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Title page

Genetic variants of *CYP3A4* and *CYP3A5* in cynomolgus and rhesus macaques.

Yasuhiro Uno, Akinori Matsushita, Naoki Osada, Shotaro Uehara, Sakae Kohara, Ryoichi Nagata, Koichiro Fukuzaki, Masahiro Utoh, Norie Murayama and Hiroshi Yamazaki

Pharmacokinetics and Bioanalysis Center (PBC), Shin Nippon Biomedical Laboratories (SNBL), Ltd., Kainan, Japan (Y.U., A.M., S.U., S.K., R.N., K.F., M.U.); Department of Biomedical Resources, National Institute of Biomedical Innovation, Ibaraki, Japan (N.O.); Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Japan (N.M., H.Y.).

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Running title page

Running title: genetic variants of macaque *CYP3A4* and *CYP3A5*

Corresponding Author: Dr. Yasuhiro Uno

Genome Research Group, Pharmacokinetics and Bioanalysis Center (PBC), Shin Nippon Biomedical Laboratories (SNBL), Ltd., 16-1 Minami Akasaka, Kainan, Wakayama 642-0017, Japan.

Phone: +81-73-483-8881, Fax: +81-73-483-7377, *E-mail address:* uno-yasuhiro@snbl.co.jp

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Abbreviations: CYP, cytochrome P450; PCR, polymerase chain reaction; SRS; substrate recognition site; UTR, untranslated region.

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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 cytochrome P450 (*CYP*) *3A4* 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 non-synonymous variants, and 34 *CYP3A5* genetic variants, including 9 non-synonymous variants. Four of these non-synonymous 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 (S437N) 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 2 out of 239 animals and 3 out 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.

Introduction

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, inter-animal differences have been noted in drug metabolism by *in vivo* analysis using dextromethorphan and *S*-mephenytoin as probe substrates (Jacqz et al., 1988), which could be, in some part, attributable to genetic variability of drug-metabolizing enzymes, as genetic variants of cytochrome P450 2C76 (*CYP2C76*) have been identified (Uno et al., 2009).

Human CYP3As are considered the major drug-metabolizing CYP subfamily, comprised of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (Gellner et al., 2001). In humans, CYP3As account for more than half of the total CYP content in human liver (Thummel and Wilkinson, 1998) and metabolize more than half of all prescription drugs, such as nefedipine, midazolam, and testosterone (Thummel and Wilkinson, 1998; Evans and Relling, 1999). Numerous inter-individual differences in drug-metabolizing capability mediated by CYP3A4 and CYP3A5 have been reported in humans, some of which are caused partially by genetic polymorphisms (see http://www.imm.ki.se/CYPalleles/). For example, hepatic CYP3A5 protein is present at detectable levels in 10-30% of Caucasians and 60% of African Americans (Hustert et al., 2001; Kuehl et al., 2001; Lin et al., 2002). This inter-individual 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 paper, 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 CYP counterparts, indicating similar drug-metabolizing properties of macaque and human CYP3A.

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 utilized to elucidate the regional and lineage differences in allele frequency of the identified variants. Metabolic activities were measured using the human CYP3A substrates, midazolam and nifedipine, to characterize potentially important variants.

Methods

Animals and genomic DNA extraction

Genomic DNA was prepared from whole blood samples 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, 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 Indonesian cynomolgus macaques were also genotyped, respectively. A 20-μl PCR reaction contained 1 ng of genomic DNA, 5 pmole 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 using an ABI PRISM BigDye Terminator v3.0 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.aspet.journals.org). Sequence data were analyzed using DNASIS Pro (Hitachi Software, Tokyo, Japan). Genetic variants of *CYP3A4* were identified by comparison with CYP3A4 cDNA sequences of cynomolgus macaque (GenBank accession no. S53047) and rhesus macaque (GenBank accession no. NM_017460), while *CYP3A5* variants were identified by comparison with CYP3A5 cDNA sequences of cynomolgus macaque (GenBank accession no. DQ074795) and rhesus macaque (GenBank accession no. 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 no. S53047) using the QuikChang XL II kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The primer pairs used were; 5′-ATGGAAAAGTGTGGGCTTTTATGATGGTC-3′ and 5′-GACCATCATAAAAGCCCACACTTTTCCAT-3′ for IVS3+1delG, 5′-GTCTGATCTGGAGCTCATGGCCCAATCAATTATCTT-3′ and 5′-AAGATAATTGATTGGGCCATGAGCTCCAGATCAGAC-3′ for c.886G>A, and 5′-CCTTACATATACACGCCCTTTGGAAATGGACCCAGAAACTGC-3′ and

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5′-GCAGTTTCTGGGTCCATTTCCAAAGGGCGTGTATATGTAAGG-3′ for c.1310G>A. Similarly, the mutation was introduced into the expression plasmid containing cynomolgus CYP3A5 cDNA (GenBank accession no. DQ074795) using the primers 5′-GGAAAGCGTTAAGTAGTTCCTAAAATTTG-3′ and 5′-CAAATTTTAGGAACTACTTAACGCTTTCC-3′ for c.625A>T, 5′-CATATACACACCCTTTGGAACTGGACCCAGAAACTGCATTGG-3′ and 5′-CCAATGCAGTTTCTGGGTCCAGTTCCAAAGGGTGTGTATATG-3′ for c.1310G>C, and 5′-TGTAGATCCCCTTGAAATTAGGCAAGCAAGGCCTTCTTCAATCAG-3′ and 5′-CTGATTGAAGAAGGCCTTGCTTGCCTAATTTCAAGGGGATCTACA-3′ for c.1437C>G. The entire sequence of the cDNA insert was confirmed by sequencing. The PCR products were cloned into a pCW vector containing human NADPH-CYP reductase cDNA. The plasmids were used for protein expression in *E. 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 CYP protein content in the membrane preparations was determined spectrally, according to the method reported by Omura and Sato (1964). The yield of NADPH-CYP 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). Briefly, a typical incubation mixture (0.25 ml) contained recombinant CYP3A4 or CYP3A5 proteins (5 pmole), 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 CH_2Cl_2, 0.2 M NaCl, and 0.1 M

Na_2CO_3. 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 RT-PCR

To examine transcripts of macaque CYP3A4 and CYP3A5, total RNA was extracted from livers of seven cynomolgus macaques and RT-PCR was carried out as described previously (Uno et al., 2006). Briefly, 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 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 5′-CACACACAGCCCAGCAAAC-3′ and 5′-CCGTCTTCATTTCAGGGTTC-3′ for CYP3A4, and 5′-CGATGGACCTCATCCCAAAT-3′ and 5′-CTCATTCTCCACTTAGGGTTCC-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 using genome samples from 78 cynomolgus macaques (38 from Indochina and 40 from Indonesia) and 34 rhesus macaques. A total of 42 variants were identified in *CYP3A4* exons, including 5 in the 5'UTR, 27 in the coding region, and 10 in the 3'UTR (Table 1). Among 12 non-synonymous variants found were, c.886G>A in substrate recognition site (SRS) 4 and c.1310G>A in the heme-binding region. A total of 34 variants were found in *CYP3A5* exons, including 5 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 non-synonymous 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, likely 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 (chi-square 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. Similarly, recent study indicated that approximately half of genetic polymorphisms were shared between the two lineages (Street et al., 2007), suggesting that genetic variants identified in either macaque lineage can be partly utilized 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 3 non-synonymous variants, and 11 *CYP3A5* variants, including 3 non-synonymous variants, appear to be unique to Indochinese cynomolgus macaques, and 11 *CYP3A4* variants, including 5 non-synonymous variants, and 10 *CYP3A5* variants, including 6 non-synonymous 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). Since 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 and 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^{-4} based on published data (Thompson et al., 2004). The higher genetic diversity of *CYP3A4* and *CYP3A5* for macaques as compared with that in humans raises the probability of greater

inter-individual variability in CYP3A-mediated drug-metabolizing capabilities of macaques as compared to humans. Moreover, in humans, genetic diversity was 2.4 fold higher in *CYP3A5* 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 *et al.* proposed that the reduced genetic diversity in macaque *CYP3A5* might have been caused by quick fixation of beneficial non-synonymous polymorphisms in either macaque lineages (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>A (V296M) in SRS4 and c.1310G>A (S437N) in the heme-binding region of *CYP3A4*, and c.1310G>C (T437S) in the heme-binding region and c.1437C>G (N479K) in SRS6 of *CYP3A5*. The analysis indicated that neither variant resulted in substantial change in metabolic activity (Table 3).

Among the genetic variants identified, c.485G>A (R162Q) and c.878T>A (L293Q) were found in Indonesian cynomolgus macaque *CYP3A4*. In human *CYP3A4*, mutations of the corresponding amino acid residues, *CYP3A4*15* (R162Q) and *CYP3A4*18* (L293P), have 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 of 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 SRS4, it is of great interest to perform functional characterization of other non-synonymous variants of macaque *CYP3A4* and *CYP3A5* found in this study.

We investigated linkage disequilibrium between the genetic variants, whose enzyme activities 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 cynomolgus macaques, S437N (CYP3A4) and T437S (CYP3A5) were in linkage disequilibrium ($P = 0.03$; chi-square test). The haplotype with 437N (CYP3A4) and 437T (CYP3A5), both derived alleles, was significantly overrepresented. Interestingly, 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 CYP-specific maximum 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 likely null alleles. Further screening of additional

genomic samples from 199 and 218 Indonesian cynomolgus macaques found additional heterozygotes for *CYP3A4* IVS3+1delG and *CYP3A5* c.625A>T, respectively. Overall, 2 out of 239 animals were heterozygous for *CYP3A4* IVS3+1delG, while 3 out 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 Indonesian cynomolgus macaques analyzed in this study.

In humans, *CYP3A5*3* (g.6986A>G) causes aberrant splicing, leading to the production of a non-functional 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 inter-animal variability of drug metabolism. To explore this possibility, RT-PCR was performed with liver samples from seven animals; however, no aberrantly spliced cynomolgus CYP3A5 transcripts were identified (data not shown). Genetic variants equivalent to *CYP3A5*3*, therefore, 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, likely due to technical difficulties. Our results present an alternative way to produce animals lacking macaque *CYP3A4* 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 CYP3A 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 cynomolgus and rhesus macaques has successfully identified 12 non-synonymous *CYP3A4* variants and 9 non-synonymous *CYP3A5* variants in cynomolgus and rhesus macaques, combined. Importantly, null genotypes were also found in cynomolgus *CYP3A4* and *CYP3A5*. Some of the genetic variants

were distributed unevenly between Indochinese and Indonesian populations of cynomolgus 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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Table 1

The genetic variants identified in cynomolgus and rhesus *CYP3A4*.

Exon	Nucleotide Change [a]	Amino Acid Change	Nucleotide Position [b]	Number of Alleles [c]			Site	Nucleotide Substitutions in CYP3A4 cDNA [d]	
				Cynomolgus		Rhesus			
				Indochina	Indonesia			Human	Rhesus
1	GGGAA(G>A)GAAAG		-87	16/76	0/80	44/66	5′ UTR		
	CTCCA(C>T)GCACA		-76	0/76	3/80	4/66	5′ UTR	T	
	ACACA(A>G)CTGAA		-46	2/76	1/80	10/66	5′ UTR	G	
	GACTC(A>G)GAGGA		-30	0/76	2/80	1/66	5′ UTR		
	GATAA(A>G)GAAGG		-15	2/76	0/80	0/66	5′ UTR	G	
	TTGGC(T>A)GTGGA		24	0/76	3/80	0/66		C	
	CTGGT(G>C)CTCCT		60	0/76	1/80	0/66			
2	GGAAA(T>C)ATTTT		147	1/76	12/80	14/68			C
3	TGGAC(G>A)TTTGA		177	1/76	0/80	0/68			
	TGGGG(G>del)TGAGT	M89X	IVS3+1	0/76	2/478 [e]	0/68			
6	AGATG(G>C)TCCCT	V146L	436	0/76	1/76	0/66			
	TCCCT(A>T)TCATT	I148F	442	0/76	1/76	0/66			
	TGAGA(A>T)ATCTG	N159Y	475	1/76	1/76	0/66			
	GAAAT(C>T)TGAGG		478	0/76	0/76	1/66			
	GAGGC(G>A)GGAAG	R162Q	485	1/76	0/76	0/66		G>A (R162Q, *15)	
7	ATGGA(C>T)GTGAT		546	0/76	0/78	1/68		T	
	GAGTG(A>G)ATATC	N192D	574	1/76	0/78	0/68			
9	CGAGT(A>G)GATTT		807	22/76	52/80	68/68		G	G
10	TGATC(T>A)GGAGC	L293Q	878	0/76	1/80	0/68		T>C(L293P,*18) A	
	AGCTC(G>A)TGGCC	V296M	886	0/76	1/80	7/68	SRS4		
	GTGGC(C>T)CAATC		891	0/76	17/80	0/68			
	ATTAT(C>T)TTCAT		903	1/76	0/80	0/68			
	ACCAC(C>G)AGCAG		930	0/76	0/80	1/68		G	
	GTTCT(T>C)TCCTT		942	0/76	0/80	10/68		C	
	TATGA(G>A)CTGGC		960	2/76	1/80	26/68		A	
	TCACC(C>G)TGATG	P325R	974	0/76	0/80	2/68			
	CTGCA(G>A)GAGGA		996	17/76	0/80	0/68			
11	ATACT(G>A)TGCTA	V350M	1048	0/76	3/80	0/66			
	GTGGT(G>A)GTGAT		1179	0/76	1/80	0/66			
12	ATACA(C>T)GCCCT	T433M	1298	1/76	0/80	0/68			
	TGGAA(G>A)TGGAC	S437N	1310	19/76	6/80	0/68	Heme		
13	CCATT(G>C)TTCTA	V490L	1468	0/76	1/80	0/66			
	GAACA(C>T)GAGAG		1565	0/76	10/80	12/66	3′ UTR		
	AATTA(C>T)TTTGT		1582	0/74	0/80	1/66	3′ UTR		
	GAAGA(C>T)GGGCT		1610	17/74	0/80	0/66	3′ UTR	T	
	TCCAA(T>C)GTACT		1624	34/74	10/80	16/66	3′ UTR		
	CTGTT(A>G)TACTT		1691	0/74	0/80	1/66	3′ UTR		
	TTATA(C>T)TTGGG		1694	0/74	0/80	3/66	3′ UTR		
	ATTTG(G>A)CTCCT		1744	16/74	0/80	0/66	3′ UTR		
	CCTGG(T>G)TCAAG		1780	3/74	0/80	0/66	3′ UTR	-	
	TCTGA(C>T)AAGAG		1825	34/74	10/80	16/66	3′ UTR	T	
	TTTCA(T>C)CCACA		1856	0/74	6/80	0/66	3′ UTR		

[a] Nucleotide changes are described as a comparison to the reference sequence of cynomolgus CYP3A4 cDNA (GenBank accession no. S53047).

[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 no. NM_017460) and single nucleotide polymorphisms reported to the human cytochrome P450 (*CYP*) allele nomenclature (http://www.cypalleles.ki.se/) for human *CYP3A4*. Allele numbers and resulting amino acid changes are described. No corresponding nucleotide for c.1780T>G allele is present in human CYP3A4 cDNA sequence (GenBank accession no. NM_017460) due to deletion of the corresponding region in the human sequence.

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

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Table 2

The genetic variants identified in cynomolgus and rhesus *CYP3A5*.

Exon	Nucleotide Change [a]	Amino Acid Change	Nucleotide Position [b]	Number of Alleles [c]			Site	Nucleotide Substitutions in CYP3A5 cDNA [d]	
				Cynomolgus		Rhesus			
				Indochina	Indonesia			Human	Rhesus
1	ACAGT(C>A)CAGCA		-66	0/76	0/78	1/68	5′ UTR		
	GCAAA(C>T)AGCAG		-58	3/76	0/78	0/68	5′ UTR		
	AGACT(C>T)AGAGG		-31	2/76	0/78	0/68	5′ UTR		
	AGGAG(A>G)CAGTT		-23	1/76	0/78	1/68	5′ UTR		
	GTGGC(G>A)ATGGA		-1	4/76	0/78	0/68	5′ UTR		
	AGCCT(G>A)GTGCT		57	1/76	0/78	0/68			
2	CTATA(C>T)GGGAC		75	9/76	0/80	0/68		T	
3	CAGGG(T>G)CTCTG		168	0/76	0/80	8/68			
	TGTTA(T>C)AAAAA		195	0/76	9/80	0/68			
	TATGG(A>G)AAAAT		207	3/76	0/80	66/68			G
4	GATCC(C>T)GACAT		261	14/76	11/80	3/68			
	CCCGA(C>G)ATGAT	D88E	264	3/76	0/80	68/68			G
5	AGAAT(T>A)CGGTC		387	64/76	80/80	66/66		A	A
	TTGCT(G>C)TCTCC		399	1/76	0/80	21/66			C
	ACCAG(C>T)GGAAA		417	1/76	1/80	0/66			
	TCAAG(G>A)AGATG	E144K	430	0/76	5/80	0/66			
6	GAGAT(A>G)TGTTG	M155V	463	2/76	0/80	38/68		G	
	ATATG(T>C)TGGTG		466	0/76	0/80	2/68			
	GGCAA(G>A)CCTGT		504	0/76	0/80	2/68			
7	TTAAG(A>T)AGTTC	K209X	625	0/76	3/516 [e]	0/68	SRS2		
	CTCTT(A>G)ACAAT		666	0/76	0/80	1/68			
8	CCTTA(T>C)CCCAG	T230I	689	3/76	0/80	68/68		C	C
10	TGATC(A>T)GGAGC	Q293L	878	0/76	1/80	0/68		T	
	TATGA(A>G)CTGGC		960	0/76	0/80	2/68			
	TGGCC(A>T)CTCAC	T323S	967	0/76	1/80	0/68			
	AGAAA(C>T)TGCAG		991	0/76	1/80	0/68			
11	GAGCC(T>C)GAGGA		1233	1/76	0/80	2/68			
	CCTGA(G>A)GAGTT		1236	1/76	1/80	0/68			
	GTTCC(G>A)CCCTG	R415H	1244	0/76	3/80	0/68			
12	TGGAA(G>C)TGGAC	T437S	1310	66/76	80/80	68/68	Heme	C	C
13	TCCCC(T>C)TGAAA		1423	0/76	3/80	0/68			
	GGCAA(C>G)CAAGG	N479K	1437	0/76	5/80	0/68	SRS6	G	
	GAGTC(A>T)AGAGA		1485	1/76	0/80	0/68			
	AGAGA(T>C)GGAAC		1491	1/76	0/80	0/68			

[a] Nucleotide changes are indicated as a comparison to the reference sequence of cynomolgus CYP3A5 cDNA (GenBank accession no. DQ074795).

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

[c] *CYP3A5* genotypes were determined for 78 cynomolgus (38 from Indochina and 40 from Indonesia) and 34 rhesus macaques.

[d] The CYP3A5 nucleotides different from cynomolgus macaque are shown for human (GenBank accession no. NM_000777) and rhesus macaque (GenBank accession no. NM_001040219). Any genetic polymorphisms of human *CYP3A5* corresponding to macaque *CYP3A5* alleles identified in this study were not reported to the human cytochrome P450 (*CYP*) allele nomenclature (http://www.cypalleles.ki.se/).

[e] For c.625A>T, allele frequency in Indonesian population is calculated with 258 animals.

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Table 3

Drug-metabolizing activity of CYP3A4 and CYP3A5 variants found in cynomolgus macaques.

Variants	Midazolam		Nifedipine Oxidation
	4-Hydroxylation	1′-Hydroxylation	
	nmol/min/nmol CYP3A		
CYP3A4:			
Reference	33	18	34
M69X	< 0.1	< 0.1	< 0.1
V296M	37	16	55
S437N	34	18	44
Human CYP3A4	13	16	35
CYP3A5:			
Reference	5.0	9.4	21
K209X	< 0.1	< 0.1	< 0.1
T437S	7.1	12	24
N479K	7.2	13	25
Human CYP3A5	3.6	17	16

For expression of reference proteins, the expression plasmids were prepared based on cynomolgus CYP3A4 and CYP3A5 cDNA sequences (GenBank accession nos. S53047 and DQ074795, respectively) with the N-terminus modifications. For protein variants, nucleotide changes were introduced into these plasmids. Expression of protein and measurement of drug-metabolizing activities were carried out using midazolam (100 μM) and nifedipine (100 μM) as described in *Methods.* Results are presented as means of duplicate determinations (the ranges were less than 15% of the values).