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CORRIGENDUM

Identification and characterization of novel sequence variations in the cytochrome *P*4502D6 (*CYP2D6*) gene in African Americans

A Gaedigk
A Bhathena
L Ndjountché
RE Pearce
SM Abdel-Rahman
SW Alander
L DiAnne Bradford
J Steven Leeder

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The authors wish to add an additional co-author as indicated in bold:

A Gaedigk, A Bhathena, L Ndjountché, RE Pearce, SM Abdel-Rahman, SW Alander, L DiAnne Bradford, **PK Rogan**[3] and J Steven Leeder

With the following affiliation:

[3]Laboratory of Human Molecular Genetics, Children's Mercy Hospital & Clinics, Kansas City, MO, USA

Correction:

The authors would like to clarify that the Human Genome Sequence Build used for the bioinformatic model mentioned on page 181 was 33 (and not 331) as described by Nalla and Rogan (Nalla VK, Rogan PK. Automated splicing mutation analysis by information theory. *Hum Mutat* 2005; **25**: 334-342). Furthermore, the Ri values given for maximum and average splice sites on the same page specifically refer to the splice donor site model.

The authors would like to apologise for any confusion caused.

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ORIGINAL ARTICLE

Identification and characterization of novel sequence variations in the cytochrome P4502D6 (*CYP2D6*) gene in African Americans

A Gaedigk[1]
A Bhathena[1]
L Ndjountché[1]
RE Pearce[1]
SM Abdel-Rahman[1]
SW Alander[1]
L DiAnne Bradford[2]
J Steven Leeder[1]

[1]*Division of Clinical Pharmacology and Experimental Therapeutics, Children's Mercy Hospital & Clinics, Kansas City, MO, USA;* [2]*Morehouse School of Medicine, Atlanta, GA, USA*

Correspondence:
Dr A Gaedigk, Division of Clinical Pharmacology & Experimental Therapeutics, Children's Mercy Hospital & Clinics, 2401 Gillham Road, Kansas City, MO 64108, USA.
Tel: +1 816 234 3941
Fax: +1 816 855 1958
E-mail: agaedigk@cmh.edu

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ABSTRACT
Cytochrome P4502D6 (*CYP2D6*) genotyping reliably predicts poor metabolizer phenotype in Caucasians, but is less accurate in African Americans. To evaluate discordance we have observed in phenotype to genotype correlation studies, select African American subjects were chosen for complete resequencing of the *CYP2D6* gene including 4.2 kb of the *CYP2D7-2D6* intergenic region. Comparisons were made to a *CYP2D6*1* reference sequence revealing novel SNPs in the upstream, coding and intervening sequences. These sequence variations, defining four functional alleles (*CYP2D6*41B*, **45A* and *B* and **46*), were characterized for their ability to influence splice site strength, transcription level or catalytic protein activity. Furthermore, their frequency was determined in a population of 251 African Americans. A -692_{TGTG} deletion (*CYP2D6*45B)* did not significantly decrease gene expression, nor could any other upstream SNP explain a genotype-discordant case. *CYP2D6*45* and **46* have a combined frequency of 4% and can be identified by a common SNP. Carriers are predicted to exhibit an extensive or intermediate CYP2D6 phenotype.
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Keywords: *CYP2D6*; SNPs; haplotype; dextromethorphan; African American

INTRODUCTION

Cytochrome P4502D6 (CYP2D6) is a major drug-metabolizing enzyme that is implicated in the metabolism of several drugs used clinically, including β-receptor antagonists, antiarrhythmic agents, morphine derivatives and, notably, an increasing number of antidepressant and antipsychotic entities.[1] This is not surprising in light of recent reports describing CYP2D6 activity towards endogenous substrates found in the human brain.[2,3] The *CYP2D6* gene locus is highly polymorphic and currently there are 51 allelic variants (*CYP2D6*1* to **51* including the ones described within this report) and over 30 additional subvariants defined.[4] As a consequence, CYP2D6 activity ranges widely within a population comprising ultrarapid (UM), extensive (EM), intermediate (IM) and poor (PM) metabolizer phenotypes. UM and PM are those most at risk for treatment failure or dose-dependent drug toxicity, respectively.

The incidence of PM subjects carrying two alleles encoding a nonfunctional or unstable enzyme is 5–10% in Caucasians, 3.5–8% in African Americans and ~1% in Asians. Caucasian populations have been extensively studied and a tight correlation between genotype and phenotype has been reported by many investigators using dextromethorphan (DM), debrisoquine or sparteine as probe

drugs. In fact, genotyping strategies comprising a relatively small number of allelic variants (*CYP2D6*3*, **4*, **5*, **6*, **7* and **8*) detect almost all PMs among European and North American Whites.[5–8] In Asians, best studied in Chinese and Japanese, about 1% of the population present as PM due to the presence of *CYP2D6*4* and **5* alleles, and a high incidence of IMs is caused by the high allele frequency of CYP2D6**10* ($f \sim 0.5$) that confers reduced activity.[9,10] Despite an initial strong relationship between phenotype and genotype data in Asian populations, the observation of discordant cases has led to the identification of six rather rare new allelic variants (*CYP2D6*14*, **18*, **21*, **36x2*, **39* and **44*) that are associated with diminished or absent activity.[4,11–16]

In contrast, studies in Black populations describe probe drug dissociation and an overall reduced activity compared to Caucasians, also referred to as a 'right-shift' of the population phenotype distribution. This shift is similar to that seen in Asians. Furthermore, cases of genotype to phenotype discordance have been described in a number of studies, but remain unresolved.[17–24] The presence of the reduced function alleles *CYP2D6*17* and **29* partially explain lower activity in Blacks, but additional nonfunctional alleles and alleles encoding protein with reduced enzymatic properties likely exist.

While the *CYP2D6* coding region has received much attention during recent years, very little is known about *CYP2D6* gene regulation and the consequences of sequence variation in the gene's upstream region.[25–27] This was primarily due to the fact that sequence data to support these investigations was limited to 1620 bp of 5′-flanking sequence deposited in M33388 commonly referred to as the 'Kimura sequence'.[28] Furthermore, sequence data for the *CYP2D7-2D6* intergenic region were incomplete, since the 'Kimura sequence' and accession X90926 (containing partial intergenic sequence and connecting with the *CYP2D7/2D8* locus sequence M33387) did not overlap. This gap persisted in all releases of the Human Genome sequence until Build 34.3 (02-10-2004), because the DNA utilized was derived from a subject homozygous for the *CYP2D6*5* gene deletion. Thus, substantial potentially important regulatory regions and sequence variations that may exist within those regions have not been thoroughly investigated.

In this study, we describe a reference sequence for *CYP2D6*1* encompassing 9 kb extending from the 3′-end of X90926 to the 3′-end of *CYP2D6* and identify the sequence variations present in the homologous 9 kb sequences derived from four novel alleles that were discovered in African American subjects. These sequence variations were assessed for their potential to alter protein activity, RNA transcription or RNA splicing *in vitro* in attempts to explain the finding of reduced activity *in vivo*.

RESULTS

Sequence Analysis of the *CYP2D7-2D6* Intergenic Region

To close the *CYP2D7-2D6* intergenic sequence gap, an 8953 bp long PCR product was generated, cloned and sequenced from a *CYP2D6*1/*5* sample that had been extensively genotyped for 25 defined allelic variants. This *CYP2D6*1* sequence was deposited in GenBank under accession number AY545216 and is being used as 'reference sequence' for all comparisons. Figure 1 shows the AY545216 sequence in comparison to M33388; both were identical within their common upstream portions, but differed in four positions within introns 1 and 2. Since these deviations occurred in all other alleles that were sequenced (including additional data not part of this report), they may represent errors in the original M33388 GenBank entry. To adhere with the widely accepted and standardized *CYP2D6* nomenclature, we refer to the nucleotide numbering in M33388 (Figure 1, positions in brackets) throughout this paper.

The AY545216 reference sequence information has permitted the redesign of primers to now amplify a 6.6 kb long PCR template for subsequent genotype analysis. The forward primer is situated upstream of the 5′-end of M33388 and encompasses the −1584C>G SNP as well as the entire *CYP2D6* coding region, allowing convenient testing of all desirable SNPs from a single template.[27]

During the course of this study, an 18 kb sequence was released in GenBank that bridges *CYP2D7* with *CYP2D6* (NG_003180 derived from clone RP4-669P10). This sequence represents *CYP2D6*2* and aligns with AY545216 in the positions that deviate from M33388 supporting the authenticity of these two new GenBank entries relative to M33388. Another recent sequence (AY480057) also closes the *CYP2D6/2D7* gap, but limits itself to 2.63 kb of this particular region. Within shared sequences, AY480057 is identical to the AY545216 *CYP2D6*1* reference sequence.

One region of intergenic sequence composed of a 22 bp long A-homopolymer at −1237 was particularly difficult to sequence. Even after extensive optimization (template length, primer position), sequence analysis remained somewhat recalcitrant. Each sequence was covered by multiple runs (forward and reverse) to obtain the best possible data. The *CYP2D6*41B* allele likely contains a $(A)_4$-insertion and NG_003180 exhibits a $(A)_5$-insertion, indicating that the number of A's may be highly variable.

Discovery, Description and Characterization of Novel *CYP2D6* Alleles

In order to resolve previously observed discordant results, DNAs from two subjects were cloned, sequenced and characterized.

Case 1

The first case initially exhibited an inconsistency in the *CYP2D6*8* genotype assay result and inclusion into data analysis had been deferred due to the inability to unambiguously assign a genotype.[29] A partial sequence analysis of exon 3 and flanking introns covering the location of the *CYP2D6*8*-associated SNP (1758G>T) revealed two new polymorphisms, namely 1757C>T, which prevented restriction digestion in the PCR-RFLP-based *CYP2D6*8* assay and 1716G>A that confers an $E_{155}K$ substitution. Subsequent

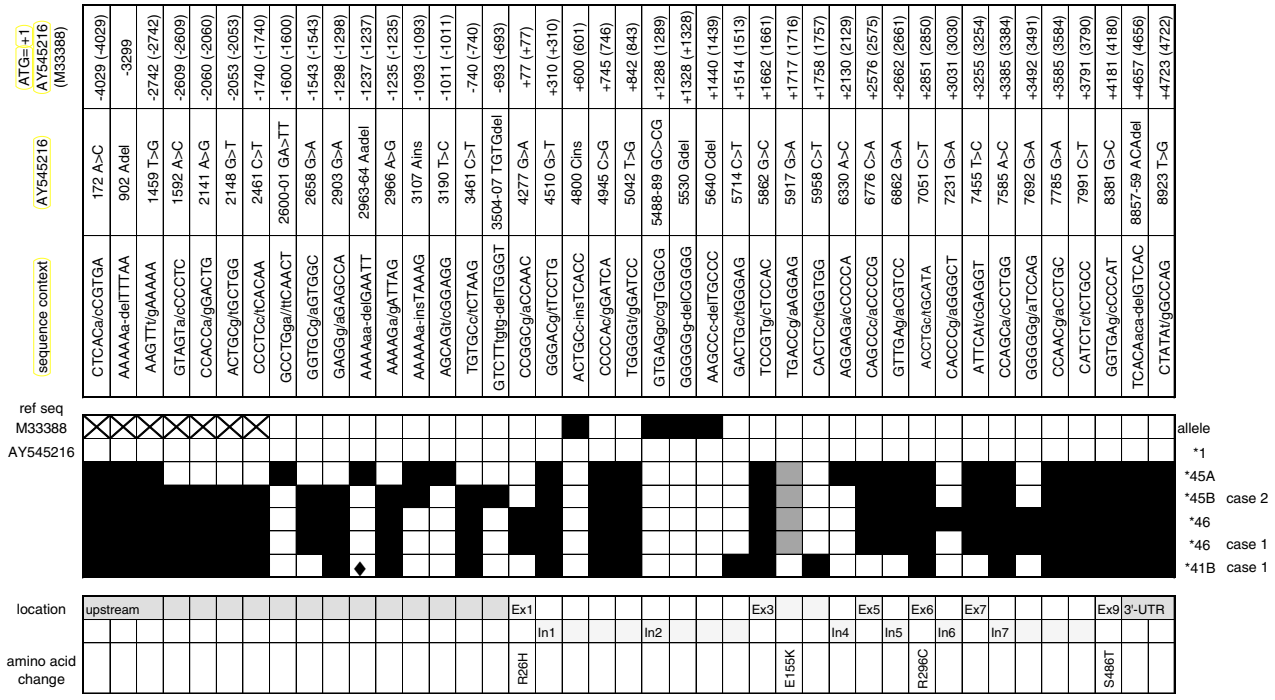

Figure 1 Sequence of six cloned *CYP2D6* alleles was determined including 4029 bp of the 5′ regulatory region. Each line in the graphic display represents the haplotype of a single allele (derived from cloned allele). Open boxes correspond to the *CYP2D6*1* reference sequences AY545216 and M33388; black boxes indicate sequence variations. X denotes sequence not part of M33388. Gray boxes indicated the common nonsynonymous SNP unique to *CYP2D6*45A*, **45B* and **46*. The sequence context line above the graph shows five nucleotides 5′ and 3′ of each variation, which is indicated in lower case; 'del' and 'ins' indicate nucleotide deletion and insertion. SNPs and their positions in AY545216 are as shown. The top row lists SNP positions with respect to the ATG start codon (*A* = +1) in AY545216 and M33388 within parentheses. Nucleotide positions as given for M33388 correspond to those used by the nomenclature web page.[4] ◆ in the *CYP2D6*41B* allele indicates a likely $(A)_4$-insertion.

sequence analysis comprising the cloned 9 kb fragments of both alleles revealed two new allelic variants, *CYP2D6*41B* and *CYP2D6*46* (Figure 1).

The 1757C>T on *CYP2D6*41B* is a synonymous SNP, but may affect RNA splicing since it is located at the −2 position of the exon 3 splice donor site. Information theory-based analysis determined an information content (Ri value) of 8.1 bits for 1757C, and hence classified it as a strong splice donor (note that the Ri values differ from those described by Rogan *et al*[30] since calculations are based on the updated model available at https://splice.cmh.edu). Neither C nor T make a positive contribution to the site's strength, but the presence of the variant 1757T slightly decreases the Ri value from 8.1 to 8.0 bits. This negligible change in the site's strength is not expected to alter mRNA splicing. In contrast, the 1513C>T SNP in intron 2 increased the strength of a cryptic site from −5.9 to 3.1. For additional SNPs present on *CYP2D6*41B*, see Figure 1. This allele was not observed in any other African American or Caucasian subject, making it a rare event with a frequency of $f < 0.001$.

The subject's second allele was designated *CYP2D6*46* and contained two nonsynonymous SNPs (77G>A, $R_{26}H$; 1716G>A, $E_{155}K$), two synonymous SNPs (2575C>A, 3254T>C) and a number of intronic and upstream SNPs as summarized in Figure 1.

The participant's EM phenotype for dextromethorphan/dextrorphan (DM/DX ratio = 0.005) and tramadol pharmacokinetic profile were comparable to those of other children carrying two functional alleles, suggesting that both new alleles were correctly spliced and encode fully functional protein (Figure 2 and Table 1).

Case 2

This case (subject 259 in Gaedigk *et al*[24]) had an initial *CYP2D6*2/*5* genotype assignment, which was revised to **5/*41* upon further testing, but remained discordant with the individual's PM phenotype (DM/DX ratio = 0.764). Cloning and sequencing of the *CYP2D6*41* allele revealed multiple sequence variations including a TGTG deletion in the 5′-upstream region at position −692 and an $E_{155}K$ substitution (1716G>A) that was also found in *CYP2D6*46* in case 1 (Figure 1). This variant allele was designated *CYP2D6*45B* by the CYP Nomenclature Committee.[4]

SNP and allele frequencies and haplotypes

To establish SNP and allele frequencies and SNP haplotypes, an African American study population comprising 251 subjects (excluding case 1, not part of this study cohort) was regenotyped for -692_{TGTG}, 77G>A ($R_{26}H$) and

1716G>A ($E_{155}K$). Only a partial association was found between -692_{TGTG} and 1716G>A, suggesting the existence of an allele that carries 1716G>A, but lacks the deletion. Such an allele was confirmed by cloning and sequencing and was designated *CYP2D6*45A* (Figure 1). The 2575C>A, 2661G>A and 3254T>C were always linked with 1716G>A. In contrast, 77A was found either in linkage with the latter SNPs or on a *CYP2D6*1* background ($n=5$; revised from *CYP2D6*1* to **43* according to *CYP2D6* nomenclature). Furthermore, 77A was also detected on two *CYP2D6*41* alleles, which are provisionally termed **41+77A* in this report. All subjects carrying -692_{TGTG} were positive for 1716G>A, 2575C>A, 266G>A and 3254T>C, but negative for 77G>A. All four new alleles also lack the intron 1 conversion that appears to be present in other *CYP2D6*2* 'backbone' alleles such as *CYP2D6*2* and **41*. Allele and SNP frequencies are given in Table 2.

In addition, SNP 3030G>A in intron 6 was found only on one of the two sequenced *CYP2D6*46* alleles (Figure 1). Genotype analysis did not reveal this SNP on any other *CYP2D6*46* or *CYP2D6*45* allele. On the other hand, an upstream SNP (−1011T>C) was present on six *CYP2D6*45A* alleles including the one that was sequenced, but was absent in three others. An AAG haplotype for −1543A, −1298A and −1235G was found in the sequenced *CYP2D6*46* and **45B* alleles and confirmed by genotyping in all such alleles. In contrast, the sequenced *CYP2D6*45A* lacked those SNPs, but genotyping confirmed their presence in the other *CYP2D6*45A* alleles, indicating that these alleles vary within their upstream region. Within positions −1740 and −4029, SNP patterns were identical for *CYP2D6*41B*, **45B* and **46* sequences, but again differed for the *CYP2D6*45A* allele. It is worth noting that none of the new alleles were positive for −1584G.

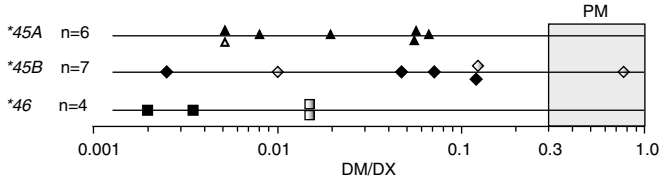

Figure 2 Genotype to phenotype correlation in heterozygous subjects with *CYP2D6*45A*, **45B* and **46* alleles. Phenotype is expressed as metabolic urinary ratio of DM/DX. PM status is defined as DM/DX>0.3 (gray rectangle). Three subjects (shaded diamonds) were genotyped as *CYP2D6*5/*45B* with DM/DX ratios of 0.01, 0.123 and 0.764 (discordant case), respectively. Two subjects were *CYP2D6*5/*46*, both with DM/DX of 0.015. The open triangle denotes the subject with a C*YP2D6*41B/*45A* genotype and a DM/DX ratio of 0.005. Symbols for subjects with similar DM/DX ratios were offset above and below the line.

In vitro *expression and activity*

To evaluate functional consequences of the nonsynonymous SNPs in *CYP2D6*45A* and **46*, pcDNA constructs coding for CYP2D6.2 ($R_{296}C$, $S_{486}T$; positive control), CYP2D6.45A ($E_{155}K$, $R_{296}C$, $S_{486}T$) and CYP2D6.46 ($R_{26}H$, $E_{155}K$, $R_{296}C$, $S_{486}T$) proteins, respectively, were expressed in COS-7 cells. Microsomal membranes were prepared and DM *O*-demethylase (CYP2D6.2, CYP2D6.45A and CYP2D6.46) and bufuralol 1′-hydroxylase activities (CYP2D6.2 and CYP2D6.46) were determined. As shown in Table 3, K_m, V_{max} and Cl_{int} (V_{max}/K_m) are comparable to CYP2D6.2 control values, suggesting that both isozymes are functional. These findings are in agreement with *in vivo* tramadol kinetic data of the subject described above (Table 1) and two *CYP2D6*5/*46* EM subjects with a DM/DX ratio of 0.015 (Figure 2).

Table 2 *CYP2D6* allele (A) and SNP (B) frequencies in $n=251$ African American subjects regenotyped for −692del, 77G>A and 1716G>A

	n	*Frequency*
(A) CYP2D6 allele		
**45A* (1716A)	9	0.018
**45B* (1716A, −692del)	8	0.016
**46* (1716A, 77A)	3	0.006
**43* (77A)	5	0.001
**41* (+77A)	2	0.004
(B) SNP		
-692_{TGTG} del	8	0.016
77G>A	10	0.020
1716G>A	20	0.040

The -692_{TGTG} deletion was associated with 1716A, while 77A was found in three different haplotypes. *CYP2D6*41* alleles positive for 77A were not sequenced and referred herein as **41+77A*.

Table 1 Total body exposure (AUC) estimates for tramadol and metabolites

	Two functional alleles (n=8)	*One functional allele (n=4)*	*Case 1*41B/*46*
Tramadol (ng h/ml)	1633.5±250.5	1677.0±954.6	1049.3
O-desmethyl tramadol M1 (ng h/ml)[a]	504.0±222.2	322.5±179.3	757.2
N-desmethyl tramadol M2 (ng h/ml)	195.0±137.0	236.4±223.1	173.9
di-*N*-*O*-desmethyl tramadol M5 (ng h/ml)[a]	128.4±61.4	92.8±53.7	233.8

Data represented as mean±SD.
[a]Metabolites mediated by CYP2D6.

Table 3 Mean kinetic parameters for dextromethorphan *O*-demethylation and bufuralol 1′-hydroxylation by CYP2D6.2, CYP2D6.45A and CYP2D6.46 expressed in COS-7 cells

Sample	*Dextromethorphan O-demethylation*			*Bufurolol 1′-hydroxylation*		
	K_m[a]	V_{max}[b]	V_{max}/K_m[c] × 1000	K_m[a]	V_{max}[b]	V_{max}/K_m[c] × 1000
CYP2D6.2	0.505	15.5	30.7	0.547	38.6	70.6
CYP2D6.45A	0.388	17.1	22.5	ND	ND	ND
CYP2D6.46	0.464	16.8	36.2	0.419	33.0	78.8

[a]Micromolar, [b]pmol/pmol P450/min and [c]μl/pmol P450/min.
ND, not determined.

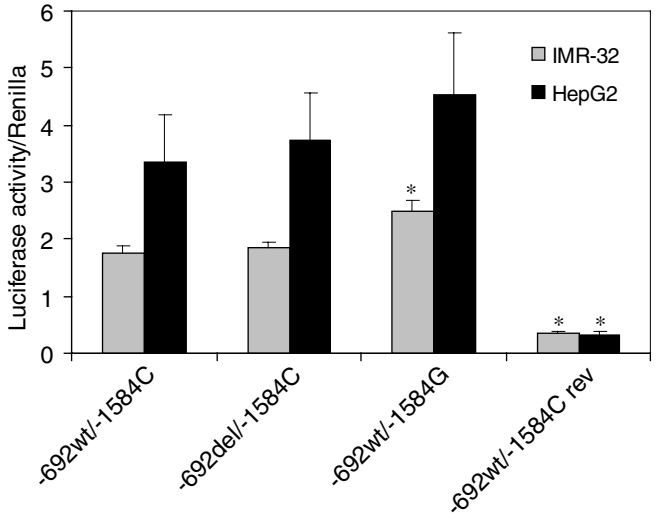

Figure 3 Luciferase reporter assays in transiently transfected HepG2 and IMR-32 cells. Constructs contain the SNPs as indicated below respective columns. The −692wt refers to TGTG at −692 and −692del to the deletion of TGTG at that position. The −692wt/−1584C 'rev' served as negative control with the promoter sequence inserted in reverse orientation. Renilla-normalized luciferase activity is expressed as the mean ± SD of triplicate determinations in a single experiment. Similar results were obtained in at least one additional transfection experiment. *Significant difference ($P \leqslant 0.001$) compared to −692wt/−1584C in IMR-32 cells and HepG2 cells, respectively.

Gene reporter assays

The -692_{TGTG} deletion was assessed by luciferase reporter assays in HepG2 and IMR-32 cells (Figure 3). The deletion was without effect in both cell lines. In addition, a search in the TRANSFAC v6.4 database did not reveal any transcription factor binding sites for the sequence within the region where the TGTG deletion is located. A construct containing −1584G was also assessed and exhibited higher luciferase activity compared to −1584C or −1584C + -692_{TGTG} controls in both cell lines. The increase, however, was significant ($P \leqslant 0.001$) in IMR-32 cells only.

Expression of CYP2D6*45B *mRNA*

To demonstrate that *CYP2D6*45B* mRNA is indeed expressed as implied by the transient transfection experiments, a bank of human prenatal liver samples was genotyped for *CYP2D6* and two prenatal specimens heterozygous for *CYP*1/*45B* and **2/*45B* genotypes were identified. In both cases, presence of *CYP2D6*45B*-derived cDNA was indirectly verified by the presence of the G>A SNP at position 1716. Additional evidence that the TGTG deletion did not affect transcription was provided by phenotyping data from two African American children. Both had the same genotype as the discordant case (*CYP2D6*5/*45B*), and had recently been phenotyped on multiple occasions with DM. In contrast to the discordant case, both children were consistently EM or IM (Figure 2).

DISCUSSION

Over recent years, we and other investigators have continuously encountered African American and African subjects who presented with genotype-discordant phenotypes and have classified them as 'outliers'.[21–24] Possible explanations for this phenomenon include drug interactions, environmental factors (eg food, natural food supplements),[31] and foremost, genetic factors, specifically the presence of unknown sequence variation(s). In order for 5–10% of a population to function phenotypically as PMs, 22–32% of alleles in that population must be nonfunctional. However, a relatively low frequency (13.3%) of nonfunctional alleles (ie *CYP2D6*3*, **4*, **5*, **6* and **40*) has been found,[24] suggesting that a significant proportion of nonfunctional alleles may have gone undetected in our African American population. The presence of additional variants was demonstrated earlier by the discovery of *CYP2D6*40* within the population now further studied[24] and *CYP2D6*42* in an 'outlier' subject who had participated in another study.[32] *CYP2D6*42* has also been found in two subjects of this cohort providing an explanation for their IM DM/DX ratio.

A better understanding of the heterogeneity of the *CYP2D6* locus in African Americans is necessary to more reliably predict phenotype from genotype data in African populations and their descendents. To that end, studies of discordant 'outlier' subjects provide the best opportunity of

identifying new allelic variants. To search for sequence variations, we expanded our focus beyond the coding and flanking intron sequences of the gene and included 4.2 kb of upstream sequence as well as the entire intronic sequences into the analysis. As summarized by Grant in a recent commentary,[33] more distant regions (potentially harboring regulatory elements) ought to be included into any thorough investigation, in addition to the immediate vicinity of the transcription start site that usually contains core promoter elements. Indeed, regulatory elements have been found within 500 bp of the *CYP2D6* transcription start site,[34] a region that appears to be devoid of SNPs (Figure 1, and unpublished data). On the other hand, the presence of SNPs within introns (outside 'flanking intronic regions') can also effectively influence mRNA splicing and hence abolish enzyme activity as demonstrated by Kuehl *et al*[35] in the evaluation of the *CYP3A5*3* allelic variant. To our knowledge, no such SNPs have been described for *CYP2D6*.

Ultimately, the study identified four new allelic variants, *CYP2D6*41B*, **45A*, **45B* and **46*. The first, *CYP2D6*41B,* constitutes a subvariant of *CYP2D6*41* since it carries one additional synonymous SNP within the coding region. Information theory-based splice site analysis, which has successfully been used in the past to accurately predict alternate splice events,[30,36,37] did not reveal a change in strength that would predict a loss or weakening of the exon 3 donor splice site. In fact, this site is 2.4-fold stronger than the average splice site. In contrast, a second SNP located in intron 2 created a cryptic splice donor site (Ri = 3.1 bits) that conceivably could be an alternative to the exon 2 donor (3.7 bits). Numerous other donor sites with bit values >3.1 are located upstream of this particular site, however, and are apparently not favored over the natural exon 2 donor site. Hence, 1513C>T likely is of minimal functional consequence. Furthermore, *in vivo* phenotyping data suggest that this allele is correctly and efficiently spliced, yielding mRNA levels comparable to those transcribed from other functional alleles such as *CYP2D6*1* or **2*. Indeed, the subject exhibited activity toward tramadol (M1 and M5 metabolite formation are mediated by CYP2D6) that was among the highest observed, whereas heterozygous subjects with one nonfunctional allele were significantly different. *CYP2D6*41B* appears to be extremely rare (only one allele observed to date); therefore, a 'default' assignment of *CYP2D6*41* in the absence of testing for 1757C>T likely predicts an accurate phenotype. As such, genotyping for this variant is neither cost-effective nor would it be expected to improve phenotype prediction.

*CYP2D6*45A*, **45B* and **46* share a novel nonsynonymous SNP (1716G>A, $E_{155}K$), two synonymous SNPs and one intronic SNP. *CYP2D6*46* carries a second amino-acid substitution ($R_{26}H$) and is therefore a separate allelic entity. Both *CYP2D6*45* and **46* encode functional protein as demonstrated by preliminary kinetic characterization of COS-7 cell expressed protein. Neither $E_{155}K$ alone nor $E_{155}K$ in combination with $R_{26}H$ appears to have an effect on activity when compared to CYP2D6.2. These *in vitro* findings are further supported by phenotype data, for example, EM status of two subjects whose activity is derived from *CYP2D6*46* only, since their second allele contains the *CYP2D6*5* gene deletion. Splice site analysis was also performed for the SNPs unique to *CYP2D6*45* and **46*, but did not indicate that any splice sites are generated by those sequence variations (data not shown). Additional studies, however, are warranted to characterize CYP2D6.45 and CYP2D6.46 proteins in more detail, for example, in comparison to other isoenzymes.

The *in vitro* kinetic data also infer that the previously described *CYP2D6*43* allele that carries $R_{26}H$, but lacks other SNPs, encodes a functional gene product. *CYP2D6*43* was initially designated **21*, but has been revised to **43* by the nomenclature committee; however, no information has been listed regarding its function.[4]

The four nucleotide deletion at position −692 in *CYP2D6*45B* is the only sequence variation across the 9 kb fragment that is unique to this allele and conceivably could lead to PM status. Since this variant was first discovered in a discordant case in conjunction with a null allele, we hypothesized that the deletion may interfere with gene expression, and thus explain the discordance and reveal regions important for *CYP2D6* regulation. To test this hypothesis, luciferase reporter constructs containing 2.6 kb of the *CYP2D6* 5′-upstream region were assessed in two cell lines, HepG2 and IMR-32. Since hepatoma-derived cell lines such as HepG2 do not express CYP2D6 or other drug-metabolizing P450 enzymes, transient transfection outcomes should be viewed with caution. Therefore, the neuroblastoma-derived IMR-32 cells were chosen as a second system as they have successfully been utilized for induction studies of another cytochrome P450 enzyme, namely CYP2E1.[38] Since results from both systems were consistent, we concluded that transcription levels likely are not affected by the -692_{TGTG} deletion, although independent confirmation in cell lines where *CYP2D6* is constitutively expressed would be desirable.

Expression of *CYP2D6*45B*-derived mRNA was also demonstrated in two prenatal liver tissue samples, further suggesting that the deletion does not affect mRNA transcription. In addition, two African American children with *CYP2D6*5/*45B* genotypes had intermediate and extensive metabolizer phenotypes (DM/DM ratios of 0.123 and 0.01) due to activity derived from their *CYP2D6*45B* allele. Taken together, the evidence is overwhelming that the -692_{TGTG} deletion does not lead to PM status and cannot explain the discordant phenotype in the investigated case. An unreported drug interaction event may have caused the discordance in this 67-year-old male participant who may not have disclosed all the medications/supplements or special diets to which he was exposed at the time of phenotyping. Unfortunately, a reassessment of his phenotype was not possible. Based on this conclusion, the frequency of PM subjects with this African American population is lower at 6.74% as originally reported (7.25%).[24]

Traditionally, PM subjects are identified by the presence of two nonfunctional alleles. Alternatively, accurate

phenotype prediction may also be achieved by exclusion of PM status by positive identification of at least one functional allele. As previously demonstrated, subjects carrying one or two −1584G alleles were EM or IM, ruling out PM status in 43% of Caucasians and 12% of African Americans studied.[27] Based on the frequency of the *CYP2D6*45*- and **46*-associated 1716G>A SNP ($f=0.04$), approximately 8% of an African American population carries at least one such allele. The assessment of both SNPs, −1584C>G and 1716G>A, allowed the exclusion of PM status in 20% of our African American population comprising 251 individuals. Future genotyping studies will include those SNPs to further evaluate their reliability to exclude PM status.

Even though the positive predictive value of a −1584G- or 1716A-containing genotype was 1.0 for the African American population investigated (assuming that the 'discordant' case was indeed nongenetic in nature and due, for example, to a drug interaction), this strategy should be applied to other populations with caution only. Additional (larger) studies in populations from different geographical locations and ethnic backgrounds are warranted to further establish the accuracy of prediction of those SNPs. Given the highly polymorphic nature of the *CYP2D* gene locus and the recombination events that have occurred between the *2D8*, *2D7* and *2D6* genes, clearly one has to anticipate that SNPs that are now defining one or more allelic variants may also be present on different haplotypes. Indeed, Raimundo *et al*[39] reported the −1584C>G SNP on a single *CYP2D6*4* and a single *CYP2D6*8* allele in Caucasians, suggesting that this SNP may rather be used as a quick initial screening tool rather than a definitive phenotype predictor.

Nonetheless, further characterization of the remaining discordant cases and resequencing of additional allelic variants and SNP correlation studies should ultimately allow reliable CYP2D6 phenotype prediction in African Americans, African populations and their descendents.

METHODS

Study Subjects

This study utilized existing DNA samples and phenotyping data from subjects enrolled in two previously conducted, IRB-approved investigations performed at the Children's Mercy Hospital and the Morehouse School of Medicine. The African American population utilized in this study included a total of 252 individuals ($n=191$ with parents and grandparents identified as African American and $n=61$ subjects who reported admixture in their parents or grandparents or descent was unknown). Genotyping data were presented previously [7,24,27,29,32] and have been updated to include results for new allelic variants described herein.

CYP2D6 Phenotyping

Briefly, a predose urine sample was collected and the study participants administered a single oral dose of DM (0.3 mg/kg as Tussin® Cough Syrup (Osco Drug, Oak Brook, IL, USA), 15 mg DM per 5 ml of solution). All urine produced during the ensuing 4 h period was collected and an aliquot stored at −20°C until analysis. The urinary concentrations of DM and its *O*-demethylated metabolite, DX, were determined using HPLC with fluorescence detection as described previously.[40] DM/DX ratios >0.3 were used to indicate a PM phenotype. In a single individual, a tramadol biodisposition profile was available in addition to a DM/DX ratio. The pharmacokinetic and statistical analyses applied to this individual have been described previously.[29]

CYP2D6 Genotyping

Blood was collected into ACD-containing vacutainers (Becton-Dickinson, Franklin Lakes, NJ, USA) and high-quality genomic DNA isolated with a QIAamp blood DNA kit (Qiagen, Valencia, CA, USA). Allele designation and nucleotide numbering is assigned according to http://www.imm.ki.se/CYPalleles/.[4]

CYP2D6 genotyping was performed as described previously[7,24,27,32] and comprised the following alleles: *CYP2D6*2*, **3*, **4*, **5*, **6*, **7*, **8*, **9*, **10*, **11*, **12*, **14*, **15*, **17*, **18*, **29*, **35*, **40*, **41* and **42*, as well as **1 × 2*, **2 × 2* and **4 × 2* gene duplications. For regenotyping purposes, a 6.6 kb fragment that encompasses the entire *CYP2D6* gene and upstream sequence was amplified by long-range PCR using JumpStart AccuTaq DNA polymerase (Sigma, St Louis, MO, USA) and primers 5′-ATG GCA GCT GCC ATA CAA TCC ACC TG (forward, AY545216: 2367–2392) and 5′-ACT GAG CCC TGG GAG GTA GGT AG (reverse, AY545216: 8931–8953). Annealing and extension were at 68°C for 7 min in the presence of 5% DMSO. The resulting PCR product was diluted at least 1000-fold to serve as template for the series of PCR-RFLP-based genotyping assays.

To identify SNPs associated with *CYP2D6*41*, **45A*, **45B* and **46*, PCR-RFLP-based assays similar in design to those developed previously were established. All assays were performed with JumpStart REDTaq DNA polymerase (Sigma, St Louis, MO, USA) and the accompanying buffer with details for the individual assays provided in Table 4. PCR reaction volumes were 8 μl and digestion volumes were 16 μl. Diagnostic restriction patterns were determined by agarose gel electrophoresis (3% agarose gels containing Synergel, Diversified Biotech, Boston, MA, USA) and documented with a Kodak 440 CF Image Station (Eastman Kodak Co., New Haven, CT, USA).

The 1757C>T SNP associated with *CYP2D6*41B* was detected while testing for *CYP2D6*8*.[24] The 1757T interferes with the restriction recognition sites partially provided by the primers such that PCR products generated from *CYP2D6*41B* remain uncut in both the wild-type and mutation-detecting assays.

Primers for PCR, genotyping and sequencing were obtained from Sigma Genosys (The Woodlands, TX, USA).

CYP2D6 Gene Cloning and Sequence Analysis

PCR fragments (9 kb long) were generated from genomic DNA with JumpStart AccuTaq DNA polymerase in the presence of 5% DMSO, a forward primer binding to 3731–3754 of X90926 that corresponds to 1–24 of AY545216 and the reverse primer used to generate the

Table 4 PCR-RFLP-based genotyping assays performed to detect SNPs associated with *CYP2D6*45A, *45B* and **46*

SNP	*Primers*		*Ann (°C)*	*Restriction enzyme*	*RFLP ref pattern (bp)*	*RFLP var pattern (bp)*
−1011T>C	AGA ATT AGG CTG GGT GGT GC	F	55	*Aci*I	662	437+225
	GAC ATG CAC AGA CGC TAT GC	R				
-692_{TGTG}del	GTC TGT GTA TGT GTG AAT ATT GTC **caT G**	F	58	*Nco*I	240	216+24
	CTT ATC TGT CAC TGG CAC TTA CC	R				
77G>A	GGC AGG TAT GGG GCT AGA AGC ACT GG	F	58	*Hha*I	226+84	310
	CAA ACC TGC TTC CCC TTC TCA GCC	R				
1716G>A	CTG GGC AAG AAG TCG CTG GAG CAG TGG G**cG gCC**	F	58	*Bsi*EI	323+32	355
	CAG AGA CTC CTC GGT CTC TCG	R	58			
	GCC CTG TGA CCA GCT GGA CAG AGC C	F		*Hind*III	271	237+34
	TTG GCG AAG GCG GCA CAA AGG CAG GCG GC**a agC T**	R				
1716G>A (cDNA)	CCA GAT CCT GGG TTT CGG G	F	58	*Hind*III	177	143+34
	TTG GCG AAG GCG GCA CAA AGG CAG GCG GC**a agC T**	R				
2575C>A	TGA GCA CAG GAT GAC CTG GGA CCC AGC **Cgg GCC**	F	56	*Apa*I	387+32	419
	GAT GGG CTC ACG CTG CAC ATC	R				
2661G>A	ACG GTG GGG GGC AAG GGT GGT GG**c TcG A**	F	62	*Xho*I	193+24	217
	CAG GTT CTC ATC ATT GAA GCT GC	R				
3030G>A	TTC TGT CCC GAG TAT GCT C	F	52	*Ava*I	246+156+7	402+7
	CGT CGT CGA TCT CCT GTT GGA CAC G	R				
3254T>C	GCT CAC ATG CCC TAC ACC ACT GCC GTG **ATg CA**	F	56	*Nsi*I	145+30	175
	GCT ATC ACC AGG TGC TGG	R				

The first column indicates SNPs and their positions. Assays are carried out on *CYP2D6*-containing long-range PCR templates, unless stated otherwise. Primers are given 5′–3′, 'F' and 'R' refer to forward and reverse. Partial restriction sites built into primers are in bold with sequence mismatches in lower case. Annealing temperatures (ann.) are as listed. For 1716G>A, two assays were used: the first allows restriction of 1716G-derived fragments, whereas the second assay allows restriction of fragments that carry 1716A. Fragment sizes are in base pairs (bp), 'ref' and 'var' refer to the reference and variant RFLP patterns, respectively.

6.6 kb fragment (see above). Primers were annealed at 63°C and extended for 10 min at 68°C. The PCR product was cloned into the pCR-XL-TOPO vector according to the instructions provided by the manufacturer (Invitrogen, Carlsbad, CA, USA). Clone inserts were characterized for integrity, and if necessary, allele identification by genotyping for at least three SNPs (eg −1584C>G, 1716C>T, 2850C>T) and by *Eco*RI digestion to assure presence of a full-length insert. For sequencing experiments, at least five plasmid clones were pooled to minimize visualization of PCR errors that may have been introduced during the amplification process. Sequencing was performed with appropriately spaced primers, DYEnamic ET dye terminator chemistry and a MegaBACE 500 capillary sequencer (Amersham Biosciences, Piscataway, NJ, USA). Sequence variations were confirmed either by direct resequencing of nested PCR products generated from newly amplified 9 kb or 6.6 kb templates or by PCR-RFLP analysis thereof. Sequence analysis for the novel alleles covered 9 kb and included: 4.2 kb upstream of the ATG start codon, coding regions, intronic regions and 535 nucleotides downstream of the TAG stop codon.

In vitro CYP2D6.45A and CYP2D6.46 Protein Expression and Activity

The 1716G>A and 77G>A nucleotide substitutions were subsequently introduced by site-directed mutagenesis into an existing CYP2D6*2 complementary DNA in the pcDNA3.1/Hygro vector (Invitrogen, Carlsbad, CA, USA) to produce CYP2D6.45 and CYP2D6.46 cDNA expression constructs. Clone integrity was established by sequence analysis prior to transfection experiments. COS-7 cell expression, microsome preparation and activity determination with DM and bufuralol were carried out as described earlier for CYP2D6.1, .2, .17 and .29.[24,41] DM ($n=10$) and bufuralol ($n=7$) concentrations ranged between 0 and 100 μM and 0 and 200 μM, respectively. Data presented for DM and bufuralol biotransformation by *CYP2D6* variants represent the mean of duplicate transfection experiments, whereas data for CYP2D6.2 (which served as positive control) are from accompanying, single transfection experiments. Microsomal incubations were performed in duplicate over the aforementioned range of substrate concentrations for each transfection experiment. Kinetic parameters for metabolite formation were estimated from the line of best fit using least-squares regression analysis of Lineweaver–Burk plots (reciprocal substrate concentration *vs* reciprocal velocity).

Characterization of the *CYP2D6*45B* -692_{TGTG} Deletion by Transient Transfection Experiments

A 2573 bp fragment was generated by long-range PCR from the genotype to phenotype discordant subject (case 2, *CYP2D6*5/*45B*), cloned with the TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA, USA) and designated −1584C/−692del. The primers were CAT **ctc GAG** CAA TGC ACA GAG ATC CAG (forward, AY545216: 1615–1641) and CCA **ctc GaG** CTC CTC TGG ACA CAC CTG G (reverse,

AY545216: 4160–4187) and contained mismatches generating *Xho*I sites as indicated in bold and lower case. Inserts with −1584C/−692wt and −1584G/−692wt but otherwise identical were subsequently created by consecutive rounds of site-directed mutagenesis. The sequence was confirmed before inserts were released by *Xho*I and cloned into the *Xho*I site of pGL3-Basic (Promega, Madison, WI, USA). A plasmid containing the −1584C/−692wt insert in reverse orientation and vector without insert served as negative controls.

The human neuroblastoma cell line IMR-32 (ATCC# CCL-127) was a gift from Dr RF Tyndale (University of Toronto, ON, Canada); HepG2 cells were purchased from ATCC. Transfection reagents were from Invitrogen, Carlsbad, CA, USA. Cells were cultured in 12-well plates to ~60% confluence and transient transfections were carried out with Lipofectamine transfection reagent according to the manufacturer's instructions. Briefly, construct plasmid (1600 ng for IMR-32, 200 ng for HepG2), 50 ng Renilla luciferase plasmid (pRL-TK, Promega, Madison, WI, USA) and 5 µl Plus reagent were incubated in 50 µl serum-free medium for 15 min at room temperature. Serum-free medium (50 µl) containing 4 µl and 2 µl Lipofectamine reagent for IMR-32 and HepG2 cells, respectively, was then added and incubated for 15 min at room temperature. After removing the growth medium, cells were overlaid with 400 µl serum-free medium and 100 µl of the transfection solution. After a 3 h incubation at 37°C, 2 ml of medium containing 10% fetal bovine serum was used to overlay the IMR-32 cells and replace the medium on the HepG2 cells. Cell extracts were prepared 24 h after transfection in Passive Lysis Buffer and luciferase activity measured on a Lumat LB 9507 luminometer using the Dual Luciferase Assay kit (Promega, Madison, WI, USA). Statistical analysis was performed with a one-way ANOVA with *post hoc* Tukey's test.

*CYP2D6*45B* Expression in Prenatal Human Liver Tissue

Prenatal human liver tissues collected with post-mortem intervals less than 4 h were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, USA and the University of Washington, Seattle, WA, USA. Use of these tissues was approved by the University of Missouri-Kansas city Pediatric Health Sciences Review Board. Total RNA was extracted from two samples genotyped as *CYP2D6*1/*45B* and **2/*45B*, respectively, with an RNeasy Mini Kit in conjunction with an on-column DNaseI treatment (Qiagen, Valencia, CA, USA). Subsequently, 1.5 µg were reverse transcribed with the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). cDNA (0.5 µl) was used for amplification and subsequent genotyping for 1716C>T (Table 4).

Automated Splice Site Analysis

Splice site analyses evaluating changes in splice site strength based on information theory-based models were recently described for *CYP2C19*, *CYP2D6* and *CYP3A5* splicing mutations.[30] The model applied to the current investigation has been updated based on the April 2003 release of the Human Genome Sequence (Build 331) and includes 111 772 donor and 108 079 acceptor sites (V Nalla and PK Rogan: Automated splice site analysis, https://splice.cmh.edu). The model implies that a sequence with an Ri value <0 bit is not a splice site, while the maximum information in the consensus sequence is 11.79 bits. The average splice site strength is 6.73 bits.

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DUALITY OF INTEREST

None declared.

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