of total 34 variants for CYP3A5 were shared by both macaque lineages. Likewise, a recent study indicated that approximately 50% of the genetic polymorphisms were shared between the two lineages (Street et al., 2007), suggesting that genetic variants identified in either macaque lineage can be partly used for analysis of the other. On the other hand, 24 and 8 of CYP3A4 alleles and 22 and 6 of CYP3A5 alleles were unique to cynomolgus and rhesus macaques, respectively. Among the variants found only in cynomolgus macaques, 10 CYP3A4 variants, including three nonsynonymous vari-
ants, and 11 CYP3A5 variants, including three nonsynonymous variants, appear to be unique to Indochinese cynomolgus macaques, and 11 CYP3A4 variants, including five nonsynonymous variants, and 10 CYP3A5 variants, including six nonsynonymous variants, appear to be unique to Indonesian cynomolgus macaques, reflecting regional differences in the prevalence of these alleles. This type of regional difference in allele frequency has also been found in rhesus macaques of Chinese and Indian origin (Ferguson et al., 2007; Hernandez et al., 2007). Moreover, in humans, genetic diversity was 2.4-fold higher in rhesus macaques (data not shown). By in-depth population genetic analysis, Osada and colleagues proposed that the reduced genetic diversity in macaque CYP3A4 might have been caused by quick

TABLE 1

<table>
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<tr>
<th>Exon</th>
<th>Nucleotide Changea</th>
<th>Amino Acid Change</th>
<th>Nucleotide Positionb</th>
<th>Number of Allelesd</th>
<th>Site</th>
<th>Nucleotide Substitutions in CYP3A4 cDNAe</th>
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<td>5 ’-UTR</td>
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<td>0/80</td>
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<td>0/80</td>
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</tr>
<tr>
<td>12</td>
<td>TGAGAG (G=) ATTTTG</td>
<td>17/6</td>
<td>0/80</td>
<td>0/80</td>
<td>6/68</td>
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</tr>
<tr>
<td>13</td>
<td>GCACAG (G=) ATTTTG</td>
<td>17/6</td>
<td>0/80</td>
<td>0/80</td>
<td>6/68</td>
<td>C</td>
</tr>
</tbody>
</table>

The genetic variants identified in cynomolgus and rhesus CYP3A4

a Nucleotide changes are described as a comparison to the reference sequence of cynomolgus CYP3A4 cDNA (GenBank accession number NM_017460).

b Position number is based on the first nucleotide of the initiation codon being 1. The upstream region is represented in negative numbers.

c CYP3A4 was resequenced in DNA from 78 cynomolgus (38 from Indochina and 40 from Indonesia) and 34 rhesus macaques.

d The corresponding nucleotides in human CYP3A4 sequence (GenBank accession number NM_000540) and single nucleotide polymorphisms reported to the human P450 allele nomenclature (http://www.cypalleles.ki.se/) for human CYP3A4: Allele numbers and resulting amino acid changes are described. No corresponding nucleotide for c.1708T>G allele is present in human CYP3A4 cDNA sequence (GenBank accession number NM_007460) due to deletion of the corresponding region in the human sequence.

e For IVS3+1delG, 239 animals were used to calculate allele frequency in Indonesian population.

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The estimated CYP3A4 diversity was 9.21 × 10−4 for Indochinese, 8.84 × 10−4 for Indonesian cynomolgus macaques, and 1.22 × 10−3 for rhesus macaques. The nucleotide diversity for human CYP3A4 was estimated to be 4.68 × 10−5 based on previously published allele frequency data (Thompson et al., 2004). The estimate for CYP3A5 was 9.70 × 10−4 for Indochinese and 6.39 × 10−4 for Indonesian cynomolgus macaques, which were slightly lower than that of rhesus macaques (1.02 × 10−3). Moreover, the nucleotide diversity for human CYP3A5 was estimated to be 1.10 × 10−3 based on published data (Thompson et al., 2004). The higher genetic diversity of CYP3A4 and CYP3A5 for macaques compared with that in humans raises the probability of greater interindividual variability in CYP3A4-mediated drug-metabolizing capabilities of macaques compared with humans. Moreover, in humans, genetic diversity was 2.4-fold higher in CYP3A4 than in CYP3A4, whereas the nucleotide diversity between the two genes was not considerably different in cynomolgus and rhesus macaques (data not shown). By in-depth population genetic analyses, Osada and colleagues proposed that the reduced genetic diversity in macaque CYP3A4 might have been caused by quick

GANTS, and 11 CYP3A5 variants, including three nonsynonymous variants, appear to be unique to Indochinese cynomolgus macaques, and 11 CYP3A4 variants, including five nonsynonymous variants, and 10 CYP3A5 variants, including six nonsynonymous variants, appear to be unique to Indonesian cynomolgus macaques, reflecting regional differences in the prevalence of these alleles. This type of regional difference in allele frequency has also been found in rhesus macaques of Chinese and Indian origin (Ferguson et al., 2007; Hernandez et al., 2007). Because such genetic heterogeneity between different populations could cause variability in drug-metabolizing properties, it would be prudent to use animals from a single population.

Nucleotide diversity per base pair in the coding region was estimated for CYP3A4 and CYP3A5 based on allele frequency. The estimated CYP3A4 diversity was 9.21 × 10−4 for Indochinese, 8.84 × 10−4 for Indonesian cynomolgus macaques, and 1.22 × 10−3 for rhesus macaques. The nucleotide diversity for human CYP3A4 was estimated to be 4.68 × 10−5 based on previously published allele frequency data (Thompson et al., 2004). The estimate for CYP3A5 was 9.70 × 10−4 for Indochinese and 6.39 × 10−4 for Indonesian cynomolgus macaques, which were slightly lower than that of rhesus macaques (1.02 × 10−3). Moreover, the nucleotide diversity for human CYP3A5 was estimated to be 1.10 × 10−3 based on published data (Thompson et al., 2004). The higher genetic diversity of CYP3A4 and CYP3A5 for macaques compared with that in humans raises the probability of greater interindividual variability in CYP3A4-mediated drug-metabolizing capabilities of macaques compared with humans. Moreover, in humans, genetic diversity was 2.4-fold higher in CYP3A4 than in CYP3A4, whereas the nucleotide diversity between the two genes was not considerably different in cynomolgus and rhesus macaques (data not shown). By in-depth population genetic analyses, Osada and colleagues proposed that the reduced genetic diversity in macaque CYP3A4 might have been caused by quick

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fixation of beneficial nonsynonymous polymorphisms in either macaque lineage (N. Osada, unpublished data). A previous study also suggested that CYP3A5 in humans may have been under different selection pressures between European and African populations (Thompson et al., 2004). These results suggest that CYP3A5 is an important gene for adaptation to local environment both in humans and macaques.

For functional characterization of genetic variants identified in macaque CYP3A4 and CYP3A5, mutant proteins were heterologously expressed in E. coli and membrane fractions were used to measure their drug-metabolizing activities using midazolam or nifedipine as substrates. Due to the importance of the SRS and the heme-binding region for protein function, we focused our analysis on genetic variants located in those functional domains, including c.886G region for protein function, we focused our analysis on genetic variability. Any genetic polymorphisms of human CYP3A5 corresponding to macaque CYP3A5 alleles identified in this study were not reported to the human P450 allele nomenclature (http://www.cypalleles.ki.se/).

### Table 2

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Nucleotide Position</th>
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<th>Site</th>
<th>CYP3A5 cDNA</th>
<th>Rhesus cDNA</th>
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* Nucleotide changes are indicated as a comparison to the reference sequence of cynomolgus CYP3A5 cDNA (GenBank accession number DQ074795).
* Position number is based on the first nucleotide of the initiation codon being +1. The upstream region is represented in negative numbers.
* CYP3A5 genotypes were determined for 78 cynomolgus (38 from Indochina and 40 from Indonesia) and 34 rhesus macaques.
* The CYP3A5 nucleotides different from cynomolgus macaque are shown for human (GenBank accession number NM_0000777) and rhesus macaque (GenBank accession number NM_001040219). Any genetic polymorphisms of human CYP3A5 corresponding to macaque CYP3A5 alleles identified in this study were not reported to the human P450 allele nomenclature (http://www.cypalleles.ki.se/).
* For c.625A>T, 258 animals were used to calculate allele frequency in Indonesian population.
been reported (see http://www.imm.ki.se/CYPalleles/). Although the functional consequence of *CYP3A4*^*15* (R162Q) has not been analyzed, *CYP3A4*^*18* (L293P) has been shown to alter the drug-metabolizing activities of human CYP3A4 enzyme. *CYP3A4*^*18* exhibited lower metabolic turnover for midazolam but higher turnover for testosterone and estrogens (Dai et al., 2001; Kang et al., 2009). Codon 293 of human CYP3A4 is located at the beginning of the conserved helix I, which is important for substrate specificity. Molecular modeling indicates that L293P modifies a straight α helix into two small α helices connected by a short loop, which essentially modifies overall tertiary structure and the arrangement of SRS regions (Kang et al., 2009). These conformational changes can account for alterations in enzymatic activity and possibly for the difference in metabolic activities between midazolam and steroid hormones (Kang et al., 2009). Influences, if any, of c.878T→A (L293Q) on the metabolic properties of macaque CYP3A4 protein could be elucidated with further investigation. Considering that the functionally important *CYP3A4*^*18* (L293P) is near but not within SR54, it is of great interest to perform functional characterization of other nonsynonymous variants of macaque CYP3A4 and CYP3A5 found in this study.

We investigated linkage disequilibrium between the genetic variants, the enzyme activities of which we analyzed, because of the close proximity of macaque CYP3A4 and CYP3A5 in the genome (~130 kb). The linkage disequilibrium between c.1310G→A (S437N) in CYP3A4 and c.1310G→C (T437S) in CYP3A5 was tested. Minor allele frequencies of the other genetic variants were quite low, and there were no animals carrying two different genetic variants. Haplotype frequency was calculated, excluding double heterozygous genotypes. We found that, in Indochinese macaqueomycus macaques, S437N (CYP3A4) and T437S (CYP3A5) were in linkage disequilibrium (*P* = 0.03; χ² test). The haplotype with 437N (CYP3A4) and 437T (CYP3A5), both derived alleles, was significantly overrepresented. It is interesting to note that 437N (CYP3A4) has greater and 437T (CYP3A5) showed smaller enzymatic activities than the ancestral alleles (Table 3). It may be possible that the two variants work with a functional compensation.

We also found potential null alleles, IVS3 + 1delG, at the exon 3/intron 3 boundary of CYP3A4 (Table 1) and c.625A>T in exon 7 of CYP3A5 (Table 2). In the presence of these alleles, a frameshift and a premature termination codon are generated, M89X for the former and K209X for the latter, resulting in the truncation of more than half of the protein, including SRSs and the heme-binding region. The P450-specific maximal peak at 450 nm was observed with the reference sequence protein but not with the mutant proteins in the reduced CO difference spectrum (data not shown). Moreover, metabolic assays revealed absence of mutant protein activities (Table 3), indicating that IVS3 + 1delG and c.625A>T are probably null alleles. Further screening of additional genomic samples from 199 and 218 Indone-
sian cynomologus macaques found additional heterozygotes for CYP3A4 IVS3 + 1delG and CYP3A5 c.625A>T, respectively. Overall, 2 of 239 animals were heterozygous for CYP3A4 IVS3 + 1delG, whereas 3 of 258 animals were heterozygous for CYP3A5 c.625A>T, leading to estimated allele frequencies of 0.0042 for the former and 0.0058 for the latter in Indone-
sian cynomologus macaques analyzed in this study.

In humans, *CYP3A5*^*3* (g.6986A→G) causes aberrant splicing, leading to the production of a nonfunctional protein due to insertion of the intron sequence in the transcribed mRNA (Kuehl et al., 2001; Lin et al., 2002). Due largely to this genotype, CYP3A5 protein expression is highly variable in human liver. If macaques possess similar genetic variants, they could be responsible for interanimal variability of drug metabolism. To explore this possibility, RT-PCR was performed with liver samples from seven animals; however, no aberrantly spliced cynomologus CYP3A5 transcripts were identified (data not shown). Therefore, genetic variants equivalent to CYP3A5*3* were not found in our macaque samples. With CYP3A5*3*, leaky splicing can occur, resulting in the partial production of functional protein. An equal amount of normal and aberrantly spliced transcripts in heterozygous liver samples was observed in the RT-PCR and sequencing results (data not shown) for macaque CYP3A4 IVS3 + 1delG, suggesting that leaky splicing does not occur with this genotype.

To examine gene function, gene knockout and knockdown have been carried out in mice but have not been reported in macaques, probably due to technical difficulties. Our results present an alternative way to produce animals lacking macaque CYP4A5 or CYP3A5 function by identifying or producing animals homozygous for the null alleles identified in this study. If macaques, like humans, show overlapping substrate specificities between CYP3A4 and CYP3A5, it would be difficult to understand the contribution of each macaque CYP3A5 to overall drug metabolism in vivo. The homozygotes are useful for investigating the functional role of macaque CYP3A4 and CYP3A5 in vivo.

In conclusion, our initial screening for genetic variants of CYP3A4 and CYP3A5 in cynomologus and rhesus macaques has successfully identified 12 nonsynonymous *CYP4A4* variants and nine nonsynony-

mous *CYP3A5* variants in cynomologus and rhesus macaques, com-
bined. It is noteworthy that null genotypes were also found in cy-
nonologus CYP3A4 and CYP3A5. Some of the genetic variants were distributed unevenly between Indone-
sian and Indone-
sian populations of cynomologus macaques, including the null genotypes identified in this study. The data presented could be a useful genetic resource when conducting drug metabolism studies in macaques.

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