CYP2D6*36 GENE ARRANGEMENTS WITHIN THE CYP2D6 LOCUS: ASSOCIATION OF CYP2D6*36 WITH POOR METABOLIZER STATUS

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ABSTRACT:

Unexplained cases of CYP2D6 genotype/phenotype discordance continue to be discovered. In previous studies, several African Americans with a poor metabolizer phenotype carried the reduced function CYP2D6*10 allele in combination with a nonfunctional allele. We pursued the possibility that these alleles harbor either a known sequence variation (i.e., CYP2D6*36 carrying a gene conversion in exon 9 along the CYP2D6*10-defining 100C>T single-nucleotide polymorphism) or novel sequences variation(s). Discordant cases were evaluated by long-range polymerase chain reaction (PCR) to test for gene rearrangement events, and a 6.6-kilobase pair PCR product encompassing the CYP2D6 gene was cloned and entirely sequenced. Thereafter, allele frequencies were determined in different study populations comprising whites, African Americans, and Asians. Analyses covering the CYP2D7 to 2D6 gene region established that CYP2D6*36 did not only exist as a gene duplication (CYP2D6*36x2) or in tandem with *10 (CYP2D6*36*10), as previously reported, but also by itself. This “single” CYP2D6*36 allele was found in nine African Americans and one Asian, but was absent in the whites tested. Ultimately, the presence of CYP2D6*36 resolved genotype/phenotype discordance in three cases. We also discovered an exon 9 conversion-positive CYP2D6*4 gene in a duplication arrangement (CYP2D6*4Nx2) and a CYP2D6*4 allele lacking 100C>T (CYP2D6*4M) in two white subjects. The discovery of an allele that carries only one CYP2D6*36 gene copy provides unequivocal evidence that both CYP2D6*36 and *36x2 are associated with a poor metabolizer phenotype. Given a combined frequency of between 0.5 and 3% in African Americans and Asians, genotyping for CYP2D6*36 should improve the accuracy of genotype-based phenotype prediction in these populations.

Cytochrome P450 2D6 (CYP2D6) is a major drug-metabolizing enzyme involved in the biotransformation of many clinically important medications, including antidepressant and antipsychotic drugs (Kirchheiner et al., 2004). CYP2D6 activity ranges considerably within a population and includes individuals with ultrarapid, extensive, intermediate (IM), and poor (PM) metabolizer status. Genetic variation within the CYP2D6 gene locus is the major contributing factor determining a subject’s activity or capability to metabolize certain drugs (Zanger et al., 2004). The CYP2D6 gene locus on chromosome 22 contains three contiguous genes, CYP2D8, CYP2D7, and CYP2D6 (Kimura et al., 1989), with only the latter encoding a functional protein (Gaedigk et al., 2005b). Not only is the CYP2D6 gene highly polymorphic, with 58 allelic variants and numerous functional protein (Gaedigk et al., 2005b). Not only is the CYP2D6 gene highly polymorphic, with 58 allelic variants and numerous subvariants reported to date (http://www.imm.ki.se/CYPalleles; accessed Jan 5, 2006), but rearrangements within the gene locus have created alleles harboring two or multiple CYP2D6 genes, deleted the entire gene, or led to the creation of fused CYP2D7/2D6 genes. Despite vast efforts characterizing CYP2D6 allelic variants in many populations of different ethnicity, subjects exhibiting a phenotype that is discordant with their determined genotype persist. Most of these cases are poor metabolizers who carry at least one functional or reduced-function allele predicting an extensive or intermediate metabolizer phenotype. Follow-up studies of such cases have revealed novel alleles such as CYP2D6*40 and *42 in African Americans (Gaedigk et al., 2002, 2003a) and CYP2D6*21 and *44 in Japanese (Yamazaki et al., 2003).

CYP2D6*36, originally termed Ch2 or *10C (http://www.imm.ki.se/CYPalleles), has been described in a tandem arrangement with CYP2D6*10B (CYP2D6*36 located upstream of CYP2D6*10B; Fig. 1) (Johansson et al., 1994). Regarding CYP2D6*36 function, Johansson et al. (1994) speculated that the 100C>T SNP (P34S) present in CYP2D6*10 and *36 is the major cause for protein destabilization and that the six-amino acid difference in exon 9 (i.e., the CYP2D7 exon 9 conversion) does not have a significant impact on CYP2D6.36 activity. Fukuda et al. (2000) later demonstrated that decreased clearance by CYP2D6.10 and CYP2D6.36 is caused not only by low protein expression, but also increased Km values. However, since the CYP2D6*36 gene did not appear to occur by itself, and activity was conferred by the downstream CYP2D6*10 gene on this allele, no further functional studies were pursued, and thus, it remained unclear whether CYP2D6*36 was an IM- or PM-associated allele. However, a discordant Japanese case was recently resolved by the discovery of a CYP2D6*36x2 gene duplication demonstrating that CYP2D6*36 is

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ABBREVIATIONS: IM, intermediate metabolizer; PM, poor metabolizer; kb, kilobase pair(s); PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; bp, base pair(s); DM/DX ratio, dextromethorphan to dextrophan ratio; UTR, untranslated region.
a loss-of-function allele as assessed by phenotyping with the probe drug debrisoquine (Chida et al., 2002).

Current genotyping protocols, including our own, define CYP2D6*10 and *36 by the presence and absence of two SNPs, 100C>T and 1846G>A, respectively. Consequently, the known *10A/B, *10x2, *36x2, and *36+*10 (tandem) alleles (Fig. 1) are not discriminated and are collectively genotyped as “CYP2D6*10”. Interestingly, three of our discordant cases had a “CYP2D6*10” in combination with a nonfunctional CYP2D6*4 allele, and we hypothesized that “CYP2D6*10” was in fact a masked CYP2D6*36x2 allele as described by Chida et al. (2002). To test this hypothesis, we conducted a detailed characterization of the CYP2D6/2D7 gene locus in phenotypic poor metabolizers with an assigned CYP2D6*10 allele.

Materials and Methods

Subjects. Study protocols, blood collection, and use of tissues were approved by the University of Missouri-Kansas City Adult and Pediatric Health Sciences Review Boards and the Morehouse School of Medicine Review Board (Atlanta, GA), respectively. All study participants gave written informed consent.

Subjects were phenotyped with dextromethorphan and genotyped previously. These studies comprised adult white Americans, n = 214 (Gaedigk et al., 1999) and African Americans, n = 281 (Gaedigk et al., 2002); African Americans with sickle cell disease (children >1 years of age), n = 126 (Alander et al., 2002); and an ethnically diverse pediatric population (subjects enrolled at 2 weeks of age), n = 155 (M. Blake, A. Gaedigk, R. E. Pearce, K. Adcock, S. Blaney, M. Christenson, L. James, G. L. Kearns, J. T. Wilson, and J. S. Leeder, manuscript in preparation).

An additional 81 genomic DNA samples were derived from an ethnically diverse tissue collection (ethnicity data were retrieved from data sheets accompanying the tissues) that was obtained from National Institute of Child Health and Human Development-supported tissue retrieval programs: the University of Maryland Brain and Tissue Bank for Developmental Disorders (Baltimore, MD) and the Central Laboratory for Human Embryology at the University of Washington (Seattle, WA). DNA samples of Asian Americans, n = 38, were collected for a previous study (Gaedigk et al., 1999) or from discarded, anticoagulated blood obtained for routine clinical management of hospitalized patients (self-reported ethnicity on hospital admission form).

DNA Isolation and CYP2D6 Genotyping. DNA was isolated from whole blood or liver tissue using DNA Blood and DNeasy Tissue Kits (QIAGEN, Valencia, CA), respectively. A subset of DNAs were isolated from cheek scrapings (buccal brushes) using Gentra reagents as recommended (Gentra Systems, Minneapolis, MN). CYP2D6 genotyping was performed as described previously (Gaedigk et al., 1999, 2002, 2003a,b, 2005a,c) and included *2 through *12, *14, *15, *17, *28, *29, *35, *40, *41, *42, *45, *46, and *1tx2, *2x2, and *4x2 gene duplications. CYP2D6 allele nomenclature throughout this report uses the recently revised allele definitions as established by the CYP2D6 nomenclature committee (http://www.imm.ki.se/CYPalleles).

Cloning and Sequencing of CYP2D6 Alleles. A 6.6-kb CYP2D6-specific fragment that encompassed the entire gene (Fig. 1, fragment C) was amplified and cloned with the pCR-XL-TOPO cloning kit (Invitrogen, Carlsbad, CA). This fragment is only derived from the most downstream gene within the locus and was also used as genotyping template. Sequence analysis of clones was performed with DYEnamic ET dye terminator chemistry and a MegaBACE 500 capillary sequencer (Amersham Biosciences Corp., Piscataway, NJ). AY545216 served as CYP2D6*1 reference sequence (Gaedigk et al., 2005a).

PCRs and Conditions. Primer sequences and additional details pertaining to all PCRs performed for the study are presented in Table 1.

Long-Range PCR. Reactions for PCR fragments A to D were carried out with JumpStart REDAccuTag LA DNA Polymerase (Sigma, St. Louis, MO) in the presence of 5% DMSO. Reaction volumes were 8 μl, and composition was as recommended. Extension times were 11, 12, 7, and 6 min, respectively. Typically, 1 to 2 μl of the PCR were analyzed by agarose gel electrophoresis. PCR A, carried out with forward and reverse primers binding to CYP2D6 exon 9 and intron 2, respectively, yielded product only from duplicated CYP2D6 genes such as *1x2, *2x2, *4x2, and *1tx2. Gene duplications such as CYP2D6*36x2 and the CYP2D6*36+*10 tandem did not amplify because of the presence of the exon 9 conversion. In contrast, a CYP2D6-specific forward primer binding to intron 6 (PCR B) allowed amplification of all duplication arrangements regardless of their exon 9 configuration (Fig. 1). Reaction D amplified a 3.6-kb fragment and was similar to that described by Chida et al. (2002) for the detection of CYP2D6*36x2. This primer set also amplified from CYP2D6*5, but produced a larger, ~5-kb-long fragment.

CYP2D6*36 Genotyping. Reaction E, a novel CYP2D6*36 assay, contained two sets of primers. The set for CYP2D6*36 amplified a 597-bp product only if the gene carrying the exon 9 conversion was located at the downstream position in the locus. The assay does not, however, discriminate between CYP2D6*36 and *36x2. The second primer set amplified an 860-bp-long CYP2D6*7 product as internal control for PCR performance (present in all samples). This PCR was carried out in a volume of 8 μl with JumpStart REDTag DNA Polymerase (Sigma). The entire reaction was separated on a 3%
agarose gel containing Synergel (Diversified Biotech, Boston, MA). This assay is referred to as the "uncut duplex assay." Fragments F-1 and F-2 (Fig. 1) were generated from diluted PCR fragments B and C, respectively, with JumpStart REDTaq DNA Polymerase. Subsequent incubation with the restriction enzyme NcoI allowed the detection of the exon 9 conversion.

CYP2D6 phenotype was determined from the urinary ratio of dextromethorphan to dextrorphan (DM/DX ratio) with PM status defined as a DM/DX ratio of ≤ 0.05 as defined by Gaedigk et al., 2003b) within 2 months of life. Each DM/DX ratio represents a phenotype assessment. Originally discordant and subsequently revised genotypes are presented. Notably, all four cases were African Americans. As shown in Table 2, three individuals, one adult and two neonates, had a CYP2D6*4/*10 genotype. The neonates had been challenged with dextromethorphan on multiple occasions (coinciding with well baby visits), indicating that the phenotype assessments truly reflected their metabolizer status. A fourth case presented with a DM/DX ratio of 0.38, just above the antimode of 0.3 at 2 weeks of age, and thus was initially classified as a poor metabolizer. However, the neonate assumed intermediate metabolism when rechallenged at 1 and 2 months of age, suggesting that this subject’s CYP2D6*10 allele may also be compromised.

To test whether any of the four subjects carried a CYP2D6*36x2 allele, we performed the PCR described by Chida et al. (2002). Indeed, all produced a 3.6-kb-long amplicon (fragment D) that harbored the conversion, whereas it was readily generated from any other CYP2D6 gene within the locus (Figs. 1 and 2D). However, fragment B, a duplication-specific, 10.5-kb product that covers the entire intergenic region as well as the "tail and head" parts of duplicated CYP2D6 genes, did not amplify in multiple attempts, whereas the fragment harboring the conversion remained uncut. Digestion patterns were resolved on 3% agarose gels containing SynNcoI (591 bp), whereas the fragment harboring the conversion remained undigested.

Results

After extensive genotype analysis, several subjects presented with a genotype/phenotype discordance. Notably, all four cases were African Americans. As shown in Table 2, three individuals, one adult and two neonates, had a CYP2D6*4/*10 genotype. The neonates had been challenged with dextromethorphan on multiple occasions (coinciding with well baby visits), indicating that the phenotype assessments truly reflected their metabolizer status. A fourth case presented with a DM/DX ratio of 0.38, just above the antimode of 0.3 at 2 weeks of age.
gene duplication events (Fig. 2B). This phenomenon is currently being further characterized.

The discovery of CYP2D6*36 in combination with another non-functional allele in phenotypic poor metabolizers demonstrated that this allele encodes a gene product that lacks appreciable activity toward dextromethorphan in vivo. To determine the frequency of CYP2D6*36 and to examine whether any alleles other than CYP2D6*36 harbor the exon 9 conversion, a total of 895 DNA samples (i.e., 1790 chromosomes) derived from either blood or tissue samples of white Americans, African Americans, Asian Americans and “other or unknown” ethnicity were genotyped using the CYP2D6*36 NcoI assay. In addition, all DNA samples positive for CYP2D6*10 were tested for CYP2D6*10x2 gene duplications and CYP2D6*36+*10 tandem arrangements by generating an ~10.5-kb amplicon (fragment B; Figs. 1 and 2B) and genotyping this fragment using a nested PCR-restriction fragment length polymorphism assay (CYP2D6*36 NcoI assay, product F-1 in Figs. 1 and 2). As summarized in Table 3, CYP2D6*36 was only present in African Americans and in one Asian subject. The CYP2D6*36+*10 tandem allele was most abundant in the Asian cohort (half of the total CYP2D6*10 were tandem alleles; Table 3), but was absent in whites and detected in only one African-American subject and in two subjects of unknown ethnicity. In three individuals, genotyping results were compatible with either a CYP2D6*36/*36+*10 or *36x2/*10 assignment. No CYP2D6*10x2 or CYP2D6*36x2 duplications were found in any subject. It is interesting that CYP2D6*36, *36x2, and the *36+*10 tandem arrangement were absent in the white populations tested. The frequencies of CYP2D6*36 (including alleles that could be either CYP2D6*36 or CYP2D6*36x2) were 0.53 to 2.5% in African Americans, 2.63% in Asians, and 3.33% in the other or unknown group.

We also designed and evaluated a novel assay to facilitate detection of CYP2D6*36 and *36x2 alleles directly from genomic DNA without the need to produce a long-range PCR product. A short 597-bp amplicon was generated alongside an internal amplification control product, only when the most downstream CYP2D6 gene contained the exon 9 conversion. This reaction was CYP2D6-specific and did not amplify from the CYP2D7 gene as demonstrated by using a CYP2D6*5/*5 DNA lacking the CYP2D6 gene on both alleles (Figs. 1 and 2E). All DNA samples that were CYP2D6*36-positive with the CYP2D6*36 NcoI assay were also positive with the novel assay, i.e., the CYP2D6*36 duplex assay.

The exon 9 conversion was also found in a single individual originally genotyped as CYP2D6*2x2/*4. As shown in Figs. 1 and 2 (lane 7), further characterization revealed that the CYP2D6*4 allele also harbored a gene duplication and that the conversion was located on both CYP2D6*4 gene copies (i.e., both *2x2 and *4x2 alleles must have generated fragment B, since F-1 amplified thereof was genotyped heterozygous for the exon 9 conversion; fragment A was derived from the CYP2D6*2x2 allele only, since F-1 was negative for the conversion; fragment C, generated from both alleles, yielded F-2 fragments with and without the conversion; and, finally, fragments D and E amplified from genomic DNA, indicating an exon 9 conversion-containing gene at the downstream position). Moreover, 1846G>A, the key CYP2D6*4 SNP, was confirmed to be present on both gene copies of the CYP2D6*4x2 allele. This was achieved by generating a PCR fragment encompassing exon 3 through 9 with primers that specifically amplified this gene structure and using it as genotyping template for 1846G>A (a *4 detection) (not shown). The presence of this novel allele was further confirmed by pedigree analysis, which showed that both offspring of our case had inherited the exon 9 conversion-negative CYP2D6*2x2 allele. This allele was designated CYP2D6*4N by the P450 nomenclature committee.

During the course of this study, we also discovered a CYP2D6*4 allele that lacked the C>T SNP at position 100. The subject was a CYP2D6*4/*4 poor metabolizer who presented with a heterozygous 100C>T genotyping result (not shown). It is noteworthy that 4180G>C was also absent. This polymorphism is otherwise present on all defined CYP2D6*4 alleles except CYP2D6*4J, according to the nomenclature web site. In addition, four other SNPs that were found on five resequenced CYP2D6*4 alleles were also missing (Fig. 3). Absence of these SNPs on the novel CYP2D6*4 variant was again confirmed by genotyping and/or resequencing DNA from the parents of the subject. This allele has been designated CYP2D6*4M by the P450 nomenclature committee.

To complete the analysis of the novel alleles, the entire 6.6-kb PCR products (fragment C) from CYP2D6*4, *4M, *4N, *10, and *10 derived from a *36+*10 tandem and *36 were cloned and sequenced. The presence of SNPs and their locations in comparison to two reference sequences, AYS45216 (CYP2D6*1 reference sequence pre-
Discussion

Three cases with a poor metabolizer phenotype and an initial discordant CYP2D6*4/*10 genotype were characterized by long-range PCR, genotyping, cloning and sequencing. Results are consistent with the presence of a “single” CYP2D6*36 gene in the most downstream position within the CYP2D6 locus. The CYP2D6*36x2 gene duplication has been characterized only in Japanese, to date, and this is the first report describing an allele carrying a single CYP2D6*36 gene copy. In addition, allele frequencies of this novel allele were determined in white, African American, and Asian population samples. This discovery not only resolved the genotype to phenotype discordance in our cases, but also provided ample evidence that CYP2D6*36 was indeed responsible for poor metabolism in vivo, as previously suggested by Fukuda et al. (2000) and Chida et al. (2002). Kinetic data on bufuralol 1'-hydroxylation and venlafaxine
O-demethylation showed significantly higher $K_m$ values for CYP2D6*36-derived protein compared with that of CYP2D6*1 and *10 gene products (Fukuda et al., 2000), and a CYP2D6*21/*36x2 subject presented as a poor metabolizer toward the probe drug debrisoquine (Chida et al., 2002). The data presented here extend these findings to dextromethorphan and suggest that CYP2D6.36 protein has limited or no appreciable activity toward other drugs known to be metabolized or bioactivated through the CYP2D6 pathway.

The fourth case presented here exhibited a metabolic ratio of DM/DX of 0.38 at 2 weeks of age and was, by strict application of the CYP2D6/*4 allelic definition, a poor metabolizer toward the probe drug debrisoquine (Chida et al., 2002). The data presented here extend these findings to dextromethorphan and suggest that CYP2D6.36 protein has limited or no appreciable activity toward other drugs known to be metabolized or bioactivated through the CYP2D6 pathway.

In conclusion, CYP2D6*36 has been overlooked in the past, and its contribution to the polymorphic expression of CYP2D6 was probably underestimated. The discovery of three poor metabolizers carrying this allele demonstrates loss of function, whereas allele frequencies between 0.5 and 3% emphasize its important contribution to immediate and poor metabolism in African Americans and Asians. It is highly recommended to include CYP2D6*36 testing for reliable phenotype prediction, especially when genotyping is performed in African Americans, subjects of African descent, and Asians.

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References


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