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REVIEW

Functionally defective or altered *CYP3A4* and *CYP3A5* single nucleotide polymorphisms and their detection with genotyping tests

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Among the four cytochrome P450 *(CYP)3A* genes, *CYP3A4* and *CYP3A5* are the most abundantly expressed in the human liver. Eighty single nucleotide polymorphisms (SNPs) of *CYP3A4/5* have been reported to the Human P450 Allele Nomenclature Committee. *CYP3A4* alleles with minimal function compared with wild type include the *CYP3A4*6 and CYP3A4*17.* Alleles with moderately decreased or altered activity include: *CYP3A4*2, *8, *11, *12, *13, *16,* and **18*. *CYP3A5* alleles with minimal function include the splice variants *CYP3A5*3, *5, *6 and CYP3A5* 10,* as well as the null allele *CYP3A5*7*. Alleles with moderately decreased catalytic activity include *CYP3A5*8 and CYP3A5*9.* This report reviews the current progress in the functional characterization of *CYP3A4* and *CYP3A5* SNPs and provides genotyping tests for possible defective variants. A combination of genotyping tests for defective *CYP3A4/CYP3A5* haplotypes will be necessary to understand the variations in the metabolism and clinical toxicity of a wide variety of clinical drugs, since these two CYP proteins have overlapping substrate specificities.

Keywords: *CYP3A4*, *CYP3A5*, genotyping, metabolism, pharmacogenetics, pharmacogenomics, polymorphisms, single nucleotide polymorphisms

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Cytochrome P450 (CYP)3A is the most abundantly expressed P450 protein in the human liver and intestine and is the predominant subfamily involved in the metabolism of clinically-used drugs, as well as many environmental compounds [1–5]. Although the literature reports various estimates of the liver expression of CYP3As, CYP3A4 and CYP3A5 are believed to be the two major CYP3As expressed in the human liver [6–10]. Although CYP3A7 is considered primarily a fetal form, expression of its mRNA has been reported in approximately 11% of adult livers [11]. However, due to the lack of commercially available antibodies specific for CYP3A7, its expression at the protein level is considered controversial (as discussed in a review by Burk and Wojnowski [12]). Similarly, the low number of transcripts in the liver for *CYP3A43* argues against its presence in this organ. Thus, CYP3A4 and CYP3A5 are the important members of this subfamily in the liver. CYP3A4 and CYP3A5 have similar structures and overlapping substrate specificities [13–16]. The *CYP3A5*1* genotype was associated with high expression of the CYP3A5 protein in the liver and small intestine, but individuals who were homozygous for *CYP3A5*3* expressed very low amounts of the CYP3A5 protein [10]. Hepatic expression levels of CYP3A4 protein vary by up to 90-fold [17,18], but *in vivo* variability in clearance is much lower, less than tenfold for several CYP3A substrates [19,20]. Although CYP3A4 has been suggested as a predominant CYP3A form in the liver and small intestine [1,4,21], another report suggests that CYP3A5 represents at least 50% of the total CYP3A content in individuals expressing *CYP3A5*1* [10]. The overlapping substrate specificities and the tissue expression of these two CYP3As hamper the establishment of associations between gene variants and phenotypic results. Individuals having defective alleles of both *CYP3A4* and *CYP3A5* would be predicted to have lower CYP3A activity than those carrying mutations in a single *CYP3A* gene. Therefore, the purpose of this paper is to review recent progress in the functional characterization of *CYP3A4* and *CYP3A5* single nucleotide polymorphisms (SNPs), and to provide a summary of the available genotyping primers for the known defective allelic variants.

CYP3A4

Molecular basis for expression and metabolism

The *CYP3A4* gene is encoded by a 27 kb sequence on human chromosome 7q21.3-q22.1 and spans 13 exons [15,22,23]. CYP3A4 consists of 502 amino acids with a molecular weight of 57 kDa [24]. The major expression site of *CYP3A4* is the liver, accounting for approximately 30% of the total P450 content, but it is also expressed in extra hepatic tissues, such as the small intestine, prostate and colon [3,21,25–27]. CYP3A4 is involved in the oxidative metabolism of a broad range of structurally diverse foreign compounds and endogenous steroid

hormones [2,3,5]. The representative therapeutic drugs metabolized by CYP3A4 include the macrolide antibiotic erythromycin, the anti-arrhythmic quinidine, the sedative-hypnotics diazepam, midazolam, and triazolam, the immune modulators cyclosporin and tacrolimus, the HIV protease inhibitors indinavir and ritonavir, the calcium channel blockers nifedipine and verapamil, and the 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor lovastatin [13,28]. Endogenous substrates include testosterone, progesterone, androstendione, cortisol, estradiol, and lithocholic acid [3,13,29,30]. Substrates bioactivated by CYP3A4 include acetaminophen, aflatoxin B1, benzo[a]pyrene-7,8-dihydrodiol, cyclophosphamide, and isofosfamide [3,15,31,32]. Wild-type forms of the *CYP3A4* gene and CYP3A4 protein [24] are now designated as *CYP3A4*1* and CYP3A4.1, respectively. The Genbank accession number for the reference sequence of *CYP3A4*1* is AF280107. CYP3A4 SNP information is organized on the home page of the Human CYP Allele Nomenclature Committee [201], and other relevant sources include the SNP database (dbSNP) home page [202] and the commercial Perlegen site [203]. Genetic variants of *CYP3A4* are assigned by the Human CYP Allele Nomenclature Committee.

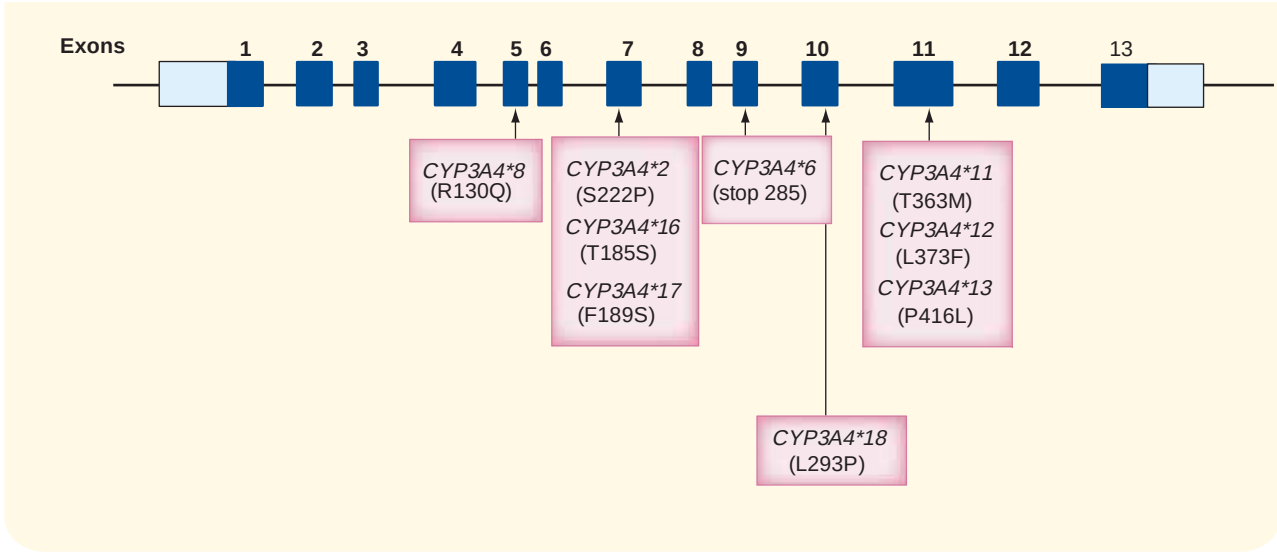
CYP3A4 *SNPs found in 3′- and 5′-UTR regions*

CYP3A4 variants found in the 5′-untranslated region (5′-UTR) include *CYP3A4*1A-F* and **1K-M*, and those in the 3′-UTR are designated as *CYP3A4*1G-J* and **1N-T*. Recently, Fukushima-Uesaka and colleagues released ten more new 5′ and 3′-UTR SNPs found in the Japanese populations [33]. The most common variant in the 5′-UTR is *CYP3A4*1B* (A-392G). Conflicting data suggested this promoter exhibited slightly higher luciferase activity (1.4–1.9-fold) than that of the wild-type A-392 construct in HepG2 and MCF7 cells [34,35], while other studies did not support this suggestion [36–38]. The other SNPs in the 5′- and 3′-UTR were found with low frequency and were not associated with transcriptional elements [39,40]. The frequency of *CYP3A4*1B* is highly variable in different racial populations with an allele frequency of 0% (Chinese, Taiwanese and Japanese) [35,41–43], 4–10% (Caucasians) [38,44,45], 9–10% (Hispanics) [41,42], and 48–80% (African–Americans) [41,42,45–47]. Studies using erythromycin [41], dextromethorphan [36] and midazolam [47] as *in vivo* probes for activity have not linked *CYP3A4*1B* with altered metabolism. There have been several studies investigating possible associations with various diseases, such as breast [37] and prostate cancer [41,42,44,48,49], and treatment-related leukemia [50]. These data suggest that the *CYP3A4*1B* marker alone cannot explain the reported association with steroid metabolism related to breast and prostate cancer. Therefore, the relationship between *CYP3A4*1B* and breast and prostate cancer is controversial and appears to be inconclusive. Other factors linked to *CYP3A4*1B* could be responsible for the possible cancer risk. A recent study [51] strongly suggested that *CYP3A4*1B* is associated with increased CYP3A5 expression due to its linkage with *CYP3A5*1A* [10]. In summary, in the current literature there appears to be insufficient evidence of linkage between *CYP3A4* SNPs in 5′- and 3′-UTRs to phenotypic variations in steroid-related diseases or drug metabolism.

CYP3A4 *SNPs found in the coding region*

A total of 18 *CYP3A4* coding variants were reported to the Human CYP Allele Nomenclature Committee. Functionally altered or defective *CYP3A4* alleles are summarized in Figure 1. *CYP3A4*2*, a S222P change, was found in a Finnish white population with a frequency of 2.7% (3 heterozygous individuals out of 162) but was absent in African–American and Chinese subjects [43]. Metabolism studies using a baculovirus expression system showed that the CYP3A4*2 protein exhibited a decreased V_{max} and intrinsic clearance for nifedipine. However, the metabolism of testosterone was not altered [43]. Although it is not definitive whether this amino acid substitution can alter the enzyme activity in testosterone metabolism, the serine to proline amino acid change could affect the three-dimensional structure of the protein, because proline is known as a helix-breaker. Therefore, it could be of value to genotype *CYP3A4*2* in clinical studies, particularly in Finnish people. Genotyping primers for *CYP3A4*2* are described in Table 1. *CYP3A4*3*, a M445T change in the heme-binding region, was found in one Chinese subject from Shanghai (1/178 individuals) [43], as well as in two Caucasian individuals (one from Eastern European Adygei [an ancient Caucasian group] and the other from Utah) [52]. A recombinant protein for the *CYP3A4*3* allele obtained from an *Escherichia coli* cDNA expression system was used to assess the catalytic activity for testosterone and the insecticide chlorpyrifos [52]. The catalytic activities of the CYP3A4*3 protein

Figure 1. *CYP3A4* alleles that exhibited altered or decreased functions compared to wild type.



Arrow heads indicate mutated locations that result in amino acid changes or a premature stop codon. Light blue boxes are 5'- and 3'-UTRs. Dark blue boxes are exons in ORF.
CYP: Cytochrome P450; ORF: Open reading frame; UTR: Untranslated region.

against these substrates were not significantly different from those of wild-type CYP3A4*1, although this change is located in the conserved heme-binding region. Eiselt and co-workers also found this allele in Caucasian DNA samples with a frequency of 0.47%, and discovered that metabolism of testosterone, progesterone and 7-benzyloxy-4-(trifluoromethyl) coumarin (7-BFC) by CYP3A4*3 was comparable to that of the wild-type protein [53]. In a recent study, kinetic parameters for nifedipine metabolism by a recombinant CYP3A4*3 protein obtained from *E. coli* were comparable to those of wild-type CYP3A4*1 [54]. All of these data suggest that the *CYP3A4*3* allele does not significantly differ from the wild type in the metabolism of testosterone, progesterone, 7-BFC, nifedipine, and chlorpyrifos, even though the amino acid change was located in the heme-binding area. *CYP3A4*4* (I118V), *CYP3A4*5*(P218R) and *CYP3A4*6* (a stop codon at amino acid 285) were found in a Chinese population [55]. In a study of 102 subjects, *CYP3A4*4* was found in three heterozygous individuals, *CYP3A4*5* was found in two heterozygotes, and the *CYP3A4*6* allele was found in one heterozygous individual [55]. *CYP3A4*6* was an A17776 insertion in exon 9, causing an early TGA stop codon in exon 9. When the ratio of urinary 6β-hydroxycortisol:free cortisol was compared to healthy Chinese population data, the authors suggested that all three alleles showed a decreased ratio [55]. Although there was a lack of data from wild-type subjects and drug usage before the ratio measurement, an individual with the *CYP3A4*6* allele showed a much lower ratio of the urinary 6ß-hydroxycortisol:free cortisol (0.88) than those with *CYP3A4*4* and *CYP3A4*5* (2.40 and 3.99, respectively). The significance of these alleles on enzyme activity needs to be further addressed. *CYP3A4*7* (G56D), *CYP3A4*8* (R130Q), *CYP3A4*9* (V170I), *CYP3A4*10* (D174H), *CYP3A4*11* (T363M), *CYP3A4*12* (L373F), and *CYP3A4*13* (P416L) were identified in Caucasian DNA samples and are functionally well-characterized *in vitro* [53]. Although most mutant and wild-type CYP3A4 proteins expressed well in a bacterial system, CYP3A4*8 and CYP3A4*13 exhibited no detectable P450 holoprotein, suggesting that these two protein products could be unstable [53]. CYP3A4*7, CYP3A4*9 and CYP3A4*10 did not differ from wild type in their expression in *E. coli* or their ability to metabolize testosterone, progesterone and 7-BFC [53]. In contrast, CYP3A4*11 was expressed more poorly in *E. coli* and had lower activity toward testosterone, progesterone

Table 1. Genotyping primers to detect *CYP3A4* alleles that exhibited altered or decreased functions compared to wild-type.

Alleles and their effects	Primers (5'-->3')	PCR size (bp)	Detection, restriction enzyme	Ref.
CYP3A4*2				
Exon 7 (S222P)	FP:CCTGTTGCATGCATAGAGG	369	Sequencing	[43]
Decreased activity	RP:GATGATGGTCACACATATC			
CYP3A4*6				
	FP:GAGCCATATTCTCAGAAGGGAGATCAAG	290	*Hinf I*	[55]
An A insertion in exon 9	RP:GTTTGTACACAGCAAGACGATACACC			
Frame shift early stop	FP:GAGCCATATTCTCAGAAGGGAGATCAAG	290	SSCP	[55]
	RP:CAAACATGTGTCGTTCTGCTATGTGG			
CYP3A4*8				
Exon 5 (R130Q)	FP:CACAACCATGGAGACCTCC	236	Sequencing	[53]
Unstable	RP:TACCTGTCCCCACCAGATTC			
CYP3A4*11				
Exon 7 (T363M)	FP:GTCTGTCTTGACTGGACATGTGG	393	Sequencing	[53]
Decreased activity	RP:GATGATGGTCACACATATCTTC			
CYP3A4*12				
Exon 11 (L373F)	FP:CAGTATGAGTTAGTCTCTGG	574	Sequencing	[53]
Altered activity	RP:CATAACTGATGACCTTCATCG			
CYP3A4*13				
Exon 11 (P416L)	FP:CAGTATGAGTTAGTCTCTGG	574	Sequencing	[53]
Unstable	RP:CATAACTGATGACCTTCATCG			
CYP3A4*16				
Exon 7 (T185S)	FP:CCTGTTGCATGCATAGAGG	369	Sequencing	[57]
Decreased activity	RP:GATGATGGTCACACATATC			
CYP3A4*17				
Exon 7 (F189S)	FP:CTGGACATGTGGGTTTCCTGT	290	*Bpm I*	[54]
Decreased activity	RP:AGCAGTTATTTTTAAGAGAGAAAGATAAAT			
CYP3A4*18				
Exon 11 (L293P)	FP:GCTTCGATCCTTTACCAGTATGA	416	Sequencing	[52]
Altered activity	RP:AGGCAGAATATGCTTGAACCAG			

Genotyping tests are not available for several alleles, CYP3A4*2, *8, *11, *11, *12, *13, *16, *and* *18. *The development of the PCR-RFLP tests or other high-throughput genetic methodologies would expedite genotyping in human samples. Sata and colleagues also provided specific amplification primers for all 13 exons of the* CYP3A4 *gene when* CYP3A4*2 *was discovered* [43].

CYP: Cytochrome P450; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; SSCP: Single-strand conformational polymorphism.

and 7-BFC compared to wild-type CYP3A4*1 [53,56]. In a cell-line system which expressed CYP3A4*11 and *16 proteins, similar levels of mRNAs for *CYP3A4*11* and *CYP3A4*16* were detected by northern blot analysis compared to wild type. However, western blot analysis demonstrated decreased levels of CYP3A4 protein, suggesting that these amino acid changes may affect protein stability. These results in a eukaryotic cell-line system agree with results obtained with the *E. coli* expression system [56]. CYP3A4*12 exhibited a significantly altered metabolic profile in testosterone and a fourfold increase in the K_m value for 1′-hydroxymidazolam formation [53]. *CYP3A4*14* (L15P) in exon 1, *CYP3A4*15* (R162Q) in exon 6 and *CYP3A4*16* (T185S) in exon 7 were identified by Lamba and colleagues [57]. This study was designed to determine the

genetic basis of *CYP3A4* variation in hepatic expression and catalytic activity using 265 individuals organized with respect to phenotype and genotype. However, not all of the individual SNPs were associated with low hepatic CYP3A4 protein expression or low CYP3A4 activity *in vivo*. Murayama and co-workers showed that CYP3A4*16 exhibited an approximate 60% decrease in testosterone 6ß-, 2ß- and 15ß-hydroxylation compared with wild-type CYP3A4*1 [56]. The effects of these coding variants on the enzyme activity against other substrates needs to be addressed. *CYP3A4*17* (F189S), *CYP3A4*18* (L293P) and *CYP3A4*19* (P467S) were found in a study of DNA from 72 different human lymphoblastoid cell lines from the Human Cell Repository, sponsored by the National Institutes of Health (Coriell Institute, NJ, USA) [52]. *CYP3A4*17* was identified in one Adygei individual from an Eastern European group as a heterozygote. *CYP3A4*18* and *CYP3A4*19* were found in one Chinese and one Indo–Pakistani, respectively, as heterozygous forms. *CYP3A4*17* displayed decreased catalytic activity compared with the wild type for both testosterone and the insecticide chlorpyrifos [52]. Kinetic analysis indicated that CYP3A4*17 exhibited a greater than 99% decrease in both V_{max} and CL_{max} for nifedipine metabolism compared to wild-type CYP3A4*1 [54]. Since CYP3A4*17 is the first defective allelic protein exhibiting a greater than 99% decrease in activity for a CYP3A substrate, 276 DNA samples from Caucasian individuals were analyzed for the *CYP3A4*17* allele, but no positives were identified. *CYP3A4*17* was originally identified in two out of nine Adygei individuals. This finding suggests that the frequency of the *CYP3A4*17* allele may be higher in certain Caucasian ethnic groups than others. Since many *CYP3A4* alleles are rare, they could be missed in a random sampling of large population studies with limited ethnic variability. Instead of limiting population studies to broad racial groups such as Asians, Caucasians and African–Americans, specific ethnic groups with ancestor information, such as Adygei, Chinese (Hong Kong), Japanese, and Indo–Pakistani, would be helpful for the estimation of genetic and phenotypic studies. CYP3A4*18 displayed a higher turnover number for testosterone and chlorpyrifos metabolism compared with wild type [52]. A second study reported that CYP3A4*18 exhibited lower Km and higher V_{max} in the metabolism of testosterone, compared with wild type [56]. However, a third study reported that this variant exhibited normal metabolism of nifedipine [54]. These differing results could reflect assay variability or multiple substrate binding sites for CYP3A4 [3,13,29]. Catalytic activity of CYP3A4*19 for testosterone, chlorpyrifos, and nifedipine was not significantly different from that of wild type [52,54].

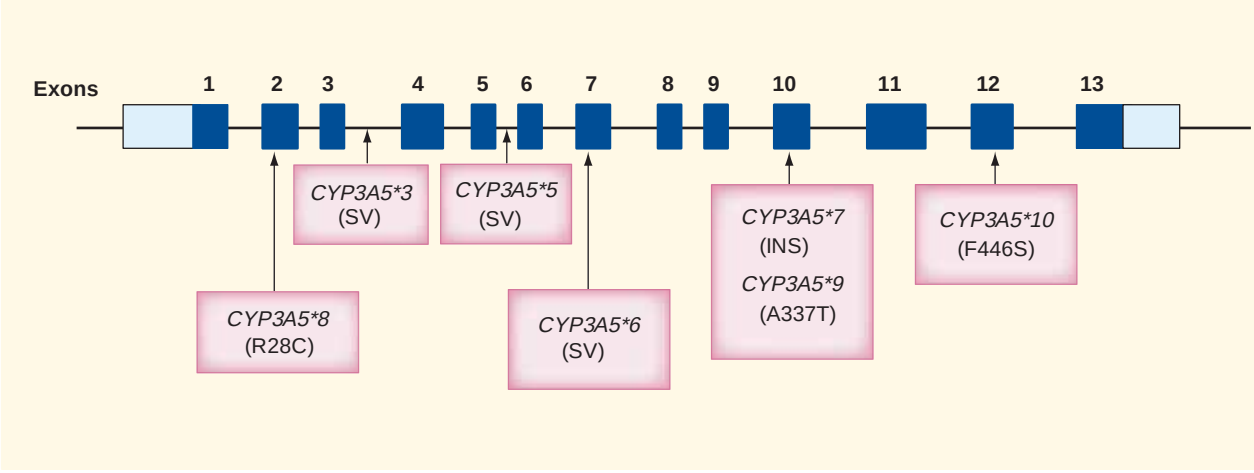
CYP3A5

Molecular basis for expression and metabolism of CYP3A5

The four *CYP3A* genes in a 231 kb length are localized in tandem on chromosome 7q21-q22.1 [15,22,23,58,59]. The *CYP3A5* gene has 13 exons encoding 502 amino acids [8,58]. The *CYP3A5*1* reference sequence is not reported, but the reference sequence of *CYP3A5*3* has been used from the accession number NG_000004.1, with the substitution of an A for base 6986 and a C for base 31611, to provide the sequence for the wild-type allele as recommended by the Human CYP Allele Nomenclature Committee [204]. CYP3A5 has been reported to be expressed at higher levels than CYP3A4 in extra hepatic tissues, such as in the lung [60], kidney [61,62], breast [63], prostate [64], and polymorphonuclear leukocytes [65]. It has been suggested that CYP3A4 and CYP3A5 share common regulatory pathways for constitutive expression [66]. Although CYP3A4 and CYP3A5 are inducible by constitutive androstane receptor (CAR) and pregnane X receptor (PXR) agonists [67], CYP3A4 is more inducible than CYP3A5 [68]. CYP3A4 contains both proximal and distal PXR elements, while CYP3A5 contains only the proximal PXR response element [66,69]. However, a recent study demonstrated a substantial induction of CYP3A5 due to this element, which may contribute to its importance in CYP3A drug metabolism [67]. Since CYP3A5 is a predominant form in the kidney, genetic polymorphisms in *CYP3A5* have been suggested to effect endogenous cortisol metabolism in the kidney, which may affect blood pressure through sodium and water retention [70,71].

There are limited catalytic studies of CYP3A5, and different laboratory conditions have been used in the catalytic characterization of CYP3A4 enzymes, such as varying amounts of nicotinamide adenine dinucleotide phosphate (NADPH)-CYP reductase, cytochrome b5, lipid compositions, and divalent cations [72–77]. CYP3A5 is less susceptible (5–19-fold) to inhibition by ketoconazole than CYP3A4 in the metabolism of midazolam, triazolam, nifedipine, and testosterone [78].

Figure 2. *CYP3A5* alleles that exhibited altered or decreased functions compared with wild type.



Arrow heads indicate mutated locations that result in amino acid changes, premature stop codon, and alternative splicing. Light blue boxes are 5'- and 3'-UTR. Dark blue boxes are exons in ORF.
CYP: Cytochrome P450; INS: Insertion; ORF: Open reading frame; SV: Splicing variant; UTR: Untranslated regions.

Although the effects of cytochrome b5 on kinetic parameters of CYP3A4 and CYP3A5 are similar [79], it is difficult to generalize which CYP3A form has stronger activity toward a particular substrate, because of the possibility of differential effects of assay conditions. However, CYP3A4 is generally believed to be more active than CYP3A5 [14], although CYP3A5 has been reported to have greater activity toward some substrates [4,14,80]. The associations between CYP3A5 expression and certain drugs may result from higher substrate specificity, rather than from its level of expression.

CYP3A5 *SNPs found in the 3'-and 5'-UTR*

CYP3A5 variants in the 5′- and 3′-UTR are *CYP3A5*1B-E* [17,81,201]. None of the 5′-UTR SNPs have been shown to be located in any known transcription factor elements [10,82]. Most of the SNPs found in 5′-UTR occur with a frequency of less than 5% [10,82]. In the 3′-UTR, however, *CYP3A5*1D* (31611C > T) is the most common variant, with a frequency of 83% in Caucasians, 60% in Asians and 40% in African–Americans [82]. This allele is highly linked to the *CYP3A5*3* allele and correlates with the racially different expression of the CYP3A5 protein. This could be another potential reason for the relatively low expression of CYP3A5*3 if this change affects mRNA stability [82].

CYP3A5 SNPs found in exons and introns

A number of SNPs occur in the coding region of *CY3A5* (Figure 2). For the functionally altered *CYP3A5* alleles, genotyping primers for restriction fragment length polymorphism (RFLP) assays, direct sequencing of SNPs, and some high-throughput assays, such as TaqMan™, that have been described in the literature for these alleles are summarized in Table 2. *CYP3A5*2* (T398N) was found in two out of five Caucasian individuals who did not express CYP3A5 [83]. Since *CYP3A5* mRNA was detected in these two individuals, CYP3A5*2 could be an unstable protein. The main reason for the variable expression of CYP3A5 in the human liver has been attributed to the *CYP3A5*3* allele [10,17,66]. The *CYP3A5*3* allele carries a mutation in intron 3 that creates a cryptic splice site and causes a premature stop codon, resulting in almost null expression of the CYP3A5 protein [10]. There are 10 haplotypes in the home page of the Human CYP Allele Nomenclature Committee (*CYP3A5*3A-J*) which are variants of the *CYP3A5*3* allele, and all of them are assumed to be associated with low expression of the CYP3A5 protein. This is the most common allele in Caucasians, and it is found in all ethnic population studies, suggesting that it is of ancient origin. CYP3A5 has been found in appreciable amounts in only 10–30% of liver samples of Caucasians and Asians [9,10]. In African–Americans, *CYP3A5**1 is the predominant allele, and the CYP3A5 protein represents at least 50% of the total CYP3A content [10], presumably exceeding the level of CYP3A4. Moreover, the presence of the *CYP3A5*3* allele probably has the most

Table 2. Genotyping primers to detect *CYP3A5* alleles which exhibit altered functions compared to wild type.

Alleles and their effect	Primers (5'-->3')	PCR size (bp)	Detection	Ref.
CYP3A5*3: *Intron 3, splicing defect*				
	FP:CTTTAAAGAGCTCTTTTGTCT**c**TCA RP:GAAGCCAGACTTTGATCATTATG	197	BseM II	[54]
	FP:CATGACTTAGTAGACAGATGAC RP:GGTCCAAACAGGGAAGA**a**ATA	293	Ssp I	[99,104]
	FP:ATGGAGAGTGGCATAGGAGATACC RP:CCATACCCCTAGTTGTACGACACA	244	Sequencing	[17]
	FP:CTTTAAAGAGCTCTTTTGTCT**c**TCA RP:CCAGGAAGCCAGACTTTGAT	200	Dde I	[97,105]
	FP:CTCTTTAAAGAGCTCTTTTGTCT**c**TCA RP:GTTGTACGACACACAGCAACC	155	Dde I	[106]
	FP:CTTTAAAGAGCTCTTTTGTC**c**T**g**CA RP:CACAGCATGTTGATCCCCATACCTA	166	Pst I	[107]
	FP:CCTGCCTTCAATTTTTCACT RP:GGTCCAAACAGGGAAGAG**g**T	196	Rsa I	[108,109]
	FP:CACGTATGTACCACCCAGCTT RP:GGAAGCCAGAACTTTGATCATT	250	Sequencing	[82]
	FP:ACTGCCCTTGCAGCATTTAG RP:TCCAAACAGGGAAGAGA**a**AT		Real-time PCR for A	[110,111]
	FP:ACTGCCCTTGCAGCATTTAG RP:TCCAAACAGGGAAGAGA**a**AC		Real-time PCR for G	[110,111]
	TCTCTTTAAAGAGCTCTTTTGTCTTTCGA TCTCTTTAAAGAGCTCTTTTGTCTTTCGG CAACCTTAGGTTCTAGTTCATTAGGGTG FAM-ATCTCTTCCCTGTTTGGACCACATTACCCTT-TAMRA		TaqMan™	[96]
	FP:GAGAGTGGCATAGGAGATACCCACGTATG RP:GGTAATGTGGTCCAAACAGGGAAGAGATTC		ASA for allele G	[48]
	FP:CATGACTTAGTAGACAGATGAC RP:CAGGGAAGAGATAC		ASA for allele G	[112]
CYP3A5*5: *Intron 5, splicing defect*				
	FP:CCATGAAGATCACCACAACT RP:CCTGTCCCCAGATTCAT**g**C	240	Nla III	[99]
	FP:CATGAAGATCACCACAACTAATGTG RP:CTTGGAAACGGACTGTGATCTTAC	252	Hsp2 II, SSCP	[90,107]

*Mismatched nucleotides with the CYP3A5 sequence are in bold and in lower case. PCR-RFLP detection for CYP3A5*3 by Dde I digestion* [97,105] *was questioned in a recent report* [54]*, because a unique sequence area was not used for primer design compared to other human CYP3As.*

ASA: Allele-specific amplification; CYP: Cytochrome P450; FAM: 6-carboxy-fluorescein; FP: Forward primer; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; R: Reverse primer; SSCP: Single-stranded conformation polymorphism; TAMRA: 6-carboxy-tetramethylrhodamine.

Table 2. Genotyping primers to detect *CYP3A5* alleles which exhibit altered functions compared to wild type.

CYP3A5*6: *Exon 7, splicing defect*				
	FP:AGGTGAGTCTAACTCAGCTTG RP:GACAGCTAAAGTGTGTGAGGG	578	Sequencing	[17]
	FP:GGTCATTGCTGTCTCCAACC RP:TCAAAAACTGGGGTAAGGAATG		Sequencing	[10]
	FP:GCTGCATGTATAGTGGAAGGAC RP:GGAATTGTACCTTTTAAGTGGATG	317	SSCP	[90]
	FP:GTGGGGTGTTGACAGCTAAAG RP:TGGAAGATGATTCAGCAGATAGT	495	Dde I	[99]
	FP:GATAGTTCTGAAAGTCTGTGGC RP:GAGAGAAATAATGGATCTAAGAAACC	268	Dde I	[106]
	FP:GTGGGTTTCTTGCTGCATGT RP:GCCCACATACTTATTGAGAG	237	Dde I	[97,105]
	FP:ACAAGACCCCTTTGTGGAGAGC**tt**TAA RP:GACGAAAGAACTGTATATTAAGTGTAT	141	Dra I	[107]
	FP:TACAGCATGGATGTGATTACTG RP:AAAGAGAGAAAGAAATAATAGCC		Sequencing	[98]
	FP:TATTGGATGCTTAGGGCAGTG RP:GATATGTGGGTTTCTTGCTGC		Sequencing	[82]
	FP:CCTTTGTGGAGAGCACT**g**AG RP:TGGTGGGGTGTTGACAGCTA		Real-time PCR for G	[111]
	GGATCTAAGAAACCAAATTTTAGGAACTGC GGATCTAAGAAACCAAATTTTAGGAACTGT GCCTACAGCATGGATGTGATTACTG FAM-AGTGCTCTCCACAAAGGGGTCTTGTGGAT-TAMRA		TaqMan	[96]
CYP3A5*7*: Exon 11, splicing defect*				
	FP:AAATACTTCACGAATACTATGATCA RP: CAGGGACATAATTGATTATCTTTG		Sequencing	[98]
CYP3A5*8: *Exon 2 (R28C), decreased activity*				
	FP:CTACAGGCATGGGCTACCATA RP:CTTGACCATTCCAGTTCCTGA		Sequencing	[82]
CYP3A5*9: *Exon 10 (A337T), decreased activity*				
	FP:CACCTTATTGGGCAAAACTG RP:AGGATCATTCAAGGCACACAC		Sequencing	[82]
CYP3A5*10: *Exon 12 (F446S), decreased activity*				
	FP:CAAGTAGGTTCTTTGGCCCAT RP:TGACCAGCCCACAAAAGTATC		Sequencing	[82]

*Mismatched nucleotides with the CYP3A5 sequence are in bold and in lower case. PCR-RFLP detection for CYP3A5*3 by Dde I digestion* [97,105] *was questioned in a recent report* [54]*, because a unique sequence area was not used for primer design compared to other human CYP3As.*

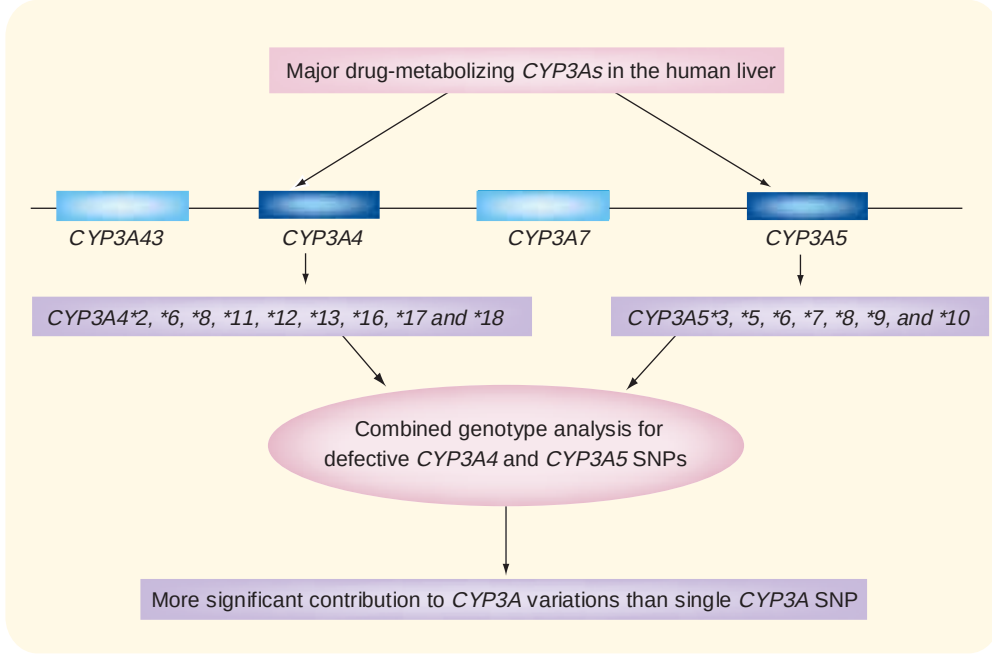
ASA: Allele-specific amplification; CYP: Cytochrome P450; FAM: 6-carboxy-fluorescein; FP: Forward primer; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; R: Reverse primer; SSCP: Single-stranded conformation polymorphism; TAMRA: 6-carboxy-tetramethylrhodamine.

significant contribution of all of the *CYP3A* alleles to the total variation in the clearance of CYP3A substrates. Although the *CYP3A5*3* variant is not sufficient to explain variable expression of total CYP3A proteins, the presence of the *CYP3A5*3* allele has been associated with a reduced clearance of CYP3A substrates, such as the lipid-lowering drugs lovastatin, simvastatin and atorvastatin [84], the immunosuppressant tacrolimus [85], cyclosporin [86,87], and midazolam [88]. Since *CYP3A5*1* is the predominant allele in African–Americans compared with other racial groups, Wojnowski and colleagues suggested there might be an increased risk to mutagenic metabolites of aflatoxin B1 in a Gambian population [89]. *CYP3A5*4* (Q200R) in exon 7 was found in 2 of 220 alleles in Chinese subjects [90]. Functional consequences of *CYP3A5*4* have not been investigated. *CYP3A4*5* was found in two Chinese subjects as a splicing variant mutated in the intron 5 splicing donor site [90]. *CYP3A5*6* is a splice variant containing a change in exon 7, which can cause deletion of this exon. This allele was found at a frequency of 13 and 16% in African–Americans [10,82]. The individuals with this allele had a lower catalytic activity for midazolam hydroxylation [10]. *CYP3A5*7*, found in African–Americans with a frequency of 10%, contains an insertion mutation which causes a premature stop codon at D348, resulting in the termination of the open reading frame [17]. One individual carrying the *CYP3A5*3/*7* alleles showed an extremely low level of CYP3A5 protein and midazolam 1'-hydroxylation. Therefore, genotyping for *CYP3A5*3* alone may not be sufficient to correlate with CYP3A5 phenotype [91]. *CYP3A5*8* (R28C) was identified in two Zaire individuals out of 24 diverse African–Americans with an allelic frequency of 4% [82]. *CYP3A5*9* (A337T) was identified in one Beijing individual out of 24 diverse Asians with an allelic frequency of 2% [82]. *CYP3A5*10* (F446S) was identified in one individual from Utah (USA) out of 24 diverse Caucasians with an allelic frequency of 2% [82]. The three allelic proteins described above were purified from a bacterial cDNA expression system [82]. CYP3A5*1 exhibited the highest maximal clearance for testosterone and the highest V_{max} for nifedipine oxidation, followed by *9, *8, and *10. In particular, CYP3A5*10 exhibited a greater than 95% decrease in the intrinsic clearance for both nifedipine and testosterone metabolism. A SNP resulting in the amino acid variant *CYP3A5*10* was found in one individual who was homozygous for *CYP3A5*3*. Although this SNP is on an allele containing the splice change, limited reverse transcription (RT)-PCR studies suggested that the livers of people who are homozygous for *CYP3A5*3* contain almost equal amounts of the wild-type and splice variant mRNA [10,66]. Therefore, although the incorrectly spliced mRNA is unstable [92,93], CYP3A5*3 and CYP3A5*10 proteins are probably expressed, albeit at a low level in the human liver. Actually, Hustert and colleagues showed that all individuals homozygous for the *CYP3A5*3* allele did express low levels of the CYP3A5 protein [17]. Thus, individuals carrying the amino acid change and the splice change that constitute the *CYP3A5*10* allele would be predicted to have lower clearance of CYP3A5 substrates than that observed in individuals carrying the *CYP3A5*3* allele.

Expert commentary

Interindividual variations in CYP3A activity are greatly influenced by drug-mediated CYP3A inhibition and induction in intestinal and hepatic tissues [13]. However, a significant role of genetic factors compared with environmental factors in interindividual variability in CYP3A4 activity was reported by Ozdemir and co-workers [94]. The accumulation of overall genetic polymorphisms with functional consequences would contribute to the correct assessment of CYP3A-mediated interindividual variations *in vivo*. Most *CYP3A4* and *CYP3A5* defective variants occur at low allelic frequencies, except for *CYP3A5*3*, **6*, and **7* [95]. In fact, many deleterious mutations may be quite rare. A confounding factor in the low frequencies is that genotyping studies are designed to represent all diverse populations, in order to avoid missing SNPs in the screening. Some *CYP3A* SNP frequencies can be high in certain ethnic groups. For example, *CYP3A4*17* was not found in 276 diverse Caucasians, but was found in two out of nine Adygei individuals [54]. Additional examples of a high incidence of SNPs in certain racial groups or specific ethnic groups can be found for *CYP3A4*1B* (no incidence in Asians, but up to 45% in African–Americans) [35,41–43,45], *CYP3A5*6* (no incidence in Asians, but 13% in African–Americans) [10,17,82,96,97], *CYP3A5*7* (10% in African–American, but no incidence in Caucasians) [17,82,98,99], and *CYP3A5*8* (two Zaire individuals in 24 diverse African–Americans) [82]. Therefore, it could be useful to carry

Figure 3. Important *CYP3A4* and *CYP3A5* alleles for the CYP3A genotyping in haplotype studies.



out genotyping/phenotyping studies in specific racial, ethnic or ancestor groups. This would give a better statistical power for the understanding of a specific SNP together with its haplotype relationship with other genes.

According to a recent comprehensive analysis, CYP3A4 expression in liver varies by up to 50- and 55-fold at the protein and mRNA level, respectively [18], and clearance variations observed *in vivo* include those seen in cortisol [100], erythromycin [100], midazolam [20], and nifedipine [101]. It is well known that preadministration of drugs can affect the expression of CYP3A in liver, adding to variability. However, the underlying genetic mutations affecting expression and clearance variations are not fully understood, indicating that further research should be performed to identify additional genetic variants of *CYP3A4*. Although screenings for human PXR variants revealed 7 missense variants [102,103], all of these variants were of too low frequency to support the high variation in CYP3A4 expression. Conceivably, mutations in other nuclear regulators or regulatory regions of receptors such as PXR might affect expression. A correlation between CYP3A4 and PXR transcripts has been reported, suggesting that expression levels of transcriptional regulators of CYP3A4 are one of the determining factors for variable CYP3A4 expression [18]. Additional sequencing of the intron and regulatory areas of *CYP3A4*, using phenotyped individuals who have also been genotyped for *CYP3A5* alleles, could reveal new and important *CYP3A4* haplotypes. To understand the contribution of low frequency *CYP3A* SNPs to phenotype, combined haplotype analysis for the known defective *CYP3A* SNPs could be more powerful than genotyping for a few *CYP3A* SNPs (Figure 3).

Outlook

The CYP3A subfamily has been studied extensively because of its considerable involvement in drug metabolism. In addition to genetic factors, interindividual variations in CYP3A activity can be affected by multiple factors including drug interactions, induction or inhibition by drugs and environmental chemicals. In addition, age, race, disease state, organ function, and dietary factors undoubtedly contribute to variability. Among the genetic factors, one of the *CYP3A* alleles, *CYP3A5*3*, provides an important explanation for low CYP3A5 expression in the liver and other tissues, and partially explains reduced catalytic activity for CYP3A5 *in vivo*. However, CYP3A5*3

Highlights

- Cytochrome P450 (CYP)3A4 and CYP3A5 are the major CYP3A enzymes involved in drug metabolism in the human liver. These two enzymes exhibit overlapping substrate specifities, similar DNA sequences and similar functions.
- Genotyping primers must be designed from the unique sequence areas after DNA sequence alignments of the four *CYP3A* genes.
- Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping tests and newer high-throughput assays are not available for several alleles (only sequencing). The development of new high-throughput methodologies for genotyping will expedite genotyping.
- Although many *CYP3A* variants have been predicted to be defective alleles *in vitro*, a thorough approach must be taken to assess these alleles in humans, and their clinical application, in order to understand the genotype/phenotype relationship.
- Most of the *CYP3A* defective coding variants occur at low allelic frequencies. The use of human subjects from the same ethnic and racial groups could provide a higher frequency for certain *CYP3A4* single nucleotide polymorphisms, and more statistical power to analyze the clinical outcome.
- Obtaining complete haplotype information for the *CYP3A4/CYP3A5* alleles is vital in understanding the impact that polymorphisms in these genes have on the effectiveness, toxicity and clinical outcome of the diverse classes of drugs metabolized by these enzymes.

alone cannot explain the total interindividual variations of CYP3A activity. To date, no single genetic defect can determine the total metabolic clearance of CYP3A substrates. Further searches for genetic variants of the *CYP3A4* gene may be necessary in the intron area and the regulatory area of *CYP3A4*. Such studies in clinically defined patients who have been genotyped for important *CYP3A5* alleles may lead to the identification of defective *CYP3A* haplotypes.

Many CYP3A variants have been reported in literature, but their functional significances have yet to be fully established. CYP3A variants characterized as being possibly defective in *in vitro* studies should be addressed in clinical studies, perhaps combined with studies of P-glycoprotein. The overlapping substrate specificity of CYP3A4 and CYP3A5 has complicated the identification of true poor-metabolizers. Complete *CYP3A4* and *CYP3A5* haplotype analysis is important, and the development of new high-throughput genetic methodologies will facilitate the ease and decrease the cost of complete haplotype analysis in the future.

The CYP3A subfamily provides major enzymes for the metabolism of endogenous steroid hormones. Hormonal disorders caused by prolonged exposure of the body to high levels of testosterone, oestrogen or cortisol can be implicated in several diseases, such as prostate cancer, breast cancer, hypertension, and Cushing's syndrome. The CYP3A family also metabolizes many pesticides [52]. Therefore, analysis of *CYP3A* haplotypes in epidemiological research could be an important aspect in understanding the physiological roles of CYP3A in the body, as well as in evaluating the hazards caused by environmental chemicals.

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